eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

ANKARA ÜNİVERSİTESİ

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JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

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JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY
Volume: 48, Issue: 3, Year: 2024

(J. Fac. Pharm. Ankara) *eISSN: 2564-6524*
ISSN: 1015-3918 (1971-2010)

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Volume: 48, Issue: 3, Year: 2024

(J. Fac. Pharm. Ankara) *eISSN: 2564-6524*
ISSN: 1015-3918 (1971-2010)

Section Editors:

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY
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(J. Fac. Pharm. Ankara) *eISSN: 2564-6524*
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Journal of Faculty of Pharmacy of Ankara University (*J. Fac. Pharm. Ankara*) is official scientific journal of Ankara University Faculty of Pharmacy. It was published between 1971 and 2010 as a print.

Journal of Faculty of Pharmacy of Ankara University is published three times (January-May-September) a year. It is an open access, peer-reviewed journal for the publication of original research reports, reviews and short communications in English or Turkish on relevant developments in pharmaceutical sciences. Proceeding of scientific meetings, conference paper, and meeting abstract may be published as special issues of supplements to the journal. All the articles appeared in this journal are published on the responsibility of the author(s). The manuscript submitted to the journal should not be published previously as a whole or in part and not be submitted elsewhere. The manuscripts should be prepared in accordance with the requirements specified.

Indexing and Abstracting

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Doi: 10.33483/jfpau.1404302

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ANTIOXIDANT ACTIVITY OF *HIPPOPHAE RHAMNOIDES* L. GROWING IN TURKEY AND INVESTIGATION OF ITS EFFECTS ON THE LIVER TOXICITY IN RATS

TÜRKİYE'DE YETİŞEN HİPPOPHAE RHAMNOİDES L. BİTKİSİNİN ANTİOKSİDAN AKTİVİTESİNİN BELİRLENMESİ VE SIÇANLARDA KARACİĞER TOKSİSİTESİ ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

Metin ÇAPA[1]*, Yücel KADIOĞLU[2], Elif ÇADIRCI[3], Mevlüt ALBAYRAK[4]

[1]Tokat Gaziosmanpaşa University, Faculty of Pharmacy, Department of Analytical Chemistry, 60400, Tokat, Türkiye
[2]Atatürk University, Faculty of Pharmacy, Department of Analytical Chemistry, 25240, Erzurum, Türkiye
[3]Atatürk Univesity, Medical Faculty, Department of Pharmacology, 25240, Erzurum, Türkiye
[4]Atatürk University, Vocational School of Health Services, Department of Pharmacy Services, 25240, Erzurum, Türkiye

ABSTRACT

Objective: *The goal of the present work was to investigate the antioxidant properties of the extracts of Hippophae rhamnoides L. and to determine their effects on liver toxicity in rats.*
Material and Method: *Metal chelation, reducing power and DPPH radical scavenging methods were used in the antioxidant activity analysis of extracts. The total phenolic content was determined using the folin-ciocalteu reagent. Plant extracts were administered orally to the rats at doses of 500 µg/kg for 2 days. Each animal group was composed of six female Albino Wistar rats with an average weight of 250 g. Microscopic examination was carried out to observe any pathological changes in the rat livers.*
Result and Discussion: *Water extract showed the highest radical scavenging activity (48.65%), reducing power (0.291 absorbance at 700 nm) and metal chelating (35.40%) at 1 mg/ml concentration. Histopathological studies showed that especially water extract reduced the severity of CCl_4-induced intoxication. Hippophae rhamnoides L. extracts were found to have antioxidant activity, and also Hippophae rhamnoides L. water extract was shown to be particularly effective in preventing liver damage.*
Keywords: *Antioxidant activity, DPPH, Hippophae rhamnoides L., rat liver toxicity, reducing power*

ÖZ

Amaç: *Bu çalışmanın amacı Hippophae rhamnoides L. meyve ekstraktlarının antioksidan özelliklerini araştırmak ve sıçanlarda oluşturulan karaciğer toksisitesi üzerindeki etkilerini belirlemektir.*
Gereç ve Yöntem: *Ekstraktların antioksidan aktivite analizinde metal iyonu kelatlama, indirgeme kuvveti ve DPPH radikal tutuklama yöntemleri kullanıldı. Toplam fenolik bileşik miktarları folin-ciocalteu ayıracı ile tespit edildi. Bitki ekstraktları 2 gün süre ile 500 µg/kg doz olacak şekilde*

* **Corresponding Author / Sorumlu Yazar:** Metin Çapa
e-mail / e-posta: metin.capa@gop.edu.tr, **Phone / Tel.:** +903562521616

Submitted / Gönderilme : 22.12.2023
Accepted / Kabul : 17.04.2024
Published / Yayınlanma : 10.09.2024

ratlara oral olarak verildi. Her bir hayvan grubu ortalama ağırlığı 250 g olan altı adet dişi Albino Wistar sıçanlardan oluşturuldu. Sıçanların karaciğerlerinde herhangi bir patolojik değişiklik olup olmadığını gözlemlemek için mikroskobik inceleme yapıldı.

Sonuç ve Tartışma: *Su ekstraktı, 1 mg/ml derişimde en yüksek radikal giderme aktivitesini (%48.65), indirgeme gücünü (700 nm'de 0.291 absorbans) ve metal iyonu kelatlamayı (%35.40) gösterdi. Histopatolojik çalışmalar özellikle su ekstraktının CCl_4 kaynaklı intoksikasyonun şiddetini azalttığını göstermiştir.*

Anahtar Kelimeler: *Antioxidan aktivite, DPPH, Hippophae rhamnoides L., indirgeme kuvveti, rat karaciğer toksisitesi*

INTRODUCTION

The use of plants for therapeutic purposes dates back to the earliest civilizations. About 5-10% of the 250.000 flowering plants grown worldwide have been studied for their active ingredients [1]. Some natural chemicals found in plants are gaining more attention and increasing demand for antioxidants that are not harmful to the human organism, as these plants have been consumed by humans and animals since ancient times [2]. *Hippophae rhamnoides* L. (HR) is a plant species belonging to the Elaeagnaceae family. HR contain more than 200 bioactive constituents, many vitamins, sterols, carotenoids, flavonoids, tocopherols, phenolics, lipids, citric acid, ascorbic acid, and more than 15 microelements (including Mn, Fe, B, F, Al, Ti, K, and so on) [3]. These biological compounds, particularly tocopherols and tocotrienols, would have an extensive range of biological activities, such as antioxidants [4,5]. Bioactive compounds in plants exert antioxidant activity through various mechanisms such as chain initiation prevention, transition metal chelation, peroxide decomposition, reducing capacity, and radical scavenging [6]. The active oxygen species hydroxyl radical ($HO^{\bullet}$), superoxide ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) are by-products of normal metabolism. These reactive oxygen species are known to have a detrimental effect. DNA damage is one of the most important factors in cancer formation [7]. Peroxidation of lipids is associated with diabetes, cancer, cardiovascular disease and aging as well as many degenerative disorders [8-11]. In conclusion, antioxidants are essential blockers of lipid peroxidation both in foods and in living cells. They are regarded as an important protection against oxidative damage. Synthetic antioxidants such as propyl gallate (PG), tertiary-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used in the food industry to prevent oxidation. They are cheaper and more effective. However, synthetic antioxidants such as BHA and BHT is considered to be carcinogenic for use in the protection of these nutrients is limited [12]. Therefore, especially in recent years, the use of plant sources of antioxidants has increased greatly [13]. These natural antioxidants have started to take an important place in scientific research [14]. Some herbs are known to have the ability to synthesize the well-known antioxidant compounds α-tocopherol (vitamin E), ascorbic acid (vitamin C) and carotenoids. A number of lipophilic compounds, including vitamin E, free fatty acids and retinol (vitamin A), have been shown to contribute to antioxidant protection [15]. These natural antioxidants can further contribute to human health by protecting DNA, proteins and membrane lipids from oxidizing damage in biological systems and preventing disease [16]. However, a very small amount of natural flora plants have been studied in terms of antioxidant determination. In this research it has been aimed to determine the antioxidant properties of the extracts of *Hippophae rhamnoides* L. (HR) plant in various solvent systems and the effects on liver toxicity in rats.

MATERIAL AND METHOD

Plant Material

The fruits of HR were collected from Tortum region (Eastern Anatolia sub. 750 m). The plant was described by Botanical Institutes, Ataturk University, Erzurum, Turkey. After the fruit parts of the plants were dried in a room environment, they were ground in a blender. Then the soxhlet apparatus was set up for extraction with diethyl ether and the extraction continued in the water bath until it became colorless. The plant residue remaining after extraction with ether was extracted with ethanol at 37ºC 3

times for 9 hours in a shaking water bath. Then, the remaining plant residue from ethanol extraction was extracted with distilled water at 37ºC 3 times for 9 hours in a shaking water bath. The resulting ether and ethanol extracts were filtered and the solvent was evaporated in the evaporator until thickening, dried in a nitrogen atmosphere and the residue was stored in a vacuum desiccator. After the water extracts were filtered, they were dried in a lyophilizer under 5μm-Hg pressure at -50ºC and stored in the freezer part of the refrigerator.

Animal Groups

The experiments were performed in accordance with the ethical guidelines approved by the Ethics Committee of the Laboratory Animal Facility (No. B.30.2.ATA.0.23.85-126-10-74). This study used a total of 30 Albino Wistar rats 12 weeks old. For the animal number calculation, a power analysis was performed using the G-Power program (v.3.1.9.7). In the power analysis of the study, it was calculated that a total of at least 10 experimental animals for 5 groups should be studied with a 5% margin of error (α err probe) and 80% power (1-β err probe). The number of animals for experiment in the groups was planned to be 6. Study of Abdel-Moneim et al. was used as a reference for the analysis [17]. Animals were provided from Medicinal and Experimental Application and Research Centre, Erzurum, Turkey (ATADEM). The animals were kept under standard laboratory conditions. They were given a regular cycle of 12 hours light and 12 hours dark at 24°C. Water and standard chow were provided to the animals. The experimental animals were Albino Wistar rats, all of which were female and their average weight was 250 g. CCl_4 was administered intraperitoneally to the animals in whom liver toxicity would be established, as 0.6 ml/kg subject [18]. Plant extracts HR was given orally as 500 μg/kg subjects. The HR extract doses to be applied were within the dose range determined according to the method of Ting et al. [19].On the first day, plant extracts or provender were given to the animals according to their diet. On the 2nd day, CCl_4 was given to the groups and 30 minutes later, plant extracts or provender were given according to their diet. On the 3rd day, excitation was performed with anesthesia under carbon monoxide (CO) gas. For pathological evaluation after excitation, tissue samples were taken in 10% formalin and transferred to Atatürk University Medical Faculty Pathology Laboratory. Liver tissues were collected and stored at -80°C until the assay was performed. In addition, blood samples were taken to be given to the Biochemistry Laboratory of Atatürk University Research Hospital for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination. Grouping of experimental animals is given below.

Group 1 was given a normal diet and served as a control.
Group 2 was given CCl_4 and served as a CCl_4 control.
Group 3 was given 0.5 mg/kg HR ether extract orally for 2 days and on the 2nd day CCl_4 was applied.
Group 4 was given 0.5 mg/kg HR ethanol extract orally for 2 days and on the 2nd day CCl_4 was applied.
Group 5 was given 0.5 mg/kg HR water extract orally for 2 days and on the 2nd day CCl_4 was applied.

Determination of Total Phenolics

Total phenolic content quantification in the extracts was carried out by Folin-Ciocalteu Reagent (FCR) [20]. 200 μl of sample (extract) solution was taken and the volume was completed to 500 μl with 0.3% HCl. 200 μl was taken from the mixture and added to 4 ml of 2% Na_2CO_3 solution. After 2 minutes, 200 μl FCR was added and after 30 minutes, the absorbance was measured at 750 nm. 2% Na_2CO_3 solution was used as a blank. The results are given as the equivalent amount of gallic acid per extract (EAG/g extract).

DPPH Radical Scavenging Activity

Free radical scavenging activities of HR plant extracts, α-tocopherol, BHA and ascorbic acid were performed using DPPH [21]. DPPH ethanol solution at a concentration of 100 μg/ml was prepared. In the method study, after taking 1 ml of plant extract solution and putting it in a test tube, 3 ml DPPH solution was added on it and kept in the dark for 30 minutes. The mixture was vortexed and its absorbance was read against ethanol at a wavelength of 517 nm. The antioxidant-free solution was used

as a control. The lower absorbance indicates the higher radical scavenging ability of the extracts. The formula shown below was used to compute the percent of radical scavenging activity.

% Radical Scavenging Activity: [(A_0-A_1/A_0) x 100]
A_1: Absorbans of mixture.
A_0: Absorbans of DPPH Solution.

Determination of Reducing Power

The extracts' reducing power was determined employing the Oyaizu method [22]. 1 ml of sample was placed in the test tube, and 2 ml of 0.2 mM phosphate buffer (pH 6.6) and 2 ml of potassium ferri cyanide [1% $K_3Fe(CN)_6$] were added and vortexed. For 20 minutes, the mixture was maintained in a 50 °C water bath. 2 ml of a 10% solution of trichloroacetic acid (TCA) was added, and the mixture was then centrifuged for 10 minutes at 2500 rpm. At the end of the centrifuge, 2 ml was taken from the clear part and 2 ml of pure water was added. Soon after waiting for 10 minutes, the absorbance of the mixture at 700 nm was measured by adding 0.5 ml $FeCl_3$ (0.1%). An increase in absorbance indicates an increased reducing power. Vitamin E and vitamin A were used as a control.

Ferrous Chelating Activity

Fe^{2+} chelating activity of the extracts was performed according to the method of Dinis et al. [23]. 10 mg/ml stock solution of the extracts was prepared in ethanol. Working solutions were prepared from this solution. 1 ml of each working solution was taken and 3.7 ml of ethanol was added on it. Then, 0.1 ml $FeCl_2$ (2mM) and 0.2 ml 5mM ferrosine were added, respectively. After 10 minutes, each sample's absorbance was compared to a $FeCl_2$ and ferrosine-free blank at 562 nm. The absorbance of each sample was measured against the $FeCl_2$ and ferrosine-free blank at 562 nm after 10 minutes. Ethylenediaminetetraacetic acid (EDTA) solution was used as reference. % Metal ion chelation was calculated by the formula below.

Chelating %: (A_0-A_1 / A_0) x100
A1: Absorbance value of the sample; A0: Absorbance value of the control

Histopathological Studies

Normal saline (physiologic saline 0.9%) was used to wash the excised liver tissue pieces. These sections were then reserved for histopathological studies. The tissues were fixed in buffered neutral formalin (%10). Then dehydrated in gradual ethanol, cleared in xylene and embedded in paraffin. Hematoxylin and eosin (H-E) stains were used to stain the prepared sections. Histopathology changes such as mononuclear cell infiltration, necrotic area, hemorrhage, dilation of sinusoids, hepatocellular degeneration and vascular congestion were studied microscopically in the sections [24].

ALT (alanine amino transaminase) and AST (aspartate amino transaminase) Analyzes of Experimental Groups

Blood samples taken from experimental animal groups were delivered to Atatürk University Research Hospital biochemistry laboratory. Results were expressed in International Units per liter (IU/l).

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Where appropriate, the differences between the groups were evaluated using the Student t test an alpha level of 0.05. Pearson correlation coefficients and p-values were used for correlations and their significance. G-Power software (v3.1.9.7) was used for Power Analysis.

RESULT AND DISCUSSION

Determination of Total Phenolics

In the determination of phenolic compounds, the results are given as equivalent amount of gallic acid. For this, the concentration-absorbance graph of the standard gallic acid was used (Figure 1). The

order of phenolic compound amount of HR plant extracts was 62.43 mg for ether extracts, 41.94 mg for water extracts and 18.94 mg for ethanol extracts (Table 1). Phenolic compounds are secondary metabolites commonly found in plants [25]. The factors caused by phenolic compounds are antioxidant properties, nutritional values and colors of foods. Phenolic compounds in plants exhibit antioxidant activity, protecting cells from free radical oxidative damage [26]. Phenolic compounds are also known to have antimutagenic, antitumor and antibacterial properties [27]. Previous studies have shown that polyphenolic compounds are compatible with antioxidant activity and have an important role in stabilizing the oxidation of lipids [28]. In the studies of Varshneya et al. [29], Phenolic compounds in extracts of HR pulp were determined as mg gallic acid equivalent, and these values for methanol, AME (70:30 water:methanol, v/h) and water extracts were 78.12, 84.28 and 19.96 mg EAG/g extract, respectively. In our study, it was determined that the water extract value of this plant was higher (41.94) and the ethanol extract value was lower (18.94).

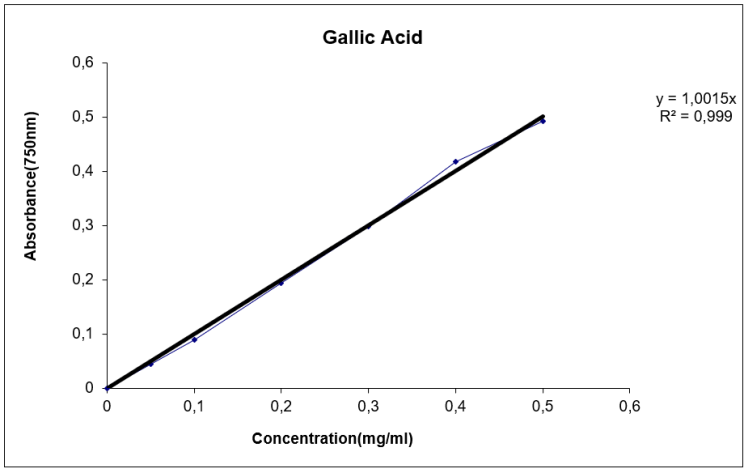

Figure 1. Standard curve of gallic acid

Table 1. Phenolic compound amounts of HR extracts as equivalent gallic acid

Plant Extract	HR Ether Extract	HR Ethanol Extract	HR Water Extract
Total Phenolics (mg EAG/g extract)	62.73±0.927	18.94±0.412	41.94±0.854

*Values were given as the mean ± SD (n=6)

DPPH Radical Scavenging Activity

Ether, ethanol and water extracts of *Hippophae rhamnoides* L. (HR) were utilized at concentrations ranging from 100 to 1000 g/ml. The results were presented in Table 2. The highest value was HR water extract of the plant (51.42%). BHA, ascorbic acid and α-tocopherol produced radical scavenging activities of 95.19%, 93.81% and 83.43%, respectively, at 100 µg/ml concentration.

DPPH radical scavenging activity was found in a concentration-dependent manner in all extracts ($p<0.05$). Finally, the % DPPH radical scavenging activity of the different extracts was calculated and presented in Table 2. The DPPH scavenging method applied in our study is very common among spectrophotometric methods used to determine the antioxidant capacity of extracts and pure compounds. This method is fast, sample analysis requires little time and does not require expensive reagents and devices. DPPH, which is a resistant free radical, takes electron or hydrogen radical from the reaction medium and turns into a durable diamagnetic molecule. For this reason, the DPPH radical is used as a substrate to determine the antioxidative properties of antioxidants. This durable chromogen free radical has high sensitivity [30]. DPPH method is a modern method that allows analysis of samples with different characteristics. A large number of pure compounds, juices, wines, tea and other extracts were

analyzed by applying this chromogen radical [31,32]. DPPH method is a modern method that allows analysis of samples with different characteristics. A large number of pure compounds, juices, wines, tea and other extracts were analyzed by applying this chromogen radical. With a DPPH concentration of 0.0004%, the IC_{50} value, which is the concentration that inhibits the DPPH radical by 50%, was determined to be 70.91 µg/ml in the study of Shivapriya et al. [33]. DPPH concentration in this study is 25 times lower than in our study. In another DPPH (0.2mM) scavenging activity study conducted by Yogendra Kumar et al. [34]. In HR leaf extracts, an activity of 47.25% was found at 0.2 mg/ml extract concentration. This value is higher than our study values (23.5-28.7%). In the studies of Varshneya et al. [29], the IC_{50} value of DPPH (0.1mM) radical removal of the water-methanol extract of the plant was determined as 143.33 µg/ml. The DPPH concentration in this study is 2/5 of that in our study. However, in our study, the IC_{50} value of the water extract of the plant was calculated by linear regression analysis and was found to be 872.56 µg/ml. Total phenolic content and DPPH radical scavenging activity were found in this increasing order, HR ethanol < HR water extract. This finding suggested that phenolic content and the ability to scavenge DPPH radicals may be closely related.

Determination of Reducing Power

The Oyaizu method was used to determine the reducing power of HR extracts [22]. The amounts of the extracts were taken in the concentration range of 100-1000 µg/ml. In our study, it was determined that all extracts have a certain reducing force activity. Results are given in Table 2. The highest activity is HR plant was detected in water extract. Retinol and α-tocopherol showed a reducing power of 0.619 and 0.245 respectively, at 100 µg/ml concentration.

Table 2. Radical scavenging activity (%) and reducing power of HR extracts

	Radical Scavenging Activity (%)			**Reducing Power (Absorbance at 700 nm)**		
Simples Concentrations	**Ether exctract**	**Ethanol exctract**	**Water exctract**	**Ether exctract**	**Ethanol exctract**	**Water exctract**
100 µg/ml	21.85±0.29	25.21±0.51	27.14±0.42	0.021±0.001	0.006±0.001	0.093±0.001
200 µg/ml	23.51±0.33	25.46±0.43	28.73±0.31	0.026±0.001	0.058±0.001	0.102±0.002
400 µg/ml	25.32±0.28	27.63±0.56	35.25±0.44	0.070±0.001	0.069±0.001	0.146±0.003
600 µg/ml	26.46±0.41	30.09±0.60	42.11±0.75	0.104±0.002	0.085±0.001	0.195±0.004
800 µg/ml	28.64±0.51	30.54±0.49	45.37±0.51	0.122±0.002	0.097±0.002	0.244±0.003
1000 µg/ml	28.39±0.42	30.61±0.59	48.65±0.94	0.154±0.002	0.109±0.002	0.291±0.005

*Values were expressed as the mean ± SD (n=6)

The reducing power of plant extracts was determined by evaluating the conversion of Fe^{3+} to Fe^{2+} using the Oyaizu method [22]. The reducing power values exhibited by the compounds are a measure of their electron donation ability [35,36]. It is known that polyphenolic compounds of plant origin have good electron and hydrogen atom donating properties. For this reason, polyphenols in plant extracts have the ability to terminate or slow down chain reactions with free radical mechanisms. Yen and Duh showed that there is a consistent correlation between the reducing forces and antioxidant activity values of methanol extracts containing yen and duh polyphenols [28]. On the other hand, Gordon stated that reductants show antioxidant activity by giving hydrogen atoms to the free radical chain [37]. The values of the reducing forces of HR extracts, ascorbic acid and tocopherol determined depending on the concentration are given in Table 2. The reducing power of the extracts at a concentration of 1 mg/ml are as follows: HR water extract (0.291)> HR ether extract (0.154)> HR ethanol extract (0.109) ($p<0.05$). Retinol and tocopherol showed reducing power of 0.619 and 0.245 respectively, at a concentration of 0.1 mg/ml. It was observed that HR water extracts showed higher reducing power at 1 mg/ml. The reduction force results of the supercritical carbon dioxide extracts of Hung et al. [19]. HR plants were measured at 700 nm in the 0.42-8.32 mg/ml concentration range and the absorbance values were recorded. The absorbance value is 0.11 at 0.42 mg/ml concentration. In our study, the absorbance value (0.146) of HR water extract at a concentration of 0.4 mg/ml is higher. For this reason, it can be said that these extracts tested have the ability to donate electrons, interact with free radicals and turn

them into more stable products, and have the ability to terminate the radical chain reaction. In addition, since the reducing power depends on the ability to deliver protons, it can be thought that there is a relationship between radical scavenging activity and reducing power. In this study, it is seen that there is a strong relationship between reducing power and radical scavenging activity (r>0.952).

Ferrous Chelating Activity

Fe^{2+} ions chelating activity of ether, ethanol and water extracts of the HR plant was performed using to the method of Dinis et al. [23]. Ferrous chelating ability of the extracts were investigated depending on the concentration. The ethanol extract demonstrated the highest ion chelating activity (56.68%) among HR plant extracts. Results are given in Table 3 and Figure 2. The Ferrous chelating activity of EDTA at 5 mg/ml concentration was 99.8%.

Table 3. Fe^{2+} Ions Chelating Activity (%) of HR extracts

Simples/Concentrations	HR Ether exctract	HR Ethanol exctract	HR Water exctract
1 mg/ml	6.53±0.12	13.56±0.29	35.40±0.61
2 mg/ml	14.07±0.18	16.58±0.36	37.88±0.74
3 mg/ml	17.28±0.24	33.86±0.37	38.13±0.55
4 mg/ml	19.19±0.35	37.48±0.59	40.58±0.60
5 mg/ml	22.31±0.49	56.68±0.86	42.81±0.81

*Values were expressed as the mean ± SD (n=6)

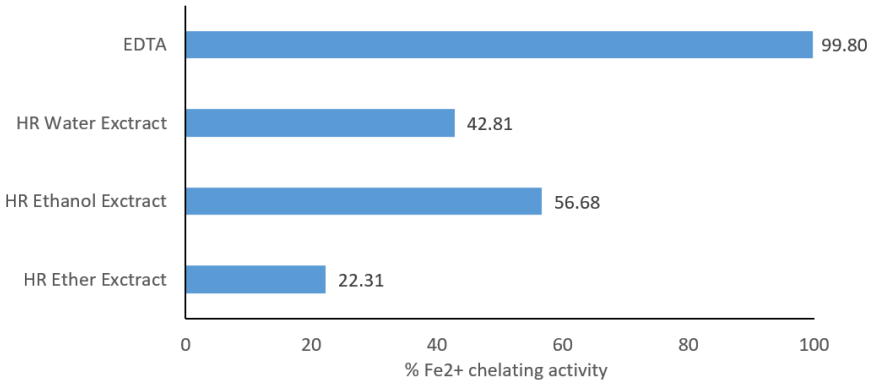

Figure 2. HR extracts and EDTA Ferrous chelating abilities at 5 mg/ml concentration

The chelating activity of metal ions is an important factor in antioxidant effect. Because Fe^{+2} ions are the most effective prooxidants [38]. Polyphenols can chelate metal ions with prooxidant properties such as iron and thus prevent the formation of free radicals caused by prooxidants [39]. Iron ions can increase lipid peroxidation by converting lipid peroxides to peroxyl and alkoxyl radicals via the Fenton process and can promote lipid peroxidation. By accepting hydrogen, these radicals can initiate lipid peroxidation chain reactions [40]. The metal ion chelating activity of the extracts is very important as they enable the capture of transition metals acting as catalysts in lipid peroxidation. It is known that even traces of metal ions accelerate the oxidation process. Therefore, compounds showing low chelating effect also have an importance in the formation of antioxidant activity [41]. It was determined that ether, ethanol and water extracts of HR plant at concentrations of 1 and 5 mg/mL showed 6.53-22.31%, 13.56-56.68% and 35.40-42.81% chelating effect, respectively. EDTA was used as a standard metal chelator in our study. At a concentration of 1 mg/ml, EDTA showed 99.5% activity. The metal chelating effect of HR extracts and EDTA decreased in the order: EDTA >HR ethanol extract> HR water extract > HR ether extract. Among HR extracts HR ethanol extract resulted the most active one (56.68%). Hung et al. found the metal ion chelation activity of the core parts of the HR plant as 7.74-38.50%, respectively, at concentrations of 0.92-18.3 mg/ml [19]. In our study, all values except HR ether extract were higher

(13.56-56.68%). The scavenging properties of phenolic compounds against radicals and reactive oxygen species such as singlet oxygen, hydroxyl radicals and free superoxide radicals make them highly effective antioxidants. [42]. A positive correlation between metal ion chelation activity with radical scavenging activity and was found, as indicated by the coefficient of determination (r=0.991). A positive correlation was found between metal ion chelation activity with radical scavenging activity and reducing power. The coefficients of correlation (r) were 0.991 and 0.889, respectively.

Histopathological Studies

Histopathology revealed liver damage in control and CCl_4-treated rats. Photomicrographs of hematoxylin-eosin-stained liver tissues are shown in Figure 3 a-f. The livers were cut into sections and stained with hematoxylin-eosin using standard techniques (200X). Histopathologic examinations such as hepatocellular degeneration, hemorrhage, mononuclear cell infiltration, vascular congestion, dilation of sinusoids, and necrotic area were recorded and scored in Table 4.

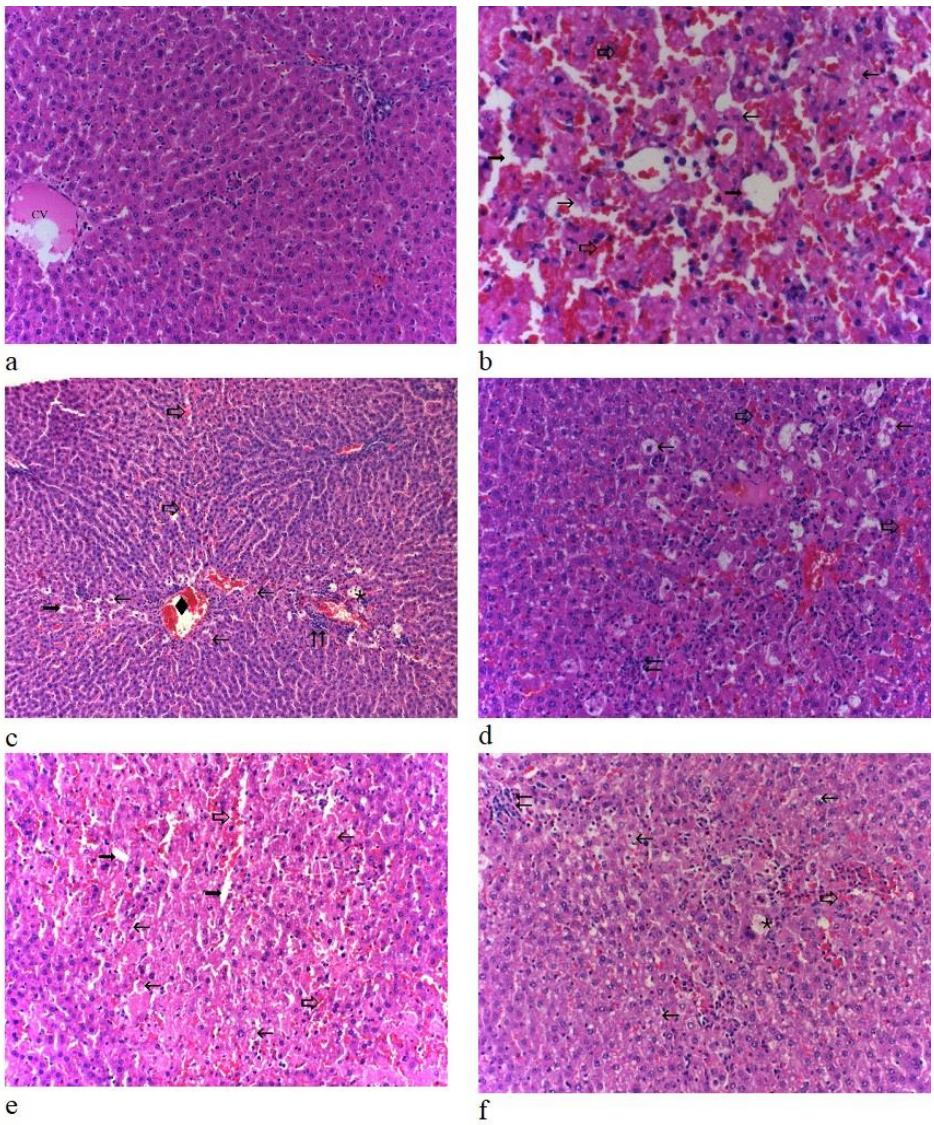

Figure 3. Effect of HR extracts on rat liver toxicity with CCl_4. (a) Control; (b-c) CCl_4 control; (d) HR Ether extract (0.5 mg/kg) + CCl_4; (e) HR Ethanol extract (0.5 mg/kg) + CCl_4; (f) HR Water extract (0.5 mg/kg) + CCl_4. Photomicrographs showing central vein (cv), hemorrhage(⇨), mononuclear cell infiltration (⇆), vascular congestion(♦), dilation of sinusoids (➞), hepatocellular degeneration (←) and necrotic area (✶)

Table 4. Histopathologic changes grading in liver sections of HR extract treated rats

Groups	Mononuclear cell infiltration	Hemorrhage	Hepatocellular degeneration	Dilation of sinusoids	Vascular congestion	Necrotic area
Control	-	-	-	-	-	-
CCl_4 Control	++	+++	+++	+++	+++	++
HR Ether Extract + CCl_4	+	++	++	++	++	++
HR Ethanol Extract + CCl_4	++	+++	++	+++	++	++
HR Water Extract + CCl_4	+	+	+	+	+	+

*Features were rated in these categories: severe (+++), moderate (++), mild (+) and none

Liver damage of CCl_4 treated rats were revealed by histopathological examinations. Photomicrographs of liver tissues stained with hematoxylin-eosin are shown in Figure 3. In the control group (Group 1), hepatocytes had normal architecture. Severe infiltration, hemorrhage, hepatocellular degeneration, dilation of sinusoids, vascular congestion and necrotic area were found in rats (Group 2) 24 hours after CCl_4 administration (Figure 3b-c). According to these results, it was determined that CCl_4-induced liver toxicity was reduced to a certain extent in rats fed with all HR extracts. Specially pretreatment of 0.5 mg/kg body weight of HR water extract reduced the severity of liver intoxication caused by CCl_4 (Figure 3f). These results clearly demonstrate the protection provided by HR water extract. It was observed that rats treated with HR water extract were less affected by CCl_4-induced toxicity.

ALT and AST Analyzes of Experimental Groups

Blood samples taken from animal groups were given to Ataturk University Research Hospital Biochemistry Laboratory for ALT and AST determination from serum. The results are presented in IU/l in Table 5.

Table 5. Effects of diets containing HR extracts on alanine amino transaminase (ALT) and aspartate amino transaminase (AST)

Item	ALT [a] (IU/l)	AST [b] (IU/l)
Group 1	45.12 ± 7.94	163.14±8.45
Group 2	516.31±24.14	2858.26±156.17
Group 3	936.72±48.36	2316.35±120.51
Group 4	841.77±39.61	3849.68±189.56
Group 5	318.48±17.97	1002.39±64.88

*Values were expressed as the mean ± SD (n=6)
[a] $p<0.05$
[b] $p<0.05$

Serum AST and ALT activities were evaluated to detect liver damage. ALT and AST values are very high in all animal groups treated with CCl_4 except the positive control group. There was a significant difference between the CCl_4 control group and the serum samples taken from the groups fed with HR extracts and administered CCl_4 ($p<0.05$).

To sum up, the data of the present study submits that extracts of HR have antioxidants. Our findings indicate that the hepatoprotective effects of HR water extracts can be attributed to the fact that they contain several components with potentially healthy biological properties, such as unsaturated fat, α-tocopherol, retinol, and carotene. Therefore, HR water extract may be effective as a hepatoprotective substance against chemically induced hepatotoxicity *in vivo*.

AUTHOR CONTRIBUTIONS

Concept: M.C., Y.K.; Design: M.C., Y.K.; Control: M.C., Y.K.; Sources: Y.K.; Materials: M.C.,

Y.K., E.C.; Data Collection and/or Processing: M.C., E.C., M.A.; Analysis and/or Interpretation: M.C., E.C., M.A.; Literature Review: M.C., Y.K.; Manuscript Writing: M.C.; Critical Review: M.C., Y.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The experiments were performed in accordance with the ethical guidelines approved by the Atatürk University Ethics Committee of the Laboratory Animal Facility (No. B.30.2.ATA.0.23.85-126-10-74).

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 803-810, 2024
Doi: 10.33483/jfpau.1441955

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE SIMULTANEOUS SPECTRAL DETERMINATION OF CANDESARTAN CILEXETIL AND HYDROCHLOROTHIAZIDE IN TABLETS USING THE TRIVARIATE CLASSICAL LEAST SQUARES METHOD

ÜÇ DEĞİŞKENLİ KLASİK EN KÜÇÜK KARELER YÖNTEMİ KULLANILARAK TABLETLERDE KANDESARTAN SİLEKSETİL VE HİDROKLOROTİAZİDİN EŞZAMANLI SPEKTRAL TAYİNİ

Asiye ÜÇER[1,2], Özgür ÜSTÜNDAĞ[2], Uğur SAKLI[2], Erdal DİNÇ[2]*

[1]Ankara Yıldırım Beyazıt University, Faculty of Pharmacy, Department of Analytical Chemistry, 06010, Etlik, Ankara, Türkiye
[2]Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06560, Yenimahalle, Ankara, Türkiye

ABSTRACT

Objective: *Candesartan cilexetil (CDS) is a member of the sartan group of drugs and is widely used to lower blood pressure. Hydrochlorothiazide (HCT) is the most commonly used diuretic group of drugs and is prescribed together with candesartan cilexetil in cases where blood pressure cannot be reduced. Pharmaceutical preparations containing these two active compounds in combination are preferred today to provide a more effective pharmacological effect. Therefore, it is of great importance to determine the quantities of these combined pharmaceutical preparations with new analytical methods that are fast, easy, and sensitive in quality control and routine analysis. In this study, a new trivariate classical least squares calibration method (TCLS) was developed for the simultaneous quantification of candesartan cilexetil (CDS) and hydrochlorothiazide (HCT) in binary mixtures and commercial tablets without using a preliminary separation step.*

Material and Method: *CDS and HCT compounds were kindly donated by National Pharm Ind., Turkey. HPLC-grade methanol (J.T. Baker, Netherlands) was used as a solvent for the spectrophotometric analysis. In the application of the TCLS method, the determination and quantification of CDS and HCT were carried out using UV spectrophotometric measurements with 1 cm quartz cells in the 200-310 nm spectral region (slit range 2 nm). The newly developed TCLS method was tested using a validation set consisting of eight synthetic mixture solutions within the working ranges of 4.0-20.0 µg/ml for CDS and HCT. Simultaneous quantification analyses of CDS and HCT were performed on ATACAND PLUS® Tablet supplied by Astra Zeneca İlaç Ltd Şti.*

Result and Discussion: *The method is based on the application of TCLS to the absorbance measurements at three different wavelength points (223.5, 240.0, and 268.5 nm). The absorptivity values ($\mu g^{-1}mlcm^{-1}$) of pure CDS and pure HCT were $6.67x10^{-2}$, $2.76x10^{-2}$, $2.33x10^{-2}$, and $11.41x10^{-2}$, $0.46x10^{-2}$, $6.42x10^{-2}$ at the selected wavelengths, respectively. Recovery values and relative standard deviation values were calculated as 97.2% and 1.61% for CDS and 99.7% and 3.67% for HCT, respectively. This method was successfully applied to the spectrophotometric quantitative analysis of tablets containing CDS and HCT, and then, a good agreement was reported.*

* **Corresponding Author / Sorumlu Yazar:** Erdal Dinç
e-mail / e-posta: dinc@ankara.edu.tr, **Phone / Tel.:** +903122126805

Submitted / Submitted : 23.02.2024
Accepted / Kabul : 22.04.2024
Published / Yayınlanma : 10.09.2024

Keywords: *Angiotensin II receptor antagonists, candesartan cilexetil, hydrochlorothiazide, quantitative tablet analysis, trivariate classical least squares method*

ÖZ

Amaç: *Kandesartan sileksetil (CDS), sartan ilaç grubuna ait olup, kan basıncını düşürmek amacıyla yaygın olarak kullanılmaktadır. Hidroklorotiazid (HCT) en sık kullanılan diüretik ilaç grubudur ve kan basıncının düşürülemediği durumlarda kandesartan sileksetil ile birlikte reçete edilir. Bu iki aktif bileşiğin kombinasyon halinde bulunduğu farmasötik preparatlar, günümüzde daha etkili bir farmakolojik etki sağlamak amacıyla tercih edilmektedir. Bu nedenle, bu kombine farmasötik preparatların miktarlarının, kalite kontrol ve rutin analizlerde hızlı, kolay ve hassas yeni analitik yöntemlerle belirlenmesi büyük önem taşımaktadır. Bu çalışmada, ikili karışımlarda ve ticari tabletlerde kandesartan sileksetil (CDS) ve hidroklorotiyazidin (HCT) bir ön ayırma adımı kullanılmadan eş zamanlı ölçümü için yeni bir üç değişkenli klasik en küçük kareler kalibrasyon yöntemi (TCLS) geliştirildi.*

Gereç ve Yöntem: *CDS ve HCT bileşikleri National Pharm Ind., Türkiye tarafından bağışlanmıştır. Spektrofotometrik analiz için solvent olarak metanol (J.T. Baker, Hollanda) kullanıldı. TCLS yönteminin uygulanmasında CDS ve HCT'nin belirlenmesi ve nicelendirilmesi, 200-310 nm spektral bölgede (slit aralığı 2 nm) 1 cm'lik kuvars hücrelerle UV spektrofotometrik ölçümler kullanılarak gerçekleştirildi. Yeni geliştirilen TCLS yöntemi, CDS ve HCT için 4.0-20.0 µg/ml çalışma aralıklarında sekiz sentetik karışım çözeltisinden oluşan bir doğrulama seti kullanılarak test edildi. Astra Zeneca İlaç Ltd Şti tarafından sağlanan ATACAND PLUS® Tablet üzerinde CDS ve HCT'nin eş zamanlı miktar tayini analizleri yapıldı.*

Sonuç ve Tartışma: *Yöntem, TCLS'nin üç farklı dalga boyu noktasında (223.5, 240.0 ve 268.5 nm) absorbans ölçümlerine uygulanmasına dayanmaktadır. Saf CDS ve saf HCT'nin absorptivite değerleri ($\mu g^{-1}mlcm^{-1}$) seçilen dalga boylarında sırasıyla $6.67x10^{-2}$, $2.76x10^{-2}$, $2.33x10^{-2}$ ve $11.41x10^{-2}$, $0.46x10^{-2}$, $6.42x10^{-2}$ idi. Geri kazanım değerleri ve bağıl standart sapma değerleri CDS için sırasıyla %97.2 ve %1.61, HCT için ise %99.7 ve %3.67 olarak hesaplandı. Bu yöntem, CDS ve HCT içeren tabletlerin spektrofotometrik kantitatif analizine başarıyla uygulandı ve ardından iyi bir uyum olduğu bildirildi.*

Anahtar Kelimeler: *Anjiyotensin II reseptör antagonistleri, hidroklorotiyazid, kandesartan sileksetil, kantitatif tablet analizi, üç değişkenli klasik en küçük kareler yöntemi*

INTRODUCTION

Angiotensin II receptor antagonists are a group of drugs, also known as the sartan family, used in the treatment of hypertension [1]. Angiotensin II, which is the basic peptide of the renin-angiotensin system, which has important physiological effects on blood pressure regulation and water and salt homeostasis, plays a key role in the cardiovascular system by playing an important role in the development of physiopathological events such as hypertension, heart and kidney failure, and atherosclerosis. Candesartan cilexetil (CDS) (see Figure 1a) is a member of this group of medicines. It helps reduce blood pressure by relaxing and widening blood vessels. Candesartan can be used to treat hypertension, left ventricular hypertrophy, isolated systolic hypertension, and diabetic nephropathy, and is also used as an alternative agent in the treatment of heart failure, myocardial infarction, systolic dysfunction, and coronary artery disease [2,3]. Hydrochlorothiazide (HCT) (see Figure 1b) belongs to a group of medicines called 'diuretics'. It is used to treat swelling due to hypertension and fluid accumulation, as well as to treat renal tubular acidosis and diabetes insipidus, and to reduce the risk of kidney stones in those with high calcium levels in the urine [4]. It helps remove water and salts such as sodium from the body through urine. This causes blood pressure to decrease. These two active ingredients are prescribed in cases where blood pressure cannot be reduced with candesartan cilexetil (CDS) or hydrochlorothiazide (HCT) alone.

It is of great importance to determine the quantities of these combined pharmaceutical preparations, which are preferred today to provide a more effective pharmacological effect, with new analytical methods that are fast, easy, and highly sensitive in quality control and routine analysis. In the literature, chromatographic separation methods such as liquid chromatography (LC) [5], high-performance liquid chromatography or ultra-performance liquid chromatography (HPLC/UPLC) [6-8],

capillary electrophoresis (CE) [9-10] and micellar electrokinetic chromatography [11], which consist of high-tech and expensive equipment for the analysis of complex samples are used. These expensive and complex methods may not always produce the expected results in the analysis processes. In addition, another disadvantage in the application of these methods is that the optimization of experimental conditions and analysis are time-consuming. On the other hand, it is noteworthy that spectrophotometric methods are also used extensively in the literature [12-13]. However, quantitative analysis of combined commercial pharmaceutical preparations is not possible with conventional spectral analysis methods because the active substances give interfering spectra in the same region [14]. In such cases, derivative spectrophotometry and its modified versions were used for a simple and rapid quantitative determination without separation steps [15-19]. However, derivative spectrophotometry requires signal-processing treatments, which need a long analysis period, to get derivative signals of zero-order spectra. Additionally, these conventional derivative methods do not yield successful results in their applications due to their disadvantages such as interference of main peaks and noise peaks, and decrease in signal/noise ratio, especially at high derivative degrees. To eliminate the drawbacks of the spectral derivative methods, numerical methods or multivariate spectral methods, such as direct absorbance measurement techniques based on a set of two or more wavelength points, provide an alternative way to quantitatively resolve complex mixtures containing two or more active compounds.

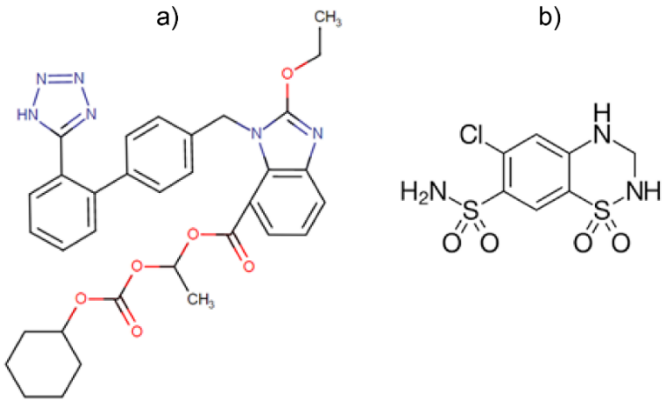

Figure 1. a) Candesartan cilexetil (CDS), **b)** Hydrochlorothiazide (HCT)

In this study, a new TCLS approach was developed, for the first time, for the quantitative analysis of a commercial pharmaceutical tablet consisting of candesartan cilexetil (CDS) and hydrochlorothiazide (HCT). The developed and validated method was successfully applied to both synthetic mixtures and commercial pharmaceutical tablets.

MATERIAL AND METHOD

Apparatus and Software

All UV spectrophotometric data were collected using a Shimadzu UV-1601 (Kyoto, Japan) double-beam UV-VIS spectrophotometer with 1 cm quartz cells. UV absorbance spectra of the samples and standard solutions were plotted in the spectral region of 200-310 nm (slit range 2 nm). After the obtained data were transferred to the Shimadzu UV computer software, the Matlab (MathWorks, Natick, MA, USA) program was used in the application of the TCLS approach to the spectra, statistical analysis, and regression analysis. All graphics and figures were drawn with Matlab. All prepared solutions were filtered with a Sartarius Minisart disposable filter with a pore size of 0.2 µm.

Chemicals and Reagents

CDS and HCT compounds were kindly donated by National Pharm Ind., Turkey. HPLC-grade methanol (J.T. Baker, Netherlands) was used as a solvent for the spectrophotometric analysis. All

samples were prepared fresh daily and stored in a dark place and a refrigerator during the analysis.

Standard, Calibration, and Validation Solutions

Stock solutions of CDS and HCT were prepared individually by dissolving 25.0 mg of active compounds in methanol in a 100 ml calibrated flask and made up to the mark with methanol. All standard and validation samples were prepared from these stock solutions. In the application of the spectral least squares method, standard solutions were prepared in the concentration range of 6.0-18.0 µg/ml for CDS and the concentration range of 4.0-16.0 µg/ml for HCT. A validation set of eight different concentrations containing these two compounds was prepared within the working ranges of 4.0-20.0 µg/ml for CDS and HCT.

Preparation of Samples

In the first application of the TCLS method in commercial pharmaceutical tablet analysis, ten tablets of ATACAND PLUS® Tablet (Astra Zeneca İlaç Ltd. Şti.) were precisely weighed and the amount corresponding to one tablet was calculated after being thoroughly powdered in a mortar. It was dissolved in methanol in a 100 ml volumetric flask. The solution content was mixed with a mechanical stirrer for 30 min and filtered through a 0.20 µm pore size membrane filter. For the analysis, 1.0 ml of the filtered solution was transferred to a 100 ml volumetric flask and the volume was made up with the same solvent.

TCLS Method and Its Application

Absorption spectra of solutions and binary mixtures prepared at different CDS and HCT concentrations were recorded. Absorptivity (a) values were calculated using absorbances measured at 223.5, 240.0 and 268.5 nm for each of the compounds in the binary mixture. Using the absorptivity (a) value, a system of equations with two unknowns for compounds in a binary mixture can be written as follows:

$$A = a.l.C,\ l\ (optical\ path\ lenght) = 1.0\ cm \quad (1)$$

Where a denote absorptivity, A denote absorbance, and C denote concentration (µg/ml).

$$A_1 = a_1C_1 + \beta_1C_2 \quad (2)$$

$$A_2 = a_2C_1 + \beta_2C_2 \quad (3)$$

$$A_3 = a_3C_1 + \beta_3C_2 \quad (4)$$

Where A_1, A_2 and A_3 denotes the absorbances of solutions of synthetic mixtures of CDS and HCT, and represent the calculated a and β absorptivity values at λ_1, λ_2 and λ_3 for CDS and HCT, respectively. C_1, and C_2 are the concentrations of CDS and HCT, respectively. The subscripts 1, 2 and 3 refer to λ_1 (223.5 nm), λ_2 (240.0 nm) and λ_3 (268.5 nm), respectively.

When written according to the matrix notation given below, the above set of equations (2, 3, and 4) greatly simplifies the matter and easily solves the equations with two unknowns:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \end{bmatrix} = \begin{bmatrix} a_1 & \beta_1 \\ a_2 & \beta_2 \\ a_3 & \beta_3 \end{bmatrix} * \begin{bmatrix} C_1 \\ C_2 \end{bmatrix} \quad (5)$$

Here, the quantification of CDS and HCT was carried out from the concentrations corresponding to the absorbance of the samples in Equation 5 at the wavelengths of λ_1, λ_2 and λ_3.

Based on Equation 5, the concentration of each compound in the binary mixture can be calculated using the multiplication of the inverse of the coefficient matrix by the absorbance measurements of samples.

RESULT VE DISCUSSION

TCLS Method and Its Application

In the spectral analysis of two-component mixture systems, the main problem is the overlapping spectral bands in the working wavelength region as in our study (see Figure 2). To solve this analytical issue, we focused mainly on the development of the new spectral numerical method, which is based on the use of the absorbance measurement at three different wavelength points, for the simultaneous quantitative analysis of two-component mixtures. This article involves verifying the feasibility and validity of a fast, easy, reliable TCLS method for the simultaneous quantification of CDS and HCT in synthetic mixtures and commercial tablets. The UV spectra of standard CDS and HCT solutions and their synthetic mixtures were plotted in the wavelength region of 200-310 nm.

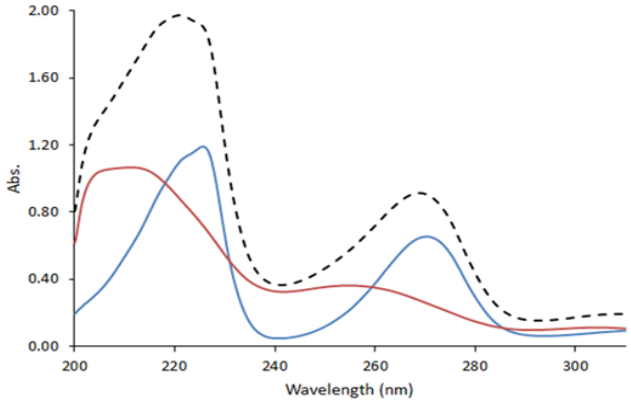

Figure 2. Absorption spectra of 10 µg/ml CDS (———) and 12 µg/ml HCT (———) solutions and commercial tablet solution (– – ·) (in methanol) containing CDS and HCT

The selection of higher sensitive wavelengths is an important parameter for the development of a multivariate spectral method for resolving complex mixtures. In this context, three different wavelengths for CDS and HCT, which correspond to two maxima of wavelength (223.5 and 268.5 nm) and a minimum (240.0 nm) were selected for the application of the new TCLS method to the analysis of CDS-HCT mixtures and pharmaceutical preparations.

In the applied method, the absorbances of the drug standards prepared in the concentration range where Beer's law is valid (6.0-18.0 µg/ml for CDC and 4.0-16.0 µg/ml for HCT) were recorded on the UV spectrophotometer at three different wavelengths (223.5, 240.0 and 268.5 nm). By using the matrix calculation approach explained in the "Experimental Section", the simultaneous quantification of two active substances (CDS and HCT) is possible by direct measurement of absorbances at 223.5, 240.0 and 268.5 nm in the UV spectrum. For both CDS and HCT, the absorptivities of the compounds corresponding to the absorbance values at three wavelengths were calculated using Equation 1. The averages of the calculated absorptivities for each concentration in the calibration set of each compound were presented in Table 1.

Table 1. Calculated absorptivities of CDS and HCT at three different wavelengths

	Absorptivity (a) x 10^{-2}		
λ (nm)	223.5	240	268.5
CDS	6.67	2.76	2.33
HCT	11.41	0.46	6.42

The same spectral procedures were applied to the tablet sample solutions. For the analytical validation of the TCLS method, a validation set consisting of eight synthetic mixture solutions at different concentrations within the 4-20 µg/ml linear working range of CDS and HCT was prepared. The accuracy and precision of the TCLS calibration were tested by analyzing the prepared validation set. Recovery values were found to be 97.2% for CDS and 99.7% for HCT. Relative standard deviation values were calculated as 1.61% for CDS and 3.67% for HCT. The results obtained by applying the TCLS approach to synthetic mixtures are presented in Table 2. As seen in Table 2, the validity of the method was reported with good accuracy and precision.

Table 2. Recovery results were obtained by applying the TCLS method to the synthetic mixtures containing different concentrations of CDS and HCT

	Synthetic Mixture (µg/ml)		Found (µg/ml)		Recovery (%)	
No.	CDS	HCT	CDS	HCT	CDS	HCT
1	6	6	5.91	6.10	98.54	101.6
2	10	6	9.72	6.05	97.19	100.8
3	14	6	13.47	6.22	96.21	103.6
4	18	6	17.19	6.27	95.51	104.4
5	8	4	7.99	3.99	99.83	99.8
6	8	8	7.86	7.65	98.28	95.7
7	8	12	7.75	11.38	96.86	94.8
8	8	16	7.63	15.43	95.37	96.5
				Mean	97.2	99.7
				SD	1.57	3.66
				RSD	1.61	3.67

SD: Standard deviation
RSD: Relative standard deviation

The commercial tablet sample was prepared five times as stated in the "Preparation of Samples" subsection. The proposed TCLS approach was applied to the analysis of the tablet samples and then, the amount of CDS and HCT in the commercial pharmaceutical preparation was determined by using the TCLS model based on the absorbance measurements at a set of three wavelengths. The determination results are presented in Table 3. The analysis results showed good agreement with the tablets' label claims. In addition to that, in the TCLS method application providing a low standard deviation and relative standard deviation, a good agreement was reported for the experimental outcomes.

Table 3. Results obtained by the applying TCLS method to the commercial tablet (Label claim in the preparation: 16 mg CDS and 12.5 mg HCT per tablet)

	mg/tablet	
Exp. No.	CDS	HCT
1	16.05	12.40
2	16.11	12.58
3	16.22	12.57
4	16.07	12.39
5	16.30	12.40
Mean	16.1	12.5
SD	0.11	0.10
RSD	0.66	0.78

SD: Standard deviation
RSD: Relative standard deviation

Conclusion

The main aim of this research article was to develop an easy, inexpensive, selective, sensitive, and validated method for the analysis of a commercial pharmaceutical preparation containing CDS and HCT. In this context, the developed TCLS approach was successfully applied to the simultaneous quantification of CDS and HCT in synthetic mixtures and tablets. In the pharmaceutical industry and R&D laboratories, the TCLS method can be used for quality control and routine analysis of binary combined preparations containing CDS and HCT active compounds.

ACKNOWLEDGEMENTS

In this paper study, the method development and its experiment procedures were performed at the Chemometric Laboratory of Faculty of Pharmacy supported by Ankara University Scientific Research Fund (Project No. 10A3336001).

AUTHOR CONTRIBUTIONS

Concept: A.Ü., E.D.; Control: A.Ü, E.D.; Design: A.Ü., Ö.Ü., E.D.; Materials: U.S., E.D.; Sources: A.Ü., Ö.Ü.; Data Collection and/or Processing: U.S., E.D.; Analysis and/or Interpretation: A.Ü., Ö.Ü., E.D.; Literature Review: A.Ü, U.S.; Manuscript Writing: A.Ü., E.D.; Critical Review: A.Ü., Ö.Ü., U.S., E.D.; Other: -

CONFLICTS OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 811-818, 2024
Doi: 10.33483/jfpau.1461131

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IN VITRO CYTOTOXIC ACTIVITIES OF PLATINUM(II) COMPLEXES CONTAINING 1*H*-BENZO[*d*]IMIDAZOLE AND 1*H*-1,3-DIAZOLE DERIVATIVES

1H-BENZO[d]İMİDAZOL VE 1H-1,3-DİAZOL TÜREVLERİ İÇEREN PLATİN(II) KOMPLEKSLERİNİN İN VİTRO SİTOTOKSİK AKTİVİTELERİ

Tuğçe YILMAZ[1], Elif ERGİN[1], Hatice ORUÇ DEMİRBAĞ[2], Semra UTKU[1]*

[1]Mersin University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 33169, Mersin, Türkiye
[2]Mersin University, Faculty of Medicine, Department of Histology and Embryology, 33110, Mersin, Türkiye

ABSTRACT

Objective: *This study aimed to synthesize and evaluate the cytotoxic activities of four platinum(II) complexes with 2-substituted or nonsubstituted 1H-benzo[d]imidazole and 1H-1,3-diazole derivatives as carrier ligands (L1-L4), which may have potent cytotoxic activity and low side effects.*
Material and Method: *K1-K4 complexes were synthesized by heating and mixing K_2PtCl_4 and the appropriate L1-L4. The chemical structures of K1-K4 were elucidated by Infrared and ^{1}H Nuclear Magnetic Resonance spectroscopic methods. In vitro, cytotoxic effects of K1-K4 complexes against prostate (DU-145), endometrial adenocarcinoma (Ishikawa), and breast cancer (MCF-7) cell lines were tested by the MTT method.*
Result and Discussion: *According to the IC_{50} values of the tested cell lines, K1 and K2 derivatives bearing unsubstituted 1H-benzo[d]imidazole (L1) and 1H-1,3-diazole (L2) were found to be the most effective compounds among these synthesized complexes.*
Keywords: *1H-1,3-diazole, 1H-benzo[d]imidazole, cisplatin, cytotoxic activity, platinum complexes*

ÖZ

Amaç: *Bu çalışmada, güçlü sitotoksik aktiviteye ve düşük yan etkilere sahip olabilecek, taşıyıcı ligand olarak 2-sübstitüe veya nonsübstitüe 1H-benzo[d]imidazol ve 1H-1,3-diazol türevleri içeren dört platin(II) kompleksinin sentezlenmesi ve sitotoksik aktivitelerinin değerlendirilmesi amaçlanmıştır.*
Gereç ve Yöntem: *K1-K4 kompleksleri, K_2PtCl_4 ve uygun taşıyıcı ligandların ısıtılıp karıştırılmasıyla sentezlenmiştir. K1-K4'ün kimyasal yapıları Infrared ve ^{1}H Nükleer Manyetik Rezonans spektroskopik yöntemleri ile aydınlatılmıştır. İn vitro olarak, K1-K4 komplekslerinin prostat (DU-145), endometrial adenokarsinom (Ishikawa) ve meme kanseri (MCF-7) hücre hatlarına karşı sitotoksik etkileri MTT yöntemi ile test edilmiştir.*
Sonuç ve Tartışma: *Test edilen hücre hatlarının IC_{50} değerlerine göre, sübstitüe olmayan 1H-benzo[d]imidazol veya 1H-1,3-diazol taşıyan K1 ve K2 türevlerinin sentezlenen bu kompleksler arasında en etkili bileşikler olduğu bulunmuştur.*
Anahtar Kelimeler: *1H-1,3-diazol, 1H-benzo[d]imidazol, platin kompleksleri, sisplatin, sitotoksik aktivite*

* **Corresponding Author / Sorumlu Yazar:** Semra Utku
e-mail / e-posta: utkusemra@mersin.edu.tr, **Phone / Tel.:** +905057572482

Submitted / Gönderilme : 05.04.2024
Accepted / Kabul : 24.04.2024
Published / Yayınlanma : 10.09.2024

INTRODUCTION

Cancer is the leading cause of death worldwide, accounting for around one out of every six deaths and affecting nearly every family. In 2022, there were an estimated 20 million new cases of cancer and 9.7 million cancer-related deaths globally. The cancer burden is expected to rise by approximately 77% by 2050, putting additional strain on healthcare systems, people, and communities [1]. Nowadays, various approaches, including radiotherapy, chemotherapy, surgery, immunotherapy, hormone therapy, and gene therapy, can be used alone or in combination for cancer treatment [2].

Cisplatin, the first platinum complex to be used clinically in the treatment of cancer patients, is the most widely prescribed chemotherapeutic drug for the treatment of testicular, ovarian, bladder, non-small lung cancer, head and neck, esophagus, advanced cervical cancer, lymphomas, metastatic osteosarcoma, and melanoma [3,4].

Despite this clinical success, cisplatin induces several toxic side effects such as nephrotoxicity, neurotoxicity, ototoxicity, nausea, and vomiting [5]. This resistance may be intrinsic or developed during prolonged treatment [6,7]. To overcome these issues, new platinum complexes have been designed and studied for their antitumor properties [8,9].

Even though thousands of complexes have been developed and evaluated, only three platinum drugs, cisplatin, carboplatin, and oxaliplatin, have been approved for clinical use worldwide. In addition, Nedaplatin, lobaplatin, and heptaplatin have only received regional approval [10,11]. These complexes, shown in Figure 1, work through a mechanism of action similar to cisplatin, involving DNA binding and transcription inhibition. They are activated intracellularly by aquation of the leaving groups and then interact with DNA to form DNA adducts by coordination of the cis-[Pt(R-NH_2)$_2$] fragment to the N-7 atom of G residue [12].

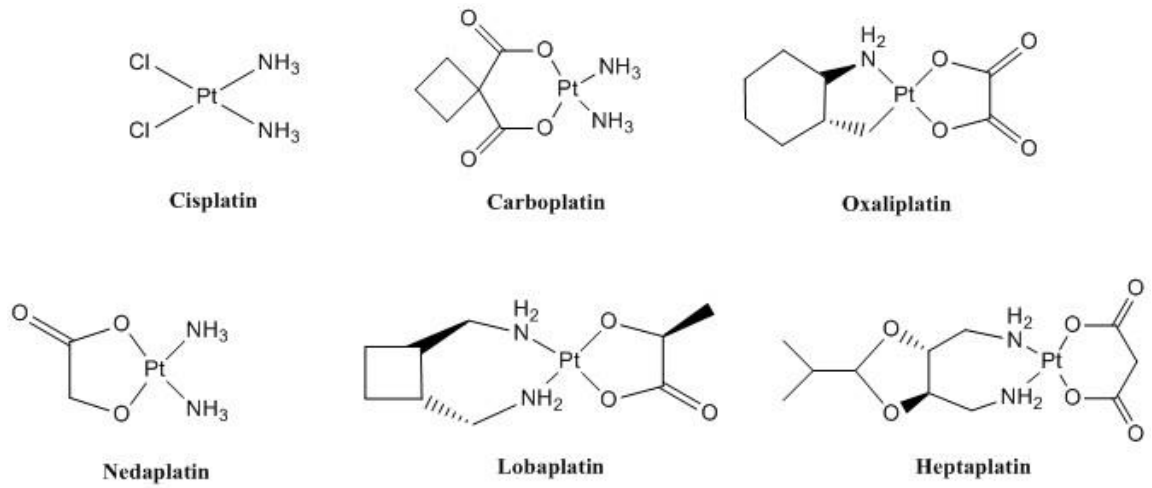

Figure 1. Structural formulas of Pt(II) anticancer metallodrugs: cisplatin, carboplatin, oxaliplatin, nedaplatin, lobaplatin, and heptaplatin

The most significant type of cisplatin-DNA binding is the intrastrand 1,2-d(GpG) cross-link. This type accounts for about 60-65% of the platinum bound to DNA. The resulting Pt-DNA adducts are responsible for distorting and bending the structure of the DNA, which in turn hinders transcription. The inhibitory effects on transcription ultimately lead to the death of the cells [3,13].

Carboplatin, a second-generation platinum (II) complex that carries a slower hydrolyzing 1,1-cyclobutane dicarboxylate ligand instead of the chlorine ligand separated in cisplatin, provided the advantage of fewer side effects in patients despite the use of higher doses than cisplatin, but it was not possible to prevent the development of cross-resistance to carboplatin [14,15]. Oxaliplatin, a third-generation platinum complex, is synthesized by replacing the ammonia and chlorine ligands in the cisplatin structure with 1,2-diaminocyclohexane and oxalate, respectively. There is no development of cross-resistance to oxaliplatin, and nephrotoxic effects are less common in patients using cisplatin and

carboplatin [16].

In our previous studies, we investigated the cytotoxic effect of K1-K4 complexes in cervix cancer (HeLa), laryngeal cancer (HEp-2), and human breast cancer (MCF-7) cell lines, and their plasmid DNA interactions are also investigated using agarose gel electrophoresis [17–19]. Furthermore, the antibacterial and antifungal activity of K1 complex has been evaluated by the macrodilution method [19].

In this study, as an extension of our investigation on the probable anticancer activity of K1-K4 complexes with 2-substituted or nonsubstituted 1*H*-benzo[d]imidazole and 1*H*-1,3-diazole as carrier ligands, were evaluated for their in vitro cytotoxic activities against prostate cancer (DU-145), endometrial adenocarcinoma (Ishikawa) and breast cancer (MCF-7) cell lines using the MTT method, which is called 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, a tetrazolium salt.

MATERIAL AND METHOD

Chemistry

1*H*-benzo[d]imidazole (L1) was synthesized by the Philips method starting from 1,2-phenylenediamine and formic acid [20]. 1*H*-1,3-diazole (L2), 2-phenyl-1*H*-benzo[d]imidazole (L3), and 2-phenyl-1*H*-1,3-diazole (L4) carrier ligands, solvents, and all starting chemicals were purchased from Sigma-Aldrich. The reactions were verified with thin-layer chromatography using silica gel plates, which were visualized under 254 nm UV light. ^{1}H NMR spectra were recorded in DMSO-d_6 on a Bruker 400 MHz FT-NMR spectrometer using tetramethylsilane as the internal standard. All chemical shifts are reported in ppm (δ). FTIR-ATR spectra were recorded on a Perkin Elmer Spectrum 400 FTIR/FTNIR spectrometer equipped with a Universal ATR Sampling Accessory and were reported in cm^{-1} units. Melting points were determined with an Electrothermal 9200 Melting Point Apparatus and are uncorrected.

Characterization of Carrier Ligands

Detailed structural analyses of carrier ligands 1*H*-benzo[d]imidazole (L1), 1*H*-1,3-diazole (L2), 2-phenyl-1*H*-benzo[d]imidazole (L3) and 2-phenyl-1*H*-1,3-diazole (L4) were carried out previously reported [17–19].

General Procedure for the Synthesis of K1-K4

To a stirred solution of L1-L4 (1.10 mmol) in ethanol-water (7:3 ml) was added dropwise an aqueous solution of K_2PtCl_4 (0.60 mmol) over 30 min at room temperature (Figure 2). The reaction mixture was heated 40-60°C for 2-6 days. The pH was adjusted to 7 and kept constant with the addition of 0.1 M $NaHCO_3$. The resulting crude precipitate formed was filtered off and washed with small portions of water, ethanol, and diethyl ether and dried in vacuo.

cis-Dichloro-bis(1*H*-benzo[d]imidazole)platinum(II) (K1). Yield:85%; Mp: >400 °C; FTIR-ATR υ (cm^{-1}): 3287-3100 (N-H, =C-H); ^{1}H-NMR (DMSO-d_6) δ: 13.44 (s, 2H, 2 x N-H), 8.84 (s, 2H, 2x Ar-H) 7.80 (d, J= 7.2 Hz, 2H, Ar-H), 7.50 (d, J= 7.2 Hz, 2H, Ar-H), 7.25-7.18 (m, 4H, 2x ArH).

cis-Dichloro-di(1*H*-1,3-diazole)platinum(II) (K2). Yield:64%; Mp: >400 °C; FTIR-ATR υ (cm-1): 3243-2888 (N-H, =C-H); ^{1}H-NMR (DMSO-d6) δ: 13.51 (s, 2H, 2x N-H), 8.30 (s, 2H, 2x Ar-H) 7.40 (s, 2H, 2x Ar-H), 7.09 (s, 2H, 2x Ar-H).

cis-Dichloro-bis(2-phenyl-1*H*-benzo[d]imidazole)platinum(II).1.5 H_2O (K3). Yield:70%; Mp: >400 °C; FTIR-ATR υ (cm-1): 3355-3055 (N-H, =C-H, O-H), 1620-1541; ^{1}H-NMR (DMSO-d6) δ: 13.34 (s, 2H, 2x N-H), 8.91-8.89 (m, 1H, ArH), 8.28-8.22 (m, 3H, 2x Ar-H), 7.91-6.79 (m, 14 H, ArH).

cis-Dichloro-bis(2-phenyl-1*H*-1,3-diazole)platinum(II) (K4). Yield:65%; Mp: >400 °C; FTIR-ATR υ (cm^{-1}): 3222-2890 **(**N-H, =C-H); ^{1}H-NMR (DMSO-d_6) δ: 13.13 (broad s, 2H, 2x NH), 8.67-7.99 (m, 4H, 2x ArH), 7.65-7.22 (m, 10H, 2x ArH).

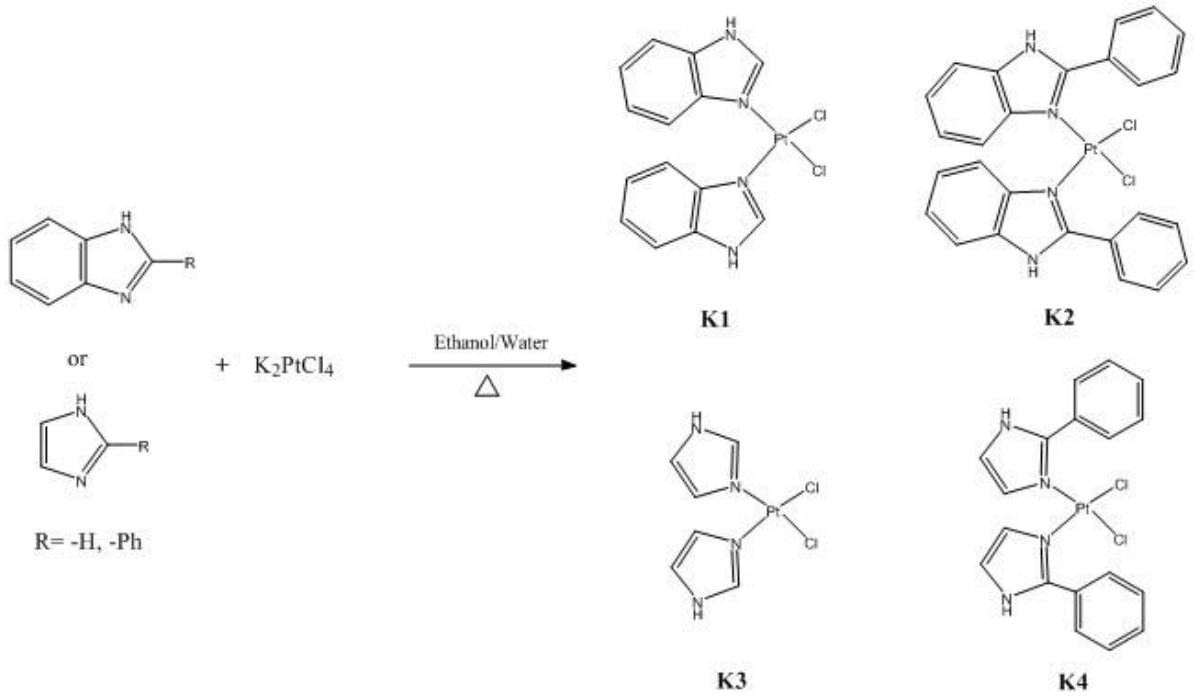

Figure 2. Synthesis of K1-K4

Biological Activity

In our study, commercially purchased MCF-7 breast cancer (The American Type Culture Collection (HTB-81, Manassas, VA, USA)), DU-145 prostate cancer (T.C. Ministry of Agriculture and Forestry Alum Institute, Cell Registration No: 00092502ATCC) and Ishikawa endometrial adenocarcinoma cell lines (Merck, ECACC-99040201, Darmstadt, Germany) were used for cytotoxicity tests. Cells were seeded in 25 cm^2 flasks using either Dulbecco's modified Eagle's medium (HyClone Laboratories, Inc., Logan, UT) or RPMI 1640 Medium (Sartorius,01-106-1A) supplemented with 10% fetal bovine serum (FBS, Gibco, 10082147), %2,5 L-glutamine (Thermo, 25030081), 1% penicillin-streptomycin (Thermo,15140130) and %1 ml amphotericin (Thermo, 15290018) mixture and then grown for 3 days at 37°C in 5% CO_2 in a humidified incubator.

The cytotoxic effect of K1-K4 and cisplatin as a positive control were tested by MTT method. MTT test was performed by ISO 10993-5 standards [21].

Cell culture was performed in 96-well plates with n=3 for each concentration and 7500 cells per well. After incubation in a humidified atmosphere of 5% CO_2 and 37°C for 24 hours, the medium was replaced with medium containing K1-K4 and cisplatin as positive control at concentrations of 2.5, 5, 10, 20, 20, 40, 80, and 160 µM and 1,56, 3.125, 6.25, 12.5, 25, 50, 100 respectively. Cells were incubated with the medicated medium for 72 hours. At the end of this time, the medium on the cells was removed, and 200 µl of medium and 50 µl of medium containing (Sigma-Aldrich, St. Louis, MO, USA)) MTT (5 mg/ml) were added to each well. Cells were incubated at 37°C for 4 hours. The medium was then removed from the cells and 200 µl of DMSO and 50 µl of glycine buffer with a pH of 10.5 was added to each well. To determine the viability of the MCF-7, DU-145, and Ishikawa cells tested, the plates containing the cells were immediately exposed to spectrophotometric measurement at a wavelength of 570 nm in an ELISA microplate reader.

The IC_{50} values of K1-K4 at concentrations of 2.5, 5, 10, 20, 40, 80, and 160 µM; cisplatin 1.56, 3.125, 6.25, 12.5, 25, 50, 100 µM are given in Table 1 and the viability values against MCF-7, DU-145 and Ishikawa cell lines are given in Table 2. The IC_{50} values were using GraphPad Prism software.

Table 1. IC_{50} (μM) values of K1-K4 and cisplatin

Complex No	MCF-7	DU-145	Ishikawa
K1 $[Pt(L1)_2Cl_2]$	3.12±0.0039	2.06±0.006	1.81±0.0102
K2 $[Pt(L2)_2Cl_2]$	1.93±0.0168	4.27±0.0042	1.90±0.0239
K3 $[Pt(L3)_2Cl_2]$	8.75±0.0158	2.16±0.0067	2.87±0.0279
K4 $[Pt(L4)_2Cl_2]$	6.52±0.0099	4.66±0.014	3.55±0.0230
Cisplatin $[Pt(NH_3)_2Cl_2]$	2.08±0.0041	1.92±0.0042	2.05±0.0068

[a] IC_{50} = 50% cytotoxic concentration against in vitro tested cells. Data are presented as mean ± SD

Table 2. K1-K4 and cisplatin % viability values against MCF-7, DU-145 and Ishikawa cell lines

		% Viability (µM ±SS)						
		160	**80**	**40**	**20**	**10**	**5**	**2.5**
MCF-7 (breast cancer)	**K1**	33.48 ±1.01	39.18 ±0.51	35.04 ±0.37	39.24 ±1.30	40.25 ±0.10	54.37 ±1.57	68.91 ± 0.58
	K2	65.54 ±1.94	65.06 ±0.19	70.48 ±2.07	71.04 ±0.62	66.38 ±1.91	71.34 ±1.50	71.80 ± 0.84
	K3	32.73 ±0.95	32.78 ±2.58	43.78 ±0.19	49.94 ±3.61	57.76 ±1.50	78.61 ±9.63	98.69 ± 3.61
	K4	32.45 ±1.11	38.63 ±0.57	39.41 ±2.01	43.35 ±1.05	59.89 ±2.77	71.74 ±3.13	79.75 ± 3.25
	Cisplatin	20.77 ±0.91	22.28 ±0.12	23.63 ±1.28	26.25 ±0.28	28.04 ±0.15	39.32 ±1.65	42.82±1.43
DU-145 (prostate cancer)	**K1**	8.79± 0.22	7.27 ± 0.18	7.95± 0.15	8.44± 0.16	9.91± 0.51	20.11± 0.32	33.63± 1.21
	K2	50.00± 1.41	62.49± 4.01	62.60± 0.54	64.14± 2.66	64.18± 2.33	70.65± 5.64	69.87± 0.63
	K3	10.69± 0.30	9.19± 0.11	9.24± 0.10	9.66± 0.28	8.78± 0.03	10.46± 0.71	20.35± 1.23
	K4	10.68± 0.16	10.81± 0.82	10.27± 0.17	10.98± 0.18	15.53± 2.19	63.56± 1.70	71.03± 0.76
	Cisplatin	7.64 ± 0.32	7.77 ± 0.05	7.94 ± 0.28	8.90 ± 0.11	10.19± 0.22	11.41± 0.40	13.73± 0.36
Ishikawa (endometrial adenocarcinoma cancer)	**K1**	5.95± 0.20	6.25 ± 0.06	7.10 ± 0.36	9.15 ± 0.44	7.45 ± 0.29	16.13± 0.53	17.87± 0.67
	K2	36.92± 0.17	41.04± 1.27	46.33± 0.16	49.09± 0.63	49.11± 0.72	49.65± 1.65	49.39± 1.31
	K3	5.74± 0.03	5.76 ± 0.18	6.02 ± 0.02	6.24 ± 0.18	10.61± 0.98	43.89± 4.61	44.67± 0.90
	K4	5.88± 0.11	6.07 ± 0.06	10.99± 0.19	13.44± 0.57	32.60± 0.44	44.34± 0.91	48.39± 3.44
	Cisplatin	5.36 ± 0.02	5.07 ± 0.07	5.59 ± 0.11	6.86 ± 0.10	13.12± 0.35	22.90± 0.46	31.55± 0.58

RESULT AND DISCUSSION

Platinum complexes represent one of the most successful families of clinically used anticancer drugs. Although cisplatin has a wide spectrum of anticancer activity, it does have significant side toxicity, and its clinical use can also be limited by the existence or development of resistance. Several thousand platinum-based compounds have been synthesized to overcome reduced toxicity and drug resistance [22,23].

In this study, to overcome the disadvantages mentioned above, the ammonia ligands in the

cisplatin structure were replaced with L1-L4 derivatives as carrier ligands, which are known to the body and have ligand properties.

The carrier ligand 1*H*-benzo[d]imidazole (L1) was synthesized using the Phillips method and its melting point was confirmed to be consistent with the literature [20].

The synthesized complexes, which were reported previously by us, were re-synthesized in this study to obtain higher yields using different temperatures and reaction times [17–19]. Our complexes synthesized in 85-64% yield were obtained in higher yields than previous studies.

The melting points of all complexes were >400 °C. The IR spectra of the K1-K4 were shown some characteristic changes when compared to those of the L1-L4 carrier ligands. In the IR spectrum of L1-L4 were observed broad bands in the region of 3400-2500 cm^{-1} due to the 2-substituted/nonsubstituted-1*H*-benzo[d]imidazole and 1*H*-1,3-diazole N-H stretching bands. K1-K4 compounds exhibited N-H stretching bands centered at 3287 and 2880 cm^{-1} sharper than that of L1-L4 carrier ligands, due to the breaking of tautomerism, indicating that the N-H group was not involved in the coordination.

The ^{1}H-NMR spectra of synthesized complexes were consistent with their corresponding protons, both in the chemical shifts and in the number of hydrogens. The spectra of the complexes were compared to those of the free ligands, and significant differences were observed. All complexes showed a large downfield shift in the imidazole N-H signal compared to their ligands, which is due to an increase in the N-H acid character after platinum binding. The N-H chemical shifts of compounds K1-K4 varied between 13.51-13.13 ppm.

The cytotoxic activities of prepared platinum (II) complexes K1-K4 and cisplatin used as reference compound were evaluated in vitro by an MTT assay. Different cancer cell lines, including MCF-7 (breast cancer cell line), DU-145 (prostate carcinoma cell line) and Ishikawa (human endometrial cancer cell line) were used in vitro cytotoxicity test. The corresponding %cell viability and IC_{50} values were shown in Table 1 and 2.

Generally, K1-K4 complexes exhibited considerable cytotoxic activities against MCF-7, DU-145, and Ishikawa cell lines. The IC_{50} values in these tested cell lines were within the range of 1.93-8.75 μM, 2.06-4.66 μM, and 1.81-3.55 μM, respectively. It is noted that complex K1 and K2 are the most effective compounds among the synthesized complexes. The cytotoxic activities of K1 and K2 complexes with the carrier ligand of 1*H*-benzo[d]imidazole and 1*H*-1,3-diazole against Ishikawa and DU-145 cell lines were tested comparable to cisplatin (IC_{50}= 1.81 and 2.06 μM, 1.90 and 4.27, 2.05 and 1.92 μM, respectively). K2 complex with 1*H*-1,3-diazole carrier ligand was tested as more effective complex than cisplatin against MCF-7 cell line (IC_{50}= 1.93 μM vs. IC_{50}=2.08 μM).

In this study, platinum compounds were synthesized in which a selection of biologically active carrier ligands were attached to Pt coordination moieties. Although these complexes exhibited better activity when compared to cisplatin in the cell lines tested, it would be early to recognize them as drug-candidate molecules since advanced anticancer activity tests have not been performed thus far.

According to our previous research, we thought that the steric hindrance of the substituted group would modify the interaction mode between platinum compounds and DNA, influencing the antitumor properties of the platin-DNA adducts and resulting in the absence of cross-resistance with cisplatin [24–26]. The increased steric hindrance of platinum complexes is expected to reduce the interaction between platinum complexes and sulfur-containing molecules while also prolonging the blood cycle, resulting in an enhanced therapeutic impact for tumors [27,28]. However, considering that the steric hindrance in the tested K3 and K4 complexes may disrupt or even prevent the interaction of the compound with DNA, we thought it would be appropriate to include the K1 and K2 complexes, which have nonsubstituted ligands, in the study. Therefore, MTT test results show that the bearing of a phenyl ring at the second position of the benzimidazole or imidazole ring increases the sterically hindered effect of platinum complexes and can reduce the moderately cytotoxic effect against tested cell lines.

ACKNOWLEDGEMENTS

This study was supported by Mersin University Scientific Research Projects Unit with project number 2022-1-TP2-4651.

AUTHOR CONTRIBUTIONS

Concept: T.Y., S.U.; Design: T.Y., E.E., H.O.D., S.U.; Control: T.Y., E.E., H.O.D., S.U.; Sources: T.Y., E.E., H.O.D., S.U.; Materials: H.O.D., S.U.; Data Collection and/or Processing: T.Y., E.E., H.O.D., S.U.; Analysis and/or Interpretation: T.Y., E.E., H.O.D., S.U.; Literature Review: T.Y., E.E., S.U.; Manuscript writing: T.Y., E.E., S.U.; Critical Review: T.Y., E.E., H.O.D., S.U.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 819-825, 2024
Doi: 10.33483/jfpau.1438607

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIMICROBIAL AND ANTIBIOFILM ACTIVITIES OF VARIOUS VEGETABLE OILS AGAINST *HELICOBACTER PYLORI*

ÇEŞİTLİ BİTKİSEL YAĞLARIN HELİCOBACTER PYLORİ'YE KARŞI ANTİMİKROBİYAL VE ANTİBİYOFİLM ETKİNLİKLERİ

Elif AYDIN[1]*

[1]Tavsanli Vocational School of Health Services, Kutahya Health Sciences University, 430200, Kutahya, Türkiye

ABSTRACT

Objective: *Vegetable oils have various biologically active components, including antibacterial, antioxidant, and anti-inflammatory properties. These oils help control nausea, vomiting, coughing and gas, as well as diarrhea and dyspepsia. It also helps to reduce stomach bloating and intestinal spasm pain. To sum up, the objective of this investigation was to assess the antibacterial and antibiofilm properties of twelve different vegetable oils against the reference strain of Helicobacter pylori, NTCC 11637.*
Material and Method: *For antibacterial activity, the minimum inhibitory concentration and the agar-well diffusion method were employed, and for antibiofilm activity, the microplate method.*
Result and Discussion: *Vegetable oils showed antimicrobial activity at concentrations of 62.5-15.625 µg/ml and antibiofilm activity at concentrations of 250-15.625 µg/ml. According to our findings, the vegetable oils we utilized may have the ability to form a novel class of Helicobacter pylori inhibitors with anti-H. pylori properties.*
Keywords: *Antimicrobial, antibiofilm, Helicobacter pylori, vegetable oils*

ÖZ

Amaç: *Bitkisel yağlar, antibakteriyel, antioksidan ve anti-enflamatuvar özellikler de dahil olmak üzere çeşitli biyolojik aktif bileşenlere sahiptir. Bu yağlar bulantı, kusma, öksürük ve gazın yanı sıra ishal ve dispepsiyi kontrol etmeye yardımcı olur. Ayrıca mide şişkinliğini ve bağırsak spazmı ağrısını azaltmaya yardımcı olur. Sonuç olarak, bu araştırmanın amacı 12 farklı bitkisel yağın Helicobacter pylori NTCC 11637 standart suşu üzerindeki antimikrobiyal ve antibiyofilm etkinliğini değerlendirmektir.*
Gereç ve Yöntem: *Antimikrobiyal etkinlik için minimum inhibitör konsantrasyon ve agar-kuyu difüzyon yöntemi, antibiyofilm etkinlik için ise mikropleyt yöntemi kullanıldı.*
Sonuç ve Tartışma: *Bitkisel yağlar 62.5-15.625 µg/ml konsantrasyonlarda antimikrobiyal, 250-15.625 µg/ml konsantrasyonlarda ise antibiyofilm etkinlik göstermiştir. Sonuçlarımız, kullandığımız bitkisel yağların, anti-Helicobacter pylori etkileri olan yeni bir Helicobacter pylori inhibitörleri sınıfının potansiyel bir bileşiği olabileceğini gösterdi.*
Anahtar Kelimeler: *Antimikrobiyal, antibiyofilm, bitkisel yağlar, Helicobacter pylori*

* **Corresponding Author / Sorumlu Yazar:** Elif Aydın
e-mail / e-posta: elif.aydin@ksbu.edu.tr, **Phone / Tel.:** +905053553846

Submitted / Gönderilme : 16.02.2024
Accepted / Kabul : 02.05.2024
Published / Yayınlanma : 10.09.2024

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped, urease-producing, gram-negative, single-flagellated bacterium that was originally identified from a human stomach in 1983. Its size ranges from 2-4 μm to 0.5-0.9 μm. This disease has the ability to infect over 50% of the global population and between 70 and 90% of people living in underdeveloped nations [2,3]. This bacterium causes gastrointestinal issues and is linked to a rapidly growing number of cases of peptic ulcer, gastritis, gastric cancer, and lymphoid tissue lymphoma connected with the gastric mucosa [4,5].

A combination antibiotic therapy including two types of antibiotics (amoxicillin, clarithromycin, levofloxacin, and metronidazole) with one proton pump inhibitor (omeprazole, lansoprazole, and pantoprazole) is used twice a day for seven days to treat *H. pylori*. This is a disease that has a low success rate despite combined treatment, and its treatment has side effects. Applied treatments fail due to age, smoking, increased bacterial load and virulence, adverse drug reactions, antibiotic resistance, and different pharmacokinetic and pharmacodynamic factors [6,7].

New therapeutic treatments or adjunctive therapies are needed to increase the rate of *H. pylori* eradication and reduce the side effects of treatment, and there is a growing interest in agents that can enhance *H. pylori* eradication, such as the use of herbal oils. At such a time, natural product sources such as plant extracts and vegetable oils provide alternative remedies to combat both resistant and non-resistant microbes [8]. The World Health Organization (WHO) states that traditional medicine is the primary source of basic healthcare for most people worldwide. Aromatic and medicinal plants are a valuable source of naturally occurring organic compounds that are frequently utilized in medicine [9]. Vegetable oils are natural, concentrated volatile aromatic compounds (VOCs) isolated from plants. Vegetable oils have some preventive/therapeutic properties such as anti-bacterial, anti-fungal, anti-viral, insecticidal and antioxidant properties. There are many plants that can be used as alternative treatments against antibiotic resistance [10]. The bioactive components of commonly used vegetable oils can have multiple antimicrobial effects, such as altering DNA and RNA synthesis, breaking down bacterial cell walls, disrupting the structure of the cell membrane, changing the levels of fatty acids, phospholipids, and protein translocation, so it is possible to use vegetable oils as antimicrobial agents against harmful microorganisms [11-13].

Studies on the use of plant organisms have accelerated recently due to the numerous adverse effects and treatment failures of synthetic medications used to treat *H. pylori* infection. Thus, this study set out to ascertain the antibacterial and antimicrobial activity of herbal oils derived from widely used medicinal plants on the gastrointestinal pathogen *H. pylori* bacteria.

MATERIAL AND METHOD

Preparation of Vegetable Oils

The 12 vegetable oils used in the study were commercially available (Biotama, Turkey) and are listed in Table 1. The initial concentration of all the different concentrations of oils used was dissolved in dimethyl sulfoxide (DMSO) (1 mg/ml) and filtered through 0.22 μM membrane filters.

H. pylori Culture

National Collection of Type Cultures *Helicobacter pylori* NTCC 11637 standard strain was used in this study. The strain was maintained in Mueller-Hinton Broth (MHB, Himedia, India) containing 20% glycerol and 10% bovine fetal serum (BFS) at -80', 10% bovine fetal serum (FBS, Serox GmbH, Mannheim, Germany), and incubated in a microaerophilic atmosphere with a ready-made kit (CampyGen, Thermo Fisher Diagnostics AG, Pratteln, Switzerland) for 72 hours at 36°C. Following incubation, 5% sheep blood was added to Columbia Agar (Himedia, India), and the mixture was incubated for 72 hours at 36°C in a microaerophilic environment [14].

Determination of Minimal Inhibitory Concentration (MIC) of Vegetable Oils on *H. pylori*

Vegetable oils' antibacterial activity was evaluated using the MIC research method described in the Clinical and Laboratory Standards Institute Manual (CLSI, 2022) [15]. The revived *H. pylori* strain

was adjusted to 1.0 with a McFarland turbidity standard. In a 96-well microplate, 80 µl of MHB supplemented with 10% BFS was applied to each well. Vegetable oil (100 µl) in each well (250-0.98 µg/ml) concentrations were transferred to the first well and diluted two-fold. Finally, 20 uL of a bacterial suspension previously standardized to 1.0 McFarland turbidity standard was dispensed into each well. One well contained only MHB supplemented with 10% BFS for negative control and one well contained bacterial suspension with MHB supplemented with 10% BFS for positive control. All micropellets were incubated for 72 h at 36°C under a 10% CO_2 atmosphere [14]. After incubation, absorbance was measured at 570 nm. The minimum inhibitory concentration (MIC) of H. pylori was determined by comparing its observable growth to that of the control.

Table 1. The names of plants and oils used in this study

	Oils	**Plants**
1	Jasmine oil	*Jasminum nudiflorum*
2	Cumin oil	*Cuminum cyminum*
3	Cinnamon oil	*Cinnamomum verum*
4	Rosemary oil	*Rosmarinus officinalis*
5	Rosehip oil	*Rosa canina*
6	Indian oil	*Ricinus communis*
7	Ylang ylang oil	*Cananga odorata*
8	Jojoba oil	*Simmondsia chinensis*
9	Safflower oil	*Carthamus tinctorius*
10	Cardamom oil	*Elettaria cardamomum*
11	Lily oil	*Lilium candidum*
12	Nettle oil	*Urtica dioica*

Evaluation of Antimicrobial Activity of Vegetable Oils on *H. pylori* by Agar-Well Diffusion Method

On Mueller-Hinton Agar (MHB, Himedia, India) with 5% sheep blood, a 1.0 McFarland turbid standardized bacterial solution was applied. Different oil concentrations ranging from 20, 40, 60, and 80 µl were applied to each well individually, and the wells were then incubated at 36°C for 72 hours in a microaerophilic environment [16]. After incubation, the presence or absence of zones was evaluated.

Evaluation of the Antibiofilm Activity of Vegetable Oils on *H. pylori*

Antibiofilm activity of vegetable oils was continued by optimizing the method used by Yu et al. [17]. In order to examine the potential suppressive impact of vegetable oil on *H. pylori* biofilm development, a 96-well microplate was filled with 80 µl of MHB supplemented with 10% BFS in each well. Two-fold serial dilutions of vegetable oil in the range of 250-0.98 µg/ml were added to the suspended *H. pylori* samples. After incubation under microaerobic conditions at 36°C for 48 h under 10% CO_2 atmosphere, bacterial suspensions were removed. To get rid of any leftover *H. pylori*, the plates were gently washed three times with 200 µl of phosphate-buffered saline (PBS). Subsequently, 200 µl of 1% crystal violet was transferred to the tube and staining was performed for 20 minutes at room temperature. Following staining, three sterile PBS washes were used to remove the stains from the crystal violet. Once the micropattern was flipped over to dry, 200 µl of a 33% glacial acetic acid solution was poured into the tube, dissolving the crystal violet rings for ten minutes. At 570 nm, the optical density of crystal violet was determined.

RESULT AND DISCUSSION

For many years, vegetable oils have been extensively utilized in a variety of industries, including natural treatment, medicine, food preservation, and medical. The scientific study of vegetable oils used in alternative medicine techniques is essential to improving the quality of care provided to patients. The U.S. Food and Drug Administration has approved the oil as safe since it often has no negative side

effects [18]. In addition, it becomes very difficult for microorganisms to gain resistance to oils. The creation of new antibacterial agents through vegetable oils holds great promise [19,20]. In light of all this information, we hypothesized that as an alternative natural treatment for *H. pylori* infection, the 12 known vegetable oils could be used.

Results of the Minimal Inhibitory Concentration (MIC)

The antimicrobial results of a total of 12 vegetable oils used in the study against *H. pylori* NTCC 11637 standard strains are given in Table 2. All oils were effective against *H. pylori* and their concentration ranges were 62.5-15.625 μg/ml. Among the oils, cumin, rosemary and coconut oil were effective at low concentrations (15.625 μg/ml).

Vegetable oils have been discovered to have varying degrees of antibacterial activity against *H. pylori* in the literature currently under publication. The vegetable oils *Eucalyptus globulus, Juniperus communis, Rosmarinus officinalis*, and *Thymus vulgaris* were discovered by Tanalp et al. to have inhibitory effects [21]. In another study, it was reported that five different essential oils extracted from plants showed inhibitory effects on drug-resistant clinical *H. pylori* isolates [22]. Likewise, Ohno et al. observed that thirteen essential oils totally prevented *H. pylori* from growing *in vitro* [23]. In our study, MIC analysis of 12 vegetable oils on *H. pylori* NTCC 11637 standard strain showed that all of them had inhibitory antibacterial effects.

Table 2. Antimicrobial values of vegetable oils on tested microorganisms

	Oils	Minimal Inhibitory Concentrations (μg/ml)	Agar-Well Diffusion (μl)
1	Jasmine oil	62.5	60
2	Cumin oil	15.625	20
3	Cinnamon oil	62.5	20
4	Rosemary oil	15.625	40
5	Rosehip oil	62.5	40
6	Indian oil	15.625	40
7	Ylang ylang oil	62.5	20
8	Jojoba oil	62.5	20
9	Safflower oil	62.5	20
10	Cardamom oil	31.25	60
11	Lily oil	62.5	40
12	Nettle oil	62.5	20

Antimicrobial Activity Results by Agar-Well Diffusion Method

With the agar well technique, like the MIC results, all oils were effective, with cumin, cinnamon, ylang ylang, jojoba, safflower oil and nettle oil being the least effective (20 μl) (Table 2). Jamine and cardamom oil were active at high concentrations (60 μl). Cumin oil was found to be more effective in both the MIC and agar well diffusion techniques.

In a study using the disk diffusion method to examine the antibacterial qualities of vegetable oils against *H. pylori*, researchers found that 30 oils affected growth with different zones of inhibition of 60 essential oils. [24]. Again, Esmaili et al. evaluated the anti-*H.pylori* activities of two different oils by the agar diffusion method and found that they were effective at low concentrations [25]. In our study, all the oils we used by agar well method showed anti-*H.pylori* activity.

Evaluation of the Antibiofilm Activity of Vegetable Oils on *H. pylori*

The inhibitory effect of vegetable oil on *H. pylori* biofilm is shown in Figure 1. Vegetable oil concentrations ranging from 250 μg/ml (2 MIC) to 15.625 μg/ml (MIC) markedly suppressed *H. pylori* biofilm formation. In the antibiofilm activity experiment, Cumin, Cinnamon, Rosemary, Ylang ylang, Jojoba and Nettle oils gave the lowest concentration of 15.625 μg/ml and Rosehip oil gave the highest concentration of 125 μg/ml.

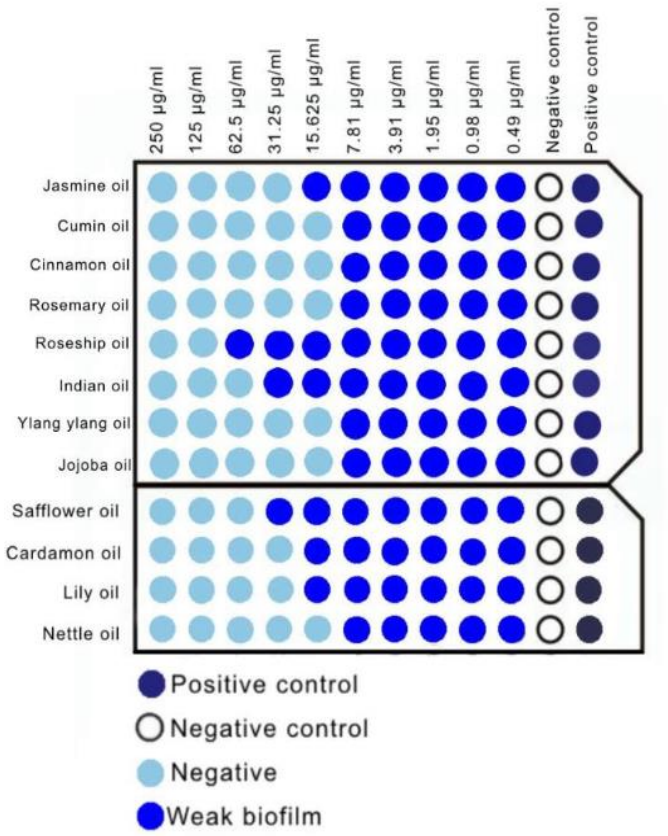

Figure 1. Antibiofilm results

Due to its capacity to create biofilms both within and outside of human hosts, *H. pylori* may offer better protection in arid environments. According to Yu et al., the *H. pylori* NCTC11637 strain's biofilm production was inhibited by Atractylodes lancea volatile oil [26]. Elbestawvy et al. also reported that Eugenol essential oil had an antibiofilm effect on *H. pylori* bacteria at 25 - 50 µg/ml [27]. In this study, we found that all the oils we examined for antibiofilm activity had a significant effect on *H. pylori*.

All these challenges to getting rid of *H. pylori* suggest that other treatments, such as traditional medicine, need to come into play. In this investigation, we investigated the anti-*H. pylori* efficacy of vegetable oils, which we think may be important for the treatment of *H. pylori* , which is difficult due to the fact that 50% of the population is infected and resistance to antimicrobial agents has increased significantly. Taken collectively, our findings imply that vegetable oils, a conventional pharmaceutical substance that is accessible, affordable, and low-cost, would be a potential option for a new class of *H. pylori* inhibitor drugs with anti-*H. pylori* efficacy. We think that more *in vitro*, *in vivo*, and molecular studies investigating antimicrobial mechanisms, especially for drug-resistant isolates, should be carried out in order to be used safely for treatment in humans. In addition, when the literature was reviewed, it was noted that there were not many studies examining the effects of vegetable oils on biofilm formation, and more studies are needed in this regard.

AUTHOR CONTRIBUTIONS

Concept: E.A.; Design: E.A.; Control: E.A.; Sources: E.A.; Materials: E.A.; Data Collection and/or Processing: E.A.; Analysis and/or Interpretation: E.A.; Literature Review: E.A.; Manuscript Writing: E.A.; Critical Review: E.A.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 826-834, 2024
Doi: 10.33483/jfpau.1458525

ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARISON OF ESTIMATED GLOMERULAR FILTRATION RATE USING DIFFERENT FORMULAS IN TURKISH POPULATION

TAHMİNİ GLOMERÜLER FİLTRASYON HIZININ FARKLI FORMÜLLERLE TÜRK POPÜLASYONUNDA KIYASLANMASI

Büşra Nur ÇATTIK[1]*, Rashida Muhammad UMAR[1]

[1]Istanbul Medipol University, Faculty of Pharmacy, Department of Clinical Pharmacy, 34815, Istanbul, Turkiye

ABSTRACT

Objective: *Creatinine-based equations are generally used in clinical practice to estimate glomerular filtration rates (GFR), but values are not usually consistent. This study aimed to evaluate the difference between estimated GFR values using different equations.*
Material and Method: *Adult Turkish patients with serum creatinine measurements between January to December 2021 and complete demographic data were included. GFR values were calculated using 5 different formulas. GFR calculated with Cockcroft-Gault were normalized to body surface area and added to the comparison. Difference between GFR values and KDIGO stages were evaluated. Albunin/creatinine ratio (ACR) of patients was also assessed.*
Result and Discussion: *A total of 305 patients with average age of 52.92 years were included. Six different GFR calculations were recorded with median values between 51.70 to 71.77 ml/min/1.73m^2. Formula of The Modification of Diet in Renal Disease with the race factor for Turkish population resulted in the lowest eGFR values. The ACR values of only 42 patients were available and it was negatively correlated to all GFR values and positively correlated to all KDIGO stages ($p<0.05$). There were noteworthy variations in GFR values, based on patient demographics and/or equations. The need for novel practical methods for estimating GFR in general and specific patient populations are necessary.*
Keywords: *Creatinine clearance, eGFR variations, GFR calculation, glomerular filtration rate, kidney function*

ÖZ

Amaç: *Glomerüler filtrasyon hızlarını (GFR) tahmin etmek için klinik uygulamada genellikle kreatinin bazlı formüller kullanılır, ancak değerler genellikle tutarlı değildir. Bu çalışmanın amacı farklı formüller kullanarak eGFR değerleri arasındaki farkı değerlendirmektir.*
Gereç ve Yöntem: *Ocak-Aralık 2021 tarihleri arasında serum kreatinin ölçümü yapılan ve demografik verileri eksiksiz olan yetişkin Türk hastalar çalışmaya dahil edildi. GFR değerleri 5 farklı formül kullanılarak hesaplandı. Cockcroft-Gault ile hesaplanan GFR, vücut yüzey alanına göre normalize edilerek karşılaştırmaya eklendi. GFR değerleri ile KDIGO evreleri arasındaki fark değerlendirildi. Hastaların albumin/kreatinin oranı (ACR) da değerlendirmeye alındı.*
Sonuç ve Tartışma: *Ortalama yaşı 52.92 yıl olan toplam 305 hasta çalışmaya dahil olmuştur. Ortanca değerleri 51.70 ila 71.77 ml/dak/1.73m^2 arasında değişen altı farklı GFR hesaplaması kaydedilmiştir. Türk popülasyonu için ırk kastsayısı içeren The Modification of Diet in Renal Disease formülü en düşük eGFR değerleri ile sonuçlanmıştır. Sadece 42 hastanın ACR verisi*

* **Corresponding Author / Sorumlu Yazar:** Büşra Nur Çattık
e-mail / e-posta: bncattik@medipol.edu.tr, **Phone / Tel.:** +902164448544

Submitted / Gönderilme : 25.03.2024
Accepted / Kabul : 15.05.2024
Published / Yayınlanma : 10.09.2024

bulunmuştur ve bu değerleri tüm GFR değerleri ile negatif, tüm KDIGO evreleri ile pozitif korelasyon göstermiştir ($p<0.05$). Hasta demografisine ve/veya denklemlere bağlı olarak GFR değerlerinde kayda değer farklılıklar görülmüştür. Genel ve spesifik hasta popülasyonlarında GFR'yi tahmin etmek için yeni pratik yöntemlere ihtiyaç vardır.
Anahtar Kelimeler: *Böbrek fonksiyonu, eGFR varyasyonları, GFR hesaplaması, glomerüler filtrasyon hızı, kreatinin klerensi*

INTRODUCTION

Glomerular filtration rate (GFR) is the indirect measurement of functional nephrons. It is considered the best overall measure of kidney function. A decline in GFR represents a malfunction of the excretion capacity which may be directly or indirectly related to the kidneys. But it is not correlated with kidney mass loss. The normal GFR value ranges between 90 to 120 ml/min/1.73 m^2 but it is considerably variable among individuals as it depends on age, sex, and body size. Measured GFR (mGFR) is the most accurate method of assessment [1]. But it is less convenient and may involve the administration of exogenous filtration markers excreted exclusively by the kidneys. In clinical setting, estimated GFR (eGFR), calculated from endogenous biomarkers excreted mostly by the kidneys, is preferred as a more convenient way of determining baseline kidney function, diagnosing kidney disease, evaluating kidney disease progression and dosing medications [2,3].

Creatinine and Cystatin-c are efficiently used endogenous filtration markers with some limitations attached to their use. In clinical practice, creatinine is more commonly used but variations in production and secretion mainly affected by individual patient characteristics, extrarenal excretion, and measurement issues are the core limitations to the use of creatinine. Cystatin C levels are also variable and its higher levels are associated with male sex, greater height and weight, higher lean body mass, higher fat mass, diabetes mellitus, higher levels of inflammatory markers, hyper- and hypothyroidism, and glucocorticoid use [3]. The equations that contain both markers give the most accurate GFR estimates [3,4]. But this is not always feasible in most clinical settings and creatinine-based estimates remain the primary approach.

Some creatinine-based estimating equations include the 2009 chronic kidney disease epidemiology (CKD- EPI) equation, the Modification of Diet in Renal Disease (MDRD) study equation, the Cockcroft-Gault (CG) equation and the recently published CKD-EPI 2021. All equations incorporate serum creatinine concentrations with different patient variables like age, weight, sex, and race. The inclusion of different variables in the different equations leads to variations in GFR values. As such the accuracy of all the equations is not universal in all patient populations. Certain formulas provide more accurate results in certain patient groups or kidney function range.

The CG equation was developed to determine creatinine clearance. Drug dosing is based on the CG as it was used in pharmacokinetic studies to establish drug dosing in kidney dysfunction [5]. But it was developed before the standardization of creatinine assays and has not been revised to suit updated versions. This leads to overestimation when used with creatinine values measured by most laboratories. Furthermore, the accuracy of eGFR values is affected by variations in body weight and body mass index [6,7]. When using the CG equation, the use of actual body weight in underweight patients, ideal body weight in patients with normal weight and adjusted body weight (0.4 correction) for overweight and obese patients give more accurate and less biased GFR values [7].

The MDRD was developed from nondiabetic patients' data, and it excludes patient weight in estimations. The equation has been re-evaluated to be used with standardized creatinine measurements and was considered to be more accurate than CG [8,9]. The chronic kidney disease epidemiology (CKD-EPI) equation was first published in 2009 and recently modified in 2021 to exclude the race parameter [10,11]. The estimation of GFR in the Turkish population was evaluated in a previous study and MDRD was established as the most suitable equation. A race factor of 0.804 was also suggested to get more accurate GFR values in this population [12]. But there is no evidence of the integration of this equation in clinical practice.

Recommendations are now put in place for the use of the most accurate method in establishing GFR for individual patients. The American Society of Nephrology (ASN) and the National Kidney

Foundation (NKF) recommend the use of the CKD-EPI 2021 equation in most clinical settings. It is considered accurate and acceptable among different populations. Though it is slightly less accurate than the CKD-EPI 2009 as it underestimates mGFR in blacks and overestimates mGFR in other individuals [3].

In this study, we aimed to investigate the difference between eGFR levels calculated using CG, MDRD, CKD-EPI equations in addition to the newly recommended CKD-EPI 2021 equation and MDRD Turkish version with the race factor of 0.804.

MATERIAL AND METHOD

In this retrospective study, adult Turkish patients with serum creatinine measurements between January and December 2021 were searched from the online record system of the Medipol Mega University Hospital in Istanbul. Patients with required demographic (age, weight, height) and health-related data were included in the study. Five serum creatinine-based equations were used to calculate the glomerular filtration rates of all patients. These equations include CG, MDRD, MDRD-TR, CKD-EPI 2009 and CKD-EPI 2021 (Table 1). In the CG equation, the adjusted body weight (0.4 correction) was used for obese patients [2,7]. GFR calculated with CG were normalized to body surface area for all patients to compare with GFR values calculated with other equations [3] and reported separately as CG-BSA. The urine albumin/creatinine ratio (ACR) was also assessed. The Kidney Disease: Improving Global Outcomes (KDIGO) kidney function classification was used to classify patients' GFR values, and ACR [13]. The GFR values and stages were compared and variations between equations were analysed.

Table 1. GFR estimation equations

Formulae	**Equation**
CG (Creatinine clearance measurement, ml/min)	(140 x age) x weight (kg)/72 x Scr (x 0.85 if female)
MDRD (GFR measurement, ml/min per 1.73 m^2)	175 x $(Scr)^{-1.154}$ x $(age)^{-0.203}$ x (0.742 if female) x (1.212 if African American)
MDRD-TR (GFR measurement, ml/min per 1.73 m^2)	175 x $(Scr)^{-1.154}$ x $(age)^{-0.203}$ x (0.742 if female) x 0.804
CKD-EPI Creatinine Equation (2009) (GFR measurement, ml/min per 1.73 m^2)	$A \times (Scr/B)^{C} \times 0.993^{age} \times$ (1.159 if black) Where: A and B are the following Female: for Scr ≤0.7, A = 144, B = 0.7, C = -0.329; and Scr >0.7, A = 144, B = 0.7, C = -1.209 Male: for Scr ≤0.9, A = 141, B = 0.9, C = -0.411; and Scr >0.9, A = 141, B = 0.9, C = -1.209
CKD-EPI Creatinine Equation (2021) (GFR measurement, ml/min per 1.73 m^2)	142 x $(Scr/A)^{B}$ x 0.9938^{age} x (1.012 if female) Where: A and B are the following Female: for Scr ≤0.7, A = 0.7, B = -0.241 and Scr >0.7, A = 0.7, B = -1.2 Male: for Scr >0.9, A = 0.9, B = -1.2 and Scr >0.9, A = 0.9, B = -1.2

CG: Cockcroft-Gault; CKD- EPI: Chronic Kidney Disease Epidemiology Equation; MDRD: Modification of Diet in Renal Disease; MDRD-TR: Modification of Diet in Renal Disease Turkish version Scr: serum creatinine

SPSS Version 25.0 was used for statistical analysis. The Kolmogorov-Smirnov test was done to determine the distribution pattern. Normal distributed continuous variables were expressed as mean ± standard deviation while not normally distributed variables were presented as median and interquartile range and ordinal and nominal data were expressed as n (%). Spearman's correlation analysis was used to analyze the relationship between continuous variables. Friedmann and Wilcoxon's tests were used to assess the difference between GFR estimates. The Mann-Whitney U test was used to analyze the

difference between eGFR median values between different groups. A *p*-value < 0.05 within a confidence interval of 95% was considered significant.

RESULT AND DISCUSSION

A total of 305 patients with complete demographic data were included in the study. The average age of patients was 52.92 years. Most (59%) of the patients were male. The average weight, BMI and serum creatinine level were 80.45 kg, 29.20 kg/m^2 and 1.68 mg/dl respectively. The adjusted body weight was calculated for 119 obese patients (BMI > 30kg/m^2). The diagnosis of acute kidney injury (AKI) and chronic kidney disease (CKD)was present in 18 and 42 patients respectively. Four patients with CKD had an AKI diagnosis. Patient demographic and health details are given in Table 2.

Table 1. Patients' demographic and health related data

Characteristics	**Median (Quartile1-Quartile3)**
Age (years)	53 (41-67)
Weight (kg)	80 (67.5-91)
Height (cm)	167 (160-173)
Body mass index (kg/m^2)	28.4 (24.7-32.7)
Body surface area (m^2)	1.92 (1.75-2.07)
Serum creatinine	1.09 (0.78-1.82)
	n (%) **305 (100)**
Gender	
Male	180 (59)
Female	125 (41)
Age group	
18-29	25 (8.2)
30-59	161 (52.8)
>60	119 (39.0)
Patients with kidney disease diagnosis	
Acute kidney diseases	18 (6)
Chronic kidney disease	42 (14)
Both	4 (1)
None	241 (79)
Weight status	
Under-weight	10 (3.3)
Normal	74 (24.3)
Over-weight	102 (33.4)
Obese	119 (39.0)

The median values of eGFR calculated using CG-ADJ, CG-BSA, MDRD, MDRD-TR, CKD-EPI 2009 and CKD-EPI 2021 equations were 70.83 ml/min, 65.82, 64.30, 51.70, 71.14 and 71.77 ml/min/1.73m^2 respectively. Similar eGFR values were obtained from CKD-EPI 2009 and CKD-EPI 2021. The lowest eGFR values were obtained from the MDRD-TR equation.

According to the KDIGO classification, 108 and 109 of the patients were in G1 stage based on the CKD-EPI 2009 and CKD-EPI 2021, while only 32 patients were in this category based on MDRD-TR. The number of patients in stage G3a was similar based on all equations. Based on MDRD-TR equation, 43 patients were in G5 stage while only 17 and 22 patients were in this category based on CG and CG-BSA respectively. The distribution of patients' KDIGO stages is given in Table 3.

Table 2. Distribution of patients based on their Kidney function stages and the presence of kidney disease

Formular	GFR Median (Quartile1-Quartile3)	KDIGO stages	Number of Patients (n)	Presence of kidney disease diagnosis				BMI			
				None (241)	AKI (18)	CKD (42)	Both (4)	<18.5 (10)	18.5-25 (74)	25-30 (102)	>30 (119)
CG	70.83 (40-111) ml/min	G1	115	113	2	0	0	1	25	42	47
		G2	71	68	1	2	0	4	15	28	24
		G3a	33	27	2	4	0	1	14	5	13
		G3b	31	19	6	5	1	1	5	11	14
		G4	38	12	5	20	1	1	8	12	17
		G5	17	2	2	11	2	2	7	4	4
CG-BSA	65.82 (36-98) ml/min/1.73 m^2	G1	95	92	2	1	0	3	25	35	32
		G2	76	76	0	0	0	2	16	29	29
		G3a	43	35	3	5	0	2	12	11	18
		G3b	27	20	4	3	0	0	7	8	12
		G4	42	16	6	18	2	2	7	14	19
		G5	22	2	3	15	2	1	7	5	9
MDRD	64.30 (37-92) ml/min/1.73 m^2	G1	86	83	2	1	0	3	22	28	33
		G2	79	79	0	0	0	3	17	28	31
		G3a	44	41	1	2	0	1	12	16	15
		G3b	34	21	6	7	0	0	8	11	15
		G4	33	11	5	15	2	1	8	8	16
		G5	29	6	4	17	2	2	7	11	9
MDRD-TR	51.70 (29-74) ml/min/1.73 m^2	G1	32	30	2	0	0	1	10	9	12
		G2	98	97	0	1	0	4	19	36	39
		G3a	45	43	1	1	0	2	16	13	14
		G3b	53	44	4	5	0	0	13	20	20
		G4	34	19	5	9	1	1	4	11	18
		G5	43	8	6	26	3	2	12	13	16
CKD-EPI 2009	71.14 (36-100) ml/min/1.73 m^2	G1	108	105	2	1	0	4	26	35	43
		G2	65	63	1	1	0	3	17	22	23
		G3a	40	37	0	3	0	0	11	15	14
		G3b	29	18	6	5	0	0	6	9	14
		G4	31	11	5	13	2	1	5	9	16
		G5	32	7	4	19	2	2	9	12	9
CKD-EPI 2021	71.77 (37-101) ml/min/1.73 m^2	G1	109	106	2	1	0	4	26	36	43
		G2	67	65	1	1	0	3	18	23	23
		G3a	39	36	0	3	0	0	10	14	15
		G3b	27	16	6	5	0	0	6	8	13
		G4	31	11	5	13	2	1	5	9	16
		G5	32	7	4	19	2	2	9	12	9

AKI: Acute kidney injury; BMI: Body mass index; CG: Cockcroft-Gault; CG-BSA: Cockcroft-Gault normalized to body surface area; CKD: Chronic kidney disease; CKD-EPI: Chronic Kidney Disease Epidemiology Equation; GFR: glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD-TR: Modification of Diet in Renal Disease Turkish version

The Mann-Whitney U was used to analyze the difference between eGFR median values and patients' BMI and age groups. There was a significant difference between the >60 age group and the other two age groups (p<0.001). There was no difference among BMI subgroups. The distribution of

patients based on KDIGO stages, their BMI and the presence of kidney disease is given in Table 3. There were two patients in G1 stage with AKI diagnosis based on all equations and one patient with CKD diagnosis based on CG-BSA, MDRD and both CKD-EPI equations. The distribution of patients with kidney disease diagnosis was similar in the G5 stage among all the equations but the highest number (n=35) of patients was based on the MDRD-TR equation.

A significant difference (p<0.001) was found between all equations when the KDIGO stages were compared. Wilcoxon test was done to clarify further the disparity between the equations. There was significant difference between all equations except between both CKD-EPI equations, and between these two equations and CG-BSA (Table 4). But when the test was repeated based on patients' BMI, a significant difference was also recorded between both CKD-EPI and CG-BSA in obese patients (p<0.008). There was no difference between CG and CG-BSA in patients with BMI<30. On the contrary, no difference was recorded between CG-BSA and MDRD (p=0.162) in obese patients.

Table 3. Variation in patients' eGFR stages calculated with different equations

KDIGO Stages	CG	CG-BSA	MDRD	MDRD-TR	CKD-EPI 2009	CKD-EPI 2021	Friedmann test p value
CG		<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	p<0.001
CG-BSA	<0.001*		0.003*	<0.001*	0.515	0.159	
MDRD	<0.001*	0.003*		<0.001*	<0.001*	<0.001*	
MDRD-TR	<0.001*	<0.001*	<0.001*		<0.001*	<0.001*	
CKD-EPI 2009	<0.001*	0.515	<0.001*	<0.001*		0.014	
CKD-EPI 2021	<0.001*	0.159	<0.001*	<0.001*	0.014		

*p<0.008 was considered significant (Bonferroni correction 0.05/6=0.008) CG: Cockcroft-Gault; CG-BSA: Cockcroft-Gault normalized to body surface area; CKD-EPI: Chronic Kidney Disease Epidemiology Equation; KDIGO: Kidney Disease: Improving Global Outcomes; MDRD: Modification of Diet in Renal Disease; MDRD-TR: Modification of Diet in Renal Disease Turkish version

The ACR was recorded for only 42 patients. The ACR of 17 patients (40.5%) was below 30 and that of 11 patients was above 300. Four of these patients had CKD and one had both AKI and CKD diagnosis (Table 5). Spearman's correlation showed a significant negative correlation between ACR and eGFR values (p<0.05) calculated using all equations with CG having the largest coefficient (-0.380) and CKD-EPI 2021 having the smallest (-0.450). Likewise, there was a positive correlation between ACR stages and all eGFR stages (p<0.05) with coefficients of 0.357, 0.392, 0.425, 0.418, 0.466, and 0.434 for CG, CG-BSA, MDRD, MDRD-TR, CKD-EPI 2009 and CKD-EPI 2021 respectively.

GFR is an essential information in the assessment of kidney function and a main determinant of drug dosage in patients with kidney dysfunction. The accurate estimation of the GFR is critical and has been a matter of debate as different equations yield variable results in diverse patient populations. The performance of equations is based on patient-related factors which have variable extent of influence on eGFR [1]. Therefore, the selection of the most suitable equation for a particular patient subset is important. In this study, we evaluated the discrepancy in GFR values calculated using different equations in Turkish patients.

MDRD is said to have an accuracy close to that of CKD-EPI [6] and is reported to be less dispersed than CG [14]. It was reported to provide the best estimate in patients with different health issues [15] and different races including the Turkish populace [12]. Altiparmak reported that the addition of a race factor of 0.804 to the 4-variable MDRD resulted in a more accurate estimation of GFR in the Turkish population and, also produced a better classification performance over various GFR equations [12]. We evaluated this formula in addition to CG, MDRD, CKD-EPI 2009 and the newly recommended CKD-EPI 2021. The eGFR values calculated using the revised MDRD-TR equation were significantly lower compared to those calculated using other equations and most patients with kidney disease diagnosis were placed in lower KDIGO stages based on this equation. We recorded a significant

difference between the MDRD and MDRD-TR. Although our study population were relatively similar to that of Altıparmak, in terms of demographic data, the average serum creatinine concentration of our patients was lower. This may have affected our results as they reported that MDRD overestimated in patients with GFR values below 30 ml/min/1.73m^2 and underestimated in others.

Table 4. Distribution of patients based on their ACR

	Frequency (n)	**A1 17**	**A2 14**	**A3 11**
Sex				
Male	28	13	7	8
Female	14	4	7	3
Age group				
18-29	3	1	2	0
30-59	19	7	5	7
>60	20	9	7	4
BMI				
<18.5	1	1	0	0
≥18.5 - <25	8	4	3	1
≥25- <30	15	5	4	6
≥30	18	7	7	4
Presence of kidney disease				
None	34	16	13	5
AKI	2	0	1	1
CKD	5	1	0	4
Both	1	0	0	1

ACR: Albumin creatinine ratio, AKI: Acute kidney injury; BMI: Body mass index; CKD: Chronic kidney disease

The CKD-EPI has been considered the best estimation equation in different populations [6]. In a study involving Turkish participants, 6-variable MDRD and CKD-EPI were found to demonstrate the best performance in estimating GFR in reference to 24-hour creatinine clearance [16]. In our study, the eGFR values from both CKD-EPI equations were statistically similar to CG-BSA with comparable median values. The median GFR from MDRD and MDRD-TR were significantly lower. We used the 4-variable MDRD in place of the 6-variable, this may have affected our results. This further highlights the importance of variability among the equations. Another study reported better performance of CG with lean body weight over MDRD and CKD-EPI in patients with metabolic diseases [17]. But the inaccuracy of all formulas to reveal a decline in GFR in patients has been emphasized [18].

GFR estimating formulas have failed to be fully accurate in overweight and obese patients and the choice of equation has been a controversial issue. Some researchers have considered the use of CG with lean body weight to be more appropriate [19,20] while others consider MDRD and CKD-EPI in patients with stable kidney function, especially for dose adjustments [2]. To compare GFR values calculated using CG with that from other formulas, adjustment to BSA is recommended [14], but this was reported to lead to underestimation of GFR in this patient population, which may have serious health consequences [21]. In our study, there was a noticeable shift in the distribution of patients based on the KDIGO stages when CG GFR values were adjusted to BSA as the GFR values dropped after adjustment. A significant proportion of our study population was overweight or obese. In the obese patients, the adjusted CG GFR values became similar to only that calculated using MDRD. After adjustment, while the difference in non-obese patients between CG and CG-BSA was neutralized, it persisted in obese patients. Routine use of gold-standard measurement methods may be indicated particularly in this patient population.

The ACR measured in spot urine is used to measure albuminuria and used to assess kidney function. In our study, this ratio was checked in only a few patients, and it correlated to patients' eGFR values and KDIGO stages. The association of lower eGFR and higher ACR in determining the risk of kidney injury in patients with or without chronic diseases is consistent [22,23]. The use of both eGFR

and ACR to assess kidney function in all general populations may provide better insight into the general kidney status although the ACR is not translated to GFR and cannot be directly used in drug dose modifications.

Our study has limitations in generalizing the results to the Turkish population, such as being conducted retrospectively with limited data and at a single center. We were not able to determine the most accurate estimation equation for the Turkish population as patients' measured GFR values were not available to be used as a standard reference. The previously recommended MDRD revised for the Turkish population resulted in the lowest GFR estimates; therefore, further research is needed.

The most accurate GFR measurement methods are not practical in clinical practice and estimation methods using endogenic biomarkers are more readily used. Our results show valuable variation in estimations based on patient demographics and/or equations. The need for novel practical methods for estimating glomerular filtration rates in general and specific patient populations are urgently needed.

AUTHOR CONTRIBUTIONS

Concept: R.M.U.; Design: B.N.Ç., R.M.U.; Control: R.M.U. Sources: - ; Materials: - ; Data Collection and/or Processing: B.N.Ç.; Analysis and/or Interpretation: B.N.Ç., R.M.U.; Literature Review: B.N.Ç., R.M.U.; Manuscript Writing B.N.Ç., R.M.U.; Critical Review: B.N.Ç., R.M.U.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The institutional review board approved this study (No:2022-389) and waived consent.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 835-839, 2024
Doi: 10.33483/jfpau.1473405

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION OF THE PREVALENCE OF EXPIRED AND UNUSED PHARMACEUTICALS IN THE ANKARA REGION

ANKARA BÖLGESİNDE SON KULLANMA TARİHİ GEÇMİŞ VE KULLANILMAMIŞ İLAÇLARIN YAYGINLIĞININ ARAŞTIRILMASI

Ongun Mehmet SAKA[1]*

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06560, Ankara, Türkiye

ABSTRACT

Objective: *Due to the increase in the market share of pharmaceuticals and the unconscious use of medicines, unused or expired medicines are being discarded in a way that harms the environment. This study aims to examine which medicines are brought to collection points as household waste in order to reduce waste medicines from the source.*
Material and Method: *The medicines collected in 12 spontaneously selected pharmacies operating in low and high income levels in the Ankara region were recorded at different periods during one year and the results were statistically evaluated. 4852 waste medicines were sorted and classified according to pharmaceutical dosage form and indication*
Result and Discussion: *Unopened 521 medicines were discarded. In addition, it was seen which drug groups create waste in socio-economic terms. In addition to the damage to the environment, it has created a foresight to prevent drug waste that creates a deficit in the budget.*
Keywords: *Environmental awareness, pharmaceutical dosage form, rational drug use, waste medicines*

ÖZ

Amaç: *İlaç Pazar payının artması ve bilinçsizce kullanılan ilaçlardan dolayı, kullanılmayan veya son kullanım tarihi geçmiş ilaçlar çevreye zarar verecek şekilde atılıyor. Bu çalışmada atık ilaçları kaynağından azaltma amacı ile hangi ilaçların evsel atık olarak toplama noktalarına getirildiğini incelemektir*
Gereç ve Yöntem: *Ankara bölgesinde, düşük ve yüksek gelir düzeyinde görev yapan spontan olarak seçilen 12 adet eczanede toplanan ilaçlar, bir yıl boyunca farklı dönemlerde kayıt altına alınıp, sonuçları istatiksel olarak değerlendirilmiştir. 4852 adet atık ilaç tasnif edilmiş ve farmasötik dosaj formu ve endikasyonuna göre sınıflandırılmıştır*
Sonuç ve Tartışma: *521 adet ilaç hiç açılmadan atılmıştır. Ayrıca sosyo-ekonomik açıdan hangi ilaç grupların atık oluşturduğu görülmüştür. Çevreye verilen zararın yanında bütçede açık oluşturan ilaç atığını engellemek için bir ön görü oluşturmuştur.*
Anahtar Kelimeler: *Akılcı ilaç kullanımı, atık ilaç, çevre bilinci, farmasötik dozaj form*

INTRODUCTION

Pharmaceuticals are a combination of materials designed to produce a therapeutic effect on humans or animals. They also have myriads of uses including disease prevention, cosmetics, diagnosis,

* **Corresponding Author / Sorumlu Yazar:** Ongun Mehmet Saka
e-mail / e-posta: omsaka@gmail.com, **Phone / Tel.:** +903122033161

Submitted / Gönderilme : 25.04.2024
Accepted / Kabul : 22.05.2024
Published / Yayınlanma : 10.09.2024

and lifestyle. These pharmaceutics help cure diseases that save lives, however they also ultimately lead to environmental contamination [1]. Pharmaceutical waste contaminants are increasing with an increase in the rate of drug production. Residues from hundreds of widely used active pharmaceutical ingredients can gain entry into the environment [2,3]. The disposal of unwanted, leftover medications to sewage and trash is the primary source of drug pollution. Thus, humans may be exposed to long-term or intermittent exposure to pharmaceutical contaminants, generally through contaminated water and foods.

The disposal of leftover medications poses an acute exposure hazard for both humans and the environment. The increases in pharmaceutical trade and the unsanitary disposal of pharmaceutical waste could pose acute and chronic poisoning risks to humans and wildlife [4,5]. Currently, there is no specific regulation regarding the management of waste pharmaceuticals in our country. However, 'the zero waste regulation' explains how pharmaceutical waste will be collected. According to this regulation, healthcare services such as hospitals, pharmacies and pharmaceutical warehouses are responsible for their pharmaceutical waste. Conversely, municipalities are responsible for the management of waste medicines generated by households [6].

The disposal of pharmaceutical waste in an unconscious manner, for example by pouring it into sinks, directly affects the environment and human health. These unconsciously discarded waste medicines cause contamination of all water resources and thus soil with chemicals. To prevent this situation, such wastes should be disposed of under control. The environmentalists and researchers have advocated the institutionalisation of 'zero waste' as a societal goal, with the intention of spurring waste management agencies to eliminate the need for waste medicine landfills. However, pharmaceutical waste can only be disposed of by incineration, as it is not possible to recycle or recover energy [7]. Therefore, the most reasonable approach is to prevent its generation, in according to the top of the waste pyramid.

According to the municipal sources of Ankara, where our study will be conducted, approximately 8.000 tons of medical waste were disposed of in 2011. In 2020, the pharmaceutical waste disposed of was 14.000 kilograms. In light of the economic burden that these leftover, unwanted pharmaceuticals will bring, it is imperative to prevent their occurrence.

In this study, in order to examine the drug use habits of the people of Ankara, waste medicines collected at random time periods in 15 pharmacies from 2 different eco-social zones were examined. During the course of a year, the waste medicines collected in different periods were classified according to their areas of use and how much of them were used was analyzed.

MATERIAL AND METHOD

Setting of the Study

This was a prospective, single-centre study conducted in two different socioeconomic population areas. The household pharmaceutical wastes collected from the public in different periods from a total of 12 pharmacies in two areas with different purchasing power were analysed. After receiving the necessary information, all waste was left in the collection unit.

Study Period

In 2020, the study was divided into winter and summer periods. A research was conducted on the medicines collected from two of the 12 pharmacies, selected at random, and examined every two months. During the examination, records were taken according to the following categories: indication groups, pharmaceutical dosage forms, and quantities used. For the purposes of comparison, the group with a high purchasing power was referred to as 'Group A' and the other group is referred to as 'Group B'.

RESULT AND DISCUSSION

During our study, 4.852 medicine boxes were examined. Table 1 presents the distribution of collected medicines by drug indication in both groups. Figures 1 and 2 illustrate the percentage distribution of waste medicines. Figure 2 compares the percentage distribution between the two groups.

Figure 3 summarizes data generated according to pharmaceutical dosage formulation. All figures were created in Excel software and comparisons were evaluated by the statistical module.

Table 1. Investigation result according to drug indications (n=4.852)

Group	Cold and Flu	Pain Killer	Antibiotics	Vitamins	Hormones	Cholesterol	Other
Group A	624 (25.59%)	692 (28.38%)	159 (6.52%)	428 (17.56%)	153 (6.28%)	103 (4.22%)	279 (11.44%)
Group B	728 (30.16%)	587 (24.32%)	138 (5.72%)	208 (8.62%)	357 (14.79%)	43 (1.78%)	353 (14.62%)

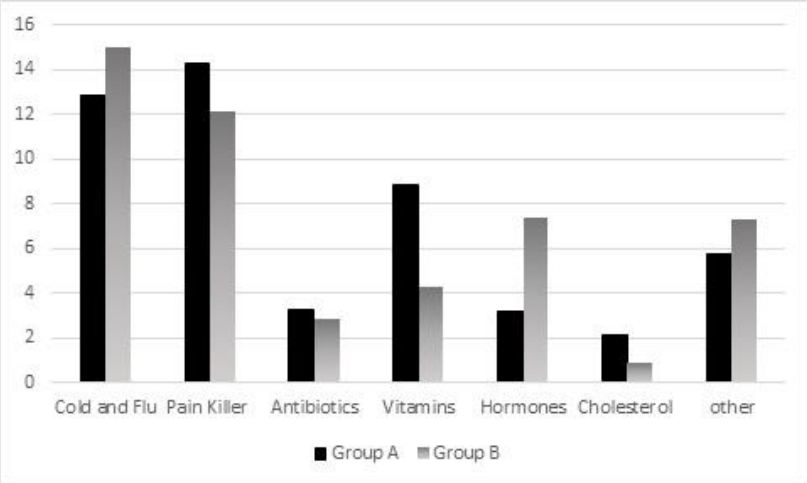

Figure 1. Percentage distribution of pharmaceutical waste according to their indications

According to the findings, although there was a 2% difference between cold medications and painkillers, no statistically significant difference was found ($p<0.05$). While the vitamin and statin group (cholesterol drugs) showed more disposal in the group with a high-income level, hormone drugs showed more disposal in the group with a lower income-level. The fact that the antibiotic group was low in both groups shows that restricting access to antibiotics in line with the rational drug policy implemented in our country is effective [5,7]. Since the period in which the research was conducted coincided with the COVID-19 pandemic, we see that the number of wastage of drugs used in cold and flu infections has increased. In this case, it is seen that our people increase their unnecessary drug intake due to their hoarding habits, and therefore the amount of waste drugs increases (15%). Therefore, different policies regarding rational drug use need to be developed.

It has also been observed that wastes belonging to the vitamin group are generally not pharmacy products. Considering that these products purchased over the internet discarded before they are used up, there is a need to control the sales of this group, whose reliability and effectiveness will be discussed. These products, which do not provide benefits or are not used for other reasons, must be licensed for sale by the necessary authorities under strict control.

When dosage forms of waste medicines are analyzed (Figure 3), it is seen that solid and liquid dosage forms take the first two places. In Group B, the amount of waste generated by liquid dosage forms is quite high. Considering that the majority of liquid dosage forms are in syrup form, it is concluded that the pediatric population is higher in this group. Wastes from creams or ointments, which constitute the semi-solid group, were found to be relatively less and in similar amounts in both groups. The fact that there were almost no products requiring a cold chain reveals that this group only took as much as they needed. Also most of collected drugs were expired (56.75%), and disposed with their original secondary container (46.25%). It is a matter of concern that 10.75% of the total quantity of waste pharmaceuticals were never used.

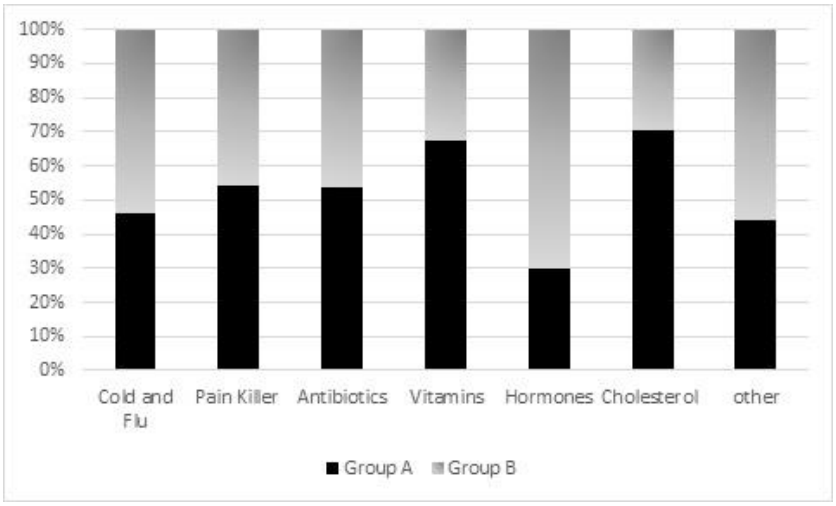

Figure 2. Percentage comparison between two groups of pharmaceutical waste according to indications

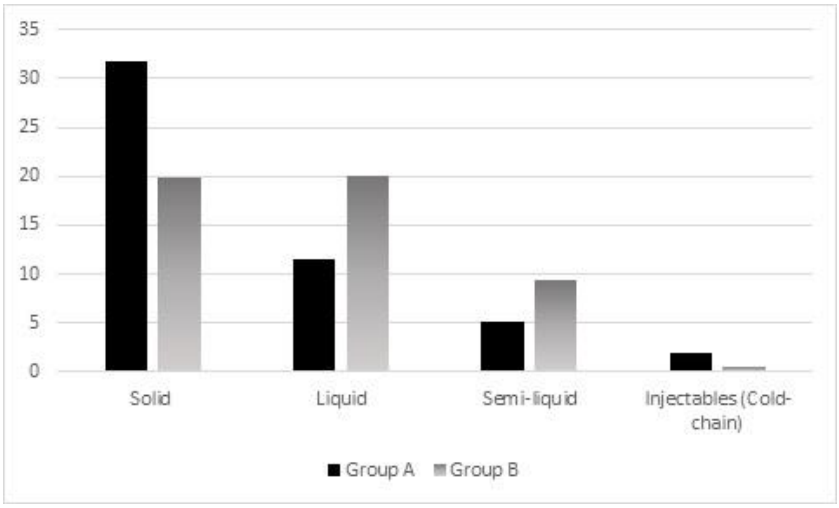

Figure 3. Percentage distribution of pharmaceutical waste according to their pharmaceutical dosage forms

Conclusion

Waste management is a crucial process for all countries. Methods such as incineration and underground burial are employed for non-recyclable wastes, yet these practices have the potential to exacerbate various environmental concerns. A review of studies conducted for the pharmacy and pharmaceutical industry revealed that waste pharmaceuticals are increasingly prevalent in Turkey, with limited disposal options. Unused or wasted pharmaceuticals represent a significant global challenge. The health sector and the consumption of health-related products are growing rapidly. Unfortunately, there is currently no process for the recovery of the waste drug molecule. Consequently, on-site separation and waste minimisation methods are being applied or attempted in Turkey and around the world with the aim of reducing pharmaceutical waste. This study aims to raise awareness and create preliminary studies for larger projects.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Mehmet Saydan, the head of Pharmabotanica Waste Drug

Management System, for his assistance in selecting a pharmacy and for sharing his expertise.

AUTHOR CONTRIBUTIONS

Concept: O.M.S.; Design: O.M.S.; Control: O.M.S.; Sources: O.M.S.; Materials: O.M.S.; Data Collection and/or Processing: O.M.S.; Analysis and/or Interpretation: O.M.S.; Literature Review: O.M.S.; Manuscript Writing: O.M.S.; Critical Review: O.M.S.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 840-852, 2024
Doi: 10.33483/jfpau.1434552

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DESIGN, SYNTHESIS, AND BIOLOGICAL ASSESSMENT OF NOVEL *N'*-(BENZYLIDENE)PROPANEHYDRAZIDES AS MTDL FOR ALZHEIMER'S DISEASE

ALZHEİMER HASTALIĞI İÇİN MTDL OLARAK YENİ N'-PROPANHİDRAZİTLERİN TASARIMI, SENTEZİ VE BİYOLOJİK DEĞERLENDİRMESİ

Burcu KILIÇ[1]*

[1]Gazi University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06330, Ankara, Türkiye

ABSTRACT

Objective: *In this study, following the multi-target directed ligands (MTDLs) strategy and drawing inspiration from the neuroprotective structure of ferulic acid, eight novel N'-(benzylidene)propanehydrazide derivatives were designed, synthesized, and tested to evaluate their cholinesterase inhibitory and antioxidant capacities.*

Material and Method: *To obtain the final compounds, first, corresponding key intermediates, 3-(substitutedamino)propanehydrazides, were prepared by the hydrolysis with hydrazine hydrate of methyl 3-(substitutedamino)propanoate intermediates. These intermediates had been prepared from the Michael addition of methyl acrylate and commercially available tertiary amine derivatives. Subsequently, the final compounds were synthesized from the reaction of the starting compounds 4-hydroxybenzaldehyde or 4-methoxybenzaldehyde and the corresponding key intermediates. Structural analysis of the synthesized and purified compounds was carried out using ^{1}H-NMR, ^{13}C-NMR, and HRMS. Then, all the final compounds were examined for their cholinesterase inhibitory effect using the modified Ellman method, their antioxidant effect using the DPPH and ORAC methods, and their metal chelator effect using UV-spectroscopy analysis. Moreover, physicochemical parameters were calculated using QikProp Schrödinger Suite 2023 to predict the druggability of all compounds.*

Result and Discussion: *Seven of the eight final compounds exhibited moderate cholinesterase inhibition at varying rates. Compounds* ***2a*** *(IC_{50} = 12.83 µM) and* ***2d*** *(IC_{50} = 16.02 µM) were identified as the most potent inhibitors for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively. Moreover, all the final compounds exhibited antioxidant activity in the ORAC assay. Chelator effects of all compounds were also observed for Cu(II), Fe(II), and Zn(II) ions at varying rates. Additionally, the final compounds demonstrated acceptable lead-like properties according to in-silico predictions.*

Keywords: *Acylhydrazone, antioxidant, Alzheimer's disease, cholinesterase inhibition, metal-chelator*

ÖZ

Amaç: *Bu çalışmada, çoklu hedefe yönelik ligand (MTDL) stratejisi izlenerek ve nöroprotektif ferulik asit yapısından ilham alınarak, sekiz yeni N'-(benziliden)propanhidrazit türevleri tasarlandı, sentezlendi, kolinesteraz inhibitör ve antioksidan kapasitelerini değerlendirmek üzere test edildi.*

Gereç ve Yöntem: *Sonuç bileşiklerin eldesi için ilk olarak, kilit ara ürünler olan 3-*

* **Corresponding Author / Sorumlu Yazar:** Burcu Kılıç
e-mail / e-posta: burcukahya@gazi.edu.tr, **Phone / Tel.:** +903122023230

Submitted / Gönderilme : 11.02.2024
Accepted / Kabul : 04.06.2024
Published / Yayınlanma : 10.09.2024

(amino)propanhidrazitler, metil 3-(sübstitüeamino)propanoat ara ürünlerinin hidrazin hidrat ile hidrolizinden hazırlandı. Bu ara ürünler ise metil akrilat ve ticari olarak mevcut tersiyer amin türevlerinin Michael katım tepkimesinden hazırlanmıştır. Hazırlanan kilit ara ürünlerin, seri hareket bileşikleri olan 4-hidroksibenzaldehit veya 4-metoksibenzaldehit ile katım tepkimesinden sonuç bileşikler sentezlenmiştir. Sentezlenen ve saflaştırılan bileşiklerin ^{1}H-NMR, ^{13}C-NMR ve HRMS analizleri ile yapı kontrolleri gerçekleştirilmiştir. Ardından, sonuç bileşiklerin modifiye Ellman yöntemiyle kolinesteraz inhibitör etki, DPPH ve ORAC yöntemleriyle antioksidan etki ve UV-spektroskopisi analiziyle de metal şelatör etki incelemeleri yapılmıştır. Ayrıca tüm bileşiklerin ilaç olabilirliklerini değerlendirebilmek amacıyla QikProp Schrodinger Suite 2023 kullanılarak fizikokimyasal parametreleri hesaplanmıştır.

Sonuç ve Tartışma: *Sekiz sonuç bileşikten yedisinin değişen oranlarda kolinesteraz inhibisyonu oluşturdukları bulunmuştur. Bileşik **2a** (IC_{50} = 12.83 µM) ve **2d** (IC_{50} = 16.02 µM) sırasıyla en iyi asetilkolinesteraz (AChE) ve bütirilkolinesteraz (BChE) inhibitörü olarak belirlenmiştir. Bununla birlikte, ORAC testinde tüm bileşikler antioksidan etki göstermiştir. Tüm bileşiklerin Cu(II), Fe(II), ve Zn(II) iyonları için değişen oranlarda şelatör etkileri de gözlenmiştir. Ayrıca, final bileşikler, in-siliko tahminlere göre kabul edilebilir öncü benzeri özellikler göstermiştir.*

Anahtar Kelimeler: *Açilhidrazon, Alzheimer hastalığı, antioksidan, kolinesteraz inhibisyonu, metal-şelatör*

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, presenting a significant global public health challenge [1]. As the aging population increases, AD prevalence rises, imposing escalating burdens on individuals, families, and healthcare systems [2]. Marked by cognitive decline, memory loss, and various behavioral symptoms, AD detrimentally impacts independence and quality of life [3]. The societal consequences extend beyond emotional and economic strain on families to a global healthcare resource burden [4].

AD exhibits distinct pathophysiological features, including acetylcholine deficiency, neuroinflammation, oxidative stress, metal ion dyshomeostasis, and protein dysregulations [5-7]. While the precise etiology remains unclear, a multifactorial interplay of genetic, environmental, and lifestyle factors contributes to disease development [8]. Genetic studies identify key genes like amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) in familial AD, with sporadic AD associated with a complex genetic landscape [9]. Medicinal chemistry plays a vital role in the search for AD therapies, focusing on designing compounds targeting specific biological pathways. Recent advances highlight potential drug targets like Aβ aggregation, tau hyperphosphorylation, neuroinflammation, and oxidative stress [10]. Medicinal chemists strive to develop compounds selectively modulating these targets for disease-modifying interventions [11,12]. Existing AD therapies primarily manage symptoms and provide temporary cognitive relief using cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists [13]. The therapeutic options within these drug classes are currently limited to donepezil, rivastigmine, galantamine, and the NMDA receptor antagonist memantine [7,14]. However, these treatments do not address fundamental disease processes, prompting the urgent need for innovative disease-modifying interventions [15,16].

Recent AD drug development emphasizes multitarget-directed ligands (MTDLs) over the traditional single-target approach [17]. Cholinesterase inhibition, particularly targeting acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), is crucial in this strategy [18,19]. While AChE has historically received more attention, recent research underscores the significance of BChE inhibition as the disease progresses [20,21]. Cholinesterases remain principal targets for MTDL development, combining inhibitory activity with neuroprotective targets [12].

Oxidative stress (OS) is identified as an early and triggering pathology in AD, contributing significantly to neurodegeneration [22,23]. Compounds with antioxidant activity are considered valuable for MTDL development against AD. The metal hypothesis implicates Fe, Cu, and Zn ions in AD pathologies, suggesting metal chelators as valuable for drug development due to their effects on various AD mechanisms [24].

Our research team has devoted research efforts to reaching MTDLs against AD during the last

decade. With this purpose, we have studied various heterocyclic scaffolds such as phthalazinone, pyridazinone, pyridazine, benzoxazolone, benzothiazolone, and thiazole [25-31]. In this study, we preferred phenol and anisole instead of heterocyclic systems, drawing inspiration from the neuroprotective ferulic acid (FA) structure [32,33]. FA serves as a potential pharmacophore that exhibits multiple pharmacological properties, such as antioxidant, neuroprotection, modulation of Aß aggregation, and anti-inflammatory effects. However, FA lacks cholinesterase inhibitor activity. Despite this, numerous studies in MTDL design have incorporated FA, often hybridizing it with known cholinesterase inhibitors. According to molecular docking results of these hybrids, FA primarily engages in π-π stacking interactions via its phenyl core with key aromatic residues (Trp286 and Tyr341 for huAChE or Trp231 and Phe329 for huBChE) at the peripheral anionic site [34,35]. Phenol and anisole connected with tertiary nitrogen-bearing structures known for their ability to interact with the catalytic site of cholinesterase enzymes [36] by using propanehydrazide bridge, which could contribute to the antioxidant and metal chelator effects [37]. As a result, we designed and synthesized novel eight *N'*-(benzylidene)propanehydrazide derivatives (Figure 1).

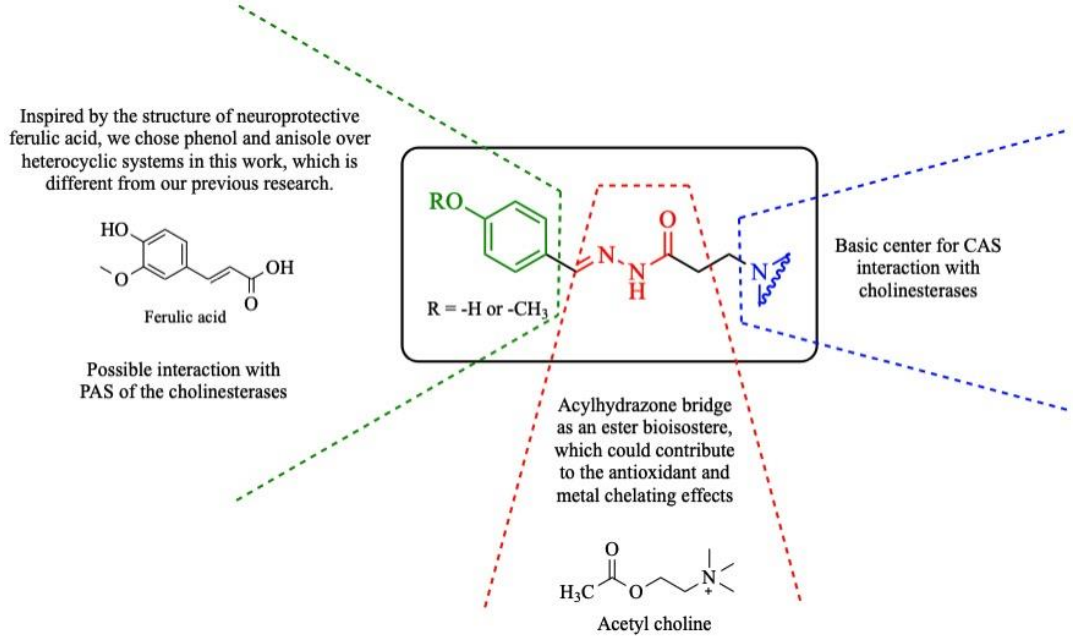

Figure 1. Design strategy and general structures of the final compounds

MATERIAL AND METHOD

Chemistry

Chemicals were acquired from commercial suppliers (Sigma Aldrich, Merck and Isolab) and Merck 60F254 plates were used for TLC. Schmelzpunkt SMP-II digital apparatus was used for melting point (mp) detection. NMR spectra were recorded using a Bruker Avance Neo 500 MHz FT-NMR spectrometer. Waters LCT Premier XE Mass Spectrometer operating in electrospray ionization (ESI) mode recruited to collect high resolution mass spectra data (HRMS). The mass spectrometer was also coupled to an AQUITY Ultra Performance Liquid Chromatography system with UV detector monitoring at 254 nm.

General method for the synthesis of 3-(substitutedamino)propanehydrazide intermediates (5a-h)

According to our previously reported method [27] corresponding amine derivative **(3a-d)** (1.0 g, 1 equivalent) in DCM (15 ml) and methyl acrylate (1.2 equivalent) was used to obtain methyl 3-(substitutedamino)propanoate intermediates **(4a-d)**. Without further purification prepared **4a-d** (1.0 equivalent) and $NH_2NH_2.H_2O$ (64%, 5.0 equivalent) were refluxed in EtOH (25 ml) for 4h. The reaction

mixture was concentrated under reduced pressure at the end of this period, then treated with diethyl ether, and the precipitated crude intermediate **(5a-d)** was filtered off and crystallized or washed with the appropriate solvent.

3-(4-Benzylpiperazin-1-yl)propanehydrazide (5a)

Recrystallized from diisopropyl ether-isopropyl alcohol. Light pinky solid. Yield: 70 %. mp: 73-75 °C. HRMS (ESI) [M + H]+ m/z for $C_{14}H_{23}N_4O$ calculated: 263.1872, found: 263.1875.

3-(4-(4-Fluorobenzyl)piperazin-1-yl)propanehydrazide (5b)

Washed with diethyl ether-petroleum ether. White solid. Yield: 46 %. mp: 58-60 °C. HRMS (ESI) [M + H]+ m/z for $C_{14}H_{22}FN_4O$ calculated: 281.1778, found: 281.1777.

3-(4-Phenylpiperidin-1-yl)propanehydrazide (5c)

Washed with diethyl ether-petroleum ether. Light pinky solid. Yield: 87 %. mp: 112-114 °C. HRMS (ESI) [M + H]+ m/z for $C_{14}H_{22}N_3O$ calculated: 248.1763, found: 248.1761.

3-(4-Benzylpiperidin-1-yl)propanehydrazide (5d)

Recrystallized from ethyl acetate. Light pinky solid. Yield: 43 %. mp: 110-112 °C. HRMS (ESI) [M +H]+ m/z for $C_{15}H_{24}N_3O$ calculated: 262.1919, found: 262.1913.

General procedure for the synthesis of *N′*-(4-hydroxy/methoxybenzylidene)propanehydrazide derivatives (1a-d and 2a-d)

4-Hydroxybenzaldehyde **(1)** or 4-methoxybenzaldehyde **(2)** (1.0 equivalent) and corresponding 3-(substitutedamino) propanehydrazide intermediate **(5a-d)** (1.0 equivalent) were dissolved in EtOH (20 ml). The mixture was either stirred at room temperature (rt) or refluxed until the starting compounds were no longer present. After completion, the mixture was concentrated under reduced pressure then diethyl ether-petroleum ether was added to the flask, precipitate was filtered. The filtered solid was recrystallized from the appropriate solvent.

***(E)*-3-(4-benzylpiperazin-1-yl)-*N′*-(4-hydroxybenzylidene)propanehydrazide (1a)**

As mentioned in the general method, compound **1** (300 mg, 2.46 mmol) and **5a** (644 mg, 2.46 mmol) were stirred overnight at rt. Tetrahydrofuran was used to recrystallize the product. White crystals. Yield: 79 %. mp: 210 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.24, 11.06 (two s, 1H, NH), 9.91, 9.88 (two s, 1H, OH), 8.04, 7.88 (two s, 1H, -N=CH-), 7.50, 7.47 (two d, J = 8.6 Hz, 2H, $H^{2,6}$), 7.39 – 7.17 (m, 5H, H^{phenyl}), 6.82, 6.81 (two d, J = 8.6 Hz, 2H, $H^{3,5}$), 3.46 (br s, 2H, piperazine-CH_2-phenyl), 2.77 (t, J = 6.6 Hz, 1H, -H_2CC=O), 2.72 – 2.58 (m, 2H, CH_2-C$\underline{H}_2$-piperazine), 2.48 – 2.21 (m, 9H, $H^{piperazine}$, -H_2CC=O). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 173.28, 167.58, 159.72, 159.51, 146.64, 143.44, 138.50, 138.48, 129.35, 129.16, 128.77, 128.62, 127.42, 125.76, 116.15, 116.10, 67.48, 62.38, 62.36, 54.12, 53.72, 52.88, 52.84, 52.71, 32.46, 30.04. HR-ESI-MS (m/z) calcd for $C_{21}H_{26}N_4O_2$ $[M+H]^+$ 367.2134, found 367.2138.

***(E)*-3-(4-(4-fluorobenzyl)piperazin-1-yl)-*N′*-(4-hydroxybenzylidene)propanehydrazide (1b)**

As mentioned in the general method, compound **1** (250 mg, 2.05 mmol) and **5b** (574 mg, 2.05 mmol) were stirred overnight at rt. Acetonitrile was used to recrystallize the product. Beige crystals. Yield: 59 %. mp: 197 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.20, 11.03 (two s, 1H, NH), 9.88 (br s, 1H, OH), 8.03, 7.87 (two s, 1H, -N=CH-), 7.50, 7.47 (two d, J = 8.7 Hz, 2H, $H^{2,6}$), 7.31 (dd, J = 14.0, 7.7 Hz, 2H, $H^{2',6'}$), 7.13 (td, J = 8.9, 3.7 Hz, 2H, $H^{3',5'}$), 6.81, 6.80 (two d, J = 8.7, 2H, $H^{3,5}$), 3.43, 3.41 (two s, 2H, piperazine-CH_2-phenyl), 2.74 (t, J = 7.3 Hz, 1H, -H_2CC=O), 2.62 – 2.55 (m, 2H, CH_2-C$\underline{H}_2$-piperazine), 2.49 – 2.30 (m, 9H, $H^{piperazine}$, -H_2CC=O). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 173.50, 167.68, 162.64, 160.71, 159.72, 159.71, 159.49, 146.59, 143.33, 134.86, 131.10, 131.04, 129.16, 128.74, 125.79, 125.74, 116.14, 116.10, 115.38, 115.22, 61.57, 54.22, 53.92, 53.05, 52.95, 32.61, 30.29. HR-ESI-MS (m/z) calcd for $C_{21}H_{25}FN_4O_2$ $[M+H]^+$ 385.2040, found 385.2041.

(*E*)-*N*'-(4-hydroxybenzylidene)-3-(4-phenylpiperidin-1-yl)propanehydrazide (1c)

As mentioned in the general method, compound **1** (250 mg, 2.05 mmol) and **5c** (506 mg, 2.05 mmol) were stirred overnight at rt. Tetrahydrofuran was used to recrystallize the product. White crystals. Yield: 44 %. mp: 241 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.23, 11.04 (two s, 1H, NH), 9.88 (br s, 1H, OH), 8.06, 7.89 (two s, 1H, -N=CH-), 7.52, 7.49 (two d, J = 8.6 Hz, 2H, $H^{2,6}$), 7.31 – 7.15 (m, 5H, H^{phenyl}), 6.82, 6.81 (two d, J = 8.6 Hz, 2H, $H^{3,5}$), 2.99 (t, J = 12.9 Hz, 2H, $H^{2piperidine(eq),}$ $H^{6piperidine(eq)}$), 2.79 (t, J = 7.2 Hz, 1H, -H_2CC=O), 2.69 – 2.60 (m, 2H, CH_2-C<u>H</u>$_2$-piperidine), 2.49 – 2.42 (m, 1H, $H^{4piperidine}$), 2.37 (t, J = 7.2 Hz, 1H, -H_2CC=O), 2.11 – 1.98 (m, 2H, $H^{2piperidine(ax)}$, $H^{6piperidine(ax)}$), 1.78 – 1.69 (m, 2H, $H^{3piperidine(eq),}$ $H^{5piperidine(eq)}$), 1.68 – 1.53 (m, 2H, $H^{3piperidine(ax),}$ $H^{5piperidine(ax)}$). HR-ESI-MS (m/z) calcd for $C_{21}H_{25}N_3O_2$ $[M+H]^+$ 352.2025, found 352.2024.

(*E*)-3-(4-benzylpiperidin-1-yl)-*N*'-(4-hydroxybenzylidene)propanehydrazide (1d)

As mentioned in the general method, compound **1** (250 mg, 2.05 mmol) and **5d** (535 mg, 2.05 mmol) were stirred overnight at rt. Tetrahydrofuran was used to recrystallize the product. Beige crystals. Yield: 53 %. mp: 224 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.21, 11.00 (two s, 1H, NH), 9.89 (br s, 1H, OH), 8.03, 7.87 (two s, 1H, -N=CH-), 7.50, 7.46 (two d, J = 8.6 Hz, 2H, $H^{2,6}$), 7.30 – 7.10 (m, 5H, H^{phenyl}), 6.81, 6.80 (two d, J = 8.6 Hz, 2H, $H^{3,5}$), 2.84 (d, J = 11.7 Hz, 2H, $H^{2piperidine(eq),}$ $H^{6piperidine(eq)}$), 2.73 (t, J = 7.2 Hz, 1H, -H_2CC=O), 2.60 – 2.52 (m, 2H, CH_2-C<u>H</u>$_2$-piperidine), 2.49, 2.47 (two d, J = 7.0 Hz, 2H, piperidine-CH_2-phenyl), 2.31 (t, J = 7.2 Hz, 1H, -H_2CC=O), 1.86 (t, J = 11.7 Hz, 2H, $H^{2piperidine(ax)}$, $H^{6piperidine(ax)}$), 1.55 – 1.38 (m, 3H, $H^{3piperidine(eq),}$ $H^{5piperidine(eq)}$, $H^{4piperidine}$), 1.22 – 1.08 (m, 2H, $H^{3piperidine(ax),}$ $H^{5piperidine(ax)}$). HR-ESI-MS (m/z) calcd for $C_{22}H_{27}N_3O_2$ $[M+H]^+$ 366.2182, found 366.2176.

(*E*)-3-(4-benzylpiperazin-1-yl)-*N*'-(4-methoxybenzylidene)propanehydrazide (2a)

As mentioned in the general method, compound **2** (223.4 µl, 1.84 mmol) and **5a** (481 mg, 1.84 mmol) were stirred overnight at rt. Tetrahydrofuran was used to recrystallize the product. White crystals. Yield: 57 %. mp: 148 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.28, 11.11 (two s, 1H, NH), 8.08, 7.92 (two s, 1H, -N=CH-), 7.62, 7.58 (two d, J = 8.8 Hz, 2H, $H^{2,6}$), 7.34 – 7.20 (m, 5H, H^{phenyl}), 6.99 (t, J = 8.8 Hz, 2H, $H^{3,5}$), 3.80, 3.79 (two s, 3H, -OCH_3), 3.45, 3.43 (two s, 2H, piperazine-CH_2-phenyl), 2.76 (t, J = 7.3 Hz, 1H, -H_2CC=O), 2.65 – 2.55 (m, 2H, CH_2-C<u>H</u>$_2$-piperazine), 2.49 – 2.07 (m, 9H, $H^{piperazine}$, -H_2CC=O). HR-ESI-MS (m/z) calcd for $C_{22}H_{28}N_4O_2$ $[M+H]^+$ 381.2291, found 381.2285.

(*E*)-3-(4-(4-fluorobenzyl)piperazin-1-yl)-*N*'-(4-methoxybenzylidene)propanehydrazide (2b)

As mentioned in the general method, compound **2** (223.4 µl, 1.84 mmol) and **5b** (514 mg, 1.84 mmol) were refluxed for 72 h. Ethylacetate-hexane was used to recrystallize the product. Light pinky crystals. Yield: 80 %. mp: 147 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.27, 11.11 (two s, 1H, NH), 8.08, 7.92 (two s, 1H, -N=CH-), 7.62, 7.58 (two d, J = 8.7 Hz, 2H, $H^{2,6}$), 7.35 – 7.25 (m, 2H, $H^{2',6'}$), 7.18 – 7.09 (m, 2H, $H^{3',5'}$), 6.99 (t, J = 8.7 Hz, 2H, $H^{3,5}$), 3.80, 3.79 (two s, 3H, -OCH_3), 3.43, 3.41 (two s, 2H, piperazine-CH_2-phenyl), 2.76 (t, J = 7.3 Hz, 1H, -H_2CC=O), 2.65 – 2.55 (m, 2H, CH_2-C<u>H</u>$_2$-piperazine), 2.49 – 2.12 (m, 9H, $H^{piperazine}$, -H_2CC=O). HR-ESI-MS (m/z) calcd for $C_{22}H_{27}FN_4O_2$ $[M+H]^+$ 399.2196, found 399.2194.

(*E*)-*N*'-(4-methoxybenzylidene)-3-(4-phenylpiperidin-1-yl)propanehydrazide (2c)

As mentioned in the general method, compound **2** (223.4 µl, 1.84 mmol) and **5c** (535 mg, 1.84 mmol) were stirred overnight at rt. Acetonitrile was used to recrystallize the product. White crystals. Yield: 75 %. mp: 165 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.30, 11.13 (two s, 1H, NH), 8.11, 7.94 (two s, 1H, -N=CH-), 7.63, 7.60 (two d, J = 8.8 Hz, 2H, $H^{2,6}$), 7.33 – 7.13 (m, 5H, H^{phenyl}), 7.00, 6.99 (two d, J = 8.8 Hz, 2H, $H^{3,5}$), 3.80, 3.79 (two s, 3H, -OCH_3), 3.00 (t, J = 11.7 Hz, 2H, $H^{2piperidine(eq),}$ $H^{6piperidine(eq)}$), 2.80 (t, J = 7.2 Hz, 1H, -H_2CC=O), 2.70 – 2.60 (m, 2H, CH_2-C<u>H</u>$_2$-piperidine), 2.49 – 2.42 (m, 1H, $H^{4piperidine}$), 2.38 (t, J = 7.2 Hz, 1H, -H_2CC=O), 2.11 – 2.00 (m, 2H, $H^{2piperidine(ax)}$, $H^{6piperidine(ax)}$), 1.78 – 1.69 (m, 2H, $H^{3piperidine(eq),}$ $H^{5piperidine(eq)}$), 1.68 – 1.55 (m, 2H, $H^{3piperidine(ax),}$ $H^{5piperidine(ax)}$). HR-ESI-MS (m/z) calcd for $C_{22}H_{27}N_3O_2$ $[M+H]^+$ 366.2182, found 366.2173.

(*E*)-3-(4-benzylpiperidin-1-yl)-*N*'-(4-methoxybenzylidene)propanehydrazide (2d)

As mentioned in the general method, compound **2** (223.4 µl, 2.05 mmol) and **5d** (479 mg, 1.84 mmol) were stirred overnight at rt. Acetonitrile was used to recrystallize the product. White crystals. Yield: 56 %. mp: 138 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.28, 11.09 (two s, 1H, NH), 8.08, 7.92 (two s, 1H, -N=CH-), 7.62, 7.58 (two d, J = 8.6 Hz, 2H, $H^{2,6}$), 7.30 – 7.10 (m, 5H, H^{phenyl}), 7.00, 6.99 (two d, J = 8.6 Hz, 2H, $H^{3,5}$), 3.80, 3.79 (two s, 3H, -OCH_3), 2.84 (t, J = 11.6 Hz, 2H, $H^{2piperidine(eq),}$ $H^{6piperidine(eq)}$), 2.74 (t, J = 7.2 Hz, 1H, -H_2CC=O), 2.61 – 2.53 (m, 2H, CH_2-C$\underline{H}_2$-piperidine), 2.49, 2.46 (two d, J = 7.0 Hz, 2H, piperidine-CH_2-phenyl), 2.32 (t, J = 7.2 Hz, 1H, -H_2CC=O), 1.86 (t, J = 11.6 Hz, 2H, $H^{2piperidine(ax)}$, $H^{6piperidine(ax)}$), 1.55 – 1.38 (m, 3H, $H^{3piperidine(eq),}$ $H^{5piperidine(eq)}$, $H^{4piperidine}$), 1.22 – 1.07 (m, 2H, $H^{3piperidine(ax),}$ $H^{5piperidine(ax)}$). HR-ESI-MS (m/z) calcd for $C_{23}H_{29}N_3O_2$ $[M+H]^+$ 380.2338, found 380.2344.

Biological Assays

Cholinesterase Inhibition Assay

AChE (electric eel) and BChE (equine serum) from Sigma Aldrich were employed in the assays, following the method previously reported by us [29,31]. To determine the IC_{50} values, GraphPad Prism software (Version 7.0) was used.

In Vitro Antioxidant Activity Assays (DPPH And ORAC-Fluorescein)

DPPH and ORAC-FL assays were conducted using our previously reported procedures [29,31]. In DPPH assay, test samples were assayed at 100 µM for 30 min incubation time. Assay was carried out in triplicate, and the mean±SD was computed.

Metal Binding Studies

Studies for the ligand-metal binding evaluation was carried out in accordance with our previously reported methodology [29,31]. The overlapping spectra of the metal-treated ligand and the control solution of the ligand were visualized, and the resulting wavelength (nm) vs. absorbance graphs were drawn.

Evaluation of In Silico Physicochemical Parameters

In order to obtain low energy conformations of the ligands as well as potential ionization states for pH 7.0 ± 2.0, LigPrep module was used. Table 3 shows the Qikprop predictions for the top-scoring states of each compound.

RESULT AND DISCUSSION

Chemistry

Route for the synthesis of intermediates and final compounds were presented in Figure 2. Corresponding key intermediates, 3-(substitutedamino)propane hydrazide intermediates **(5a-d)**, were prepared from the hydrolization with hydrazine hydrate of methyl 3-(substitutedamino)propanoate intermediates **(4a-d)** have been prepared from [27] the Michael addition of methyl acrylate and appropriate amine derivative **(3a-d)**. Finally, from the reaction of starting compound 4-hydroxybenzaldehyde **(1)** or 4-methoxybenzaldehyde **(2)** and corresponding key intermediate **(5a-d)**, *N'*-(4-hydroxybenzylidene) propanehydrazides **(1a-d)** and *N'*-(4-methoxybenzylidene) propanehydrazides **(2a-d)** were synthesized.

Eight final compounds were synthesized in this study. ^{1}H NMR and high-resolution mass spectra (LC-HRMS) were used for the chemical structure and purity verification of the compounds. Additionally, ^{13}C NMR data of the **1a** and **1b** were presented as representatives of the series. The data obtained from all analyses of the compounds matched the proposed structures. Acylhydrazones, in other words, (-C(O)-N-N=CH) structure can potentially have *E/Z* geometrical isomers (C=N), *cis/trans* amide conformers (C(O)-NH). During the characterization of title compounds with TLC and LC-HRMS techniques, only the presence of the *E* isomers, less hindered and favored ones was confirmed.

Additionally, energy calculations of the isomers confirm this observation (Figure 3). As a result of diastereomeric nature of title compounds (*E*, *cis* and *E*, *trans*), explicit sets of certain protons or duplication were observed in ^{1}H NMR spectra with a similar pattern for all compounds (Figure 4).

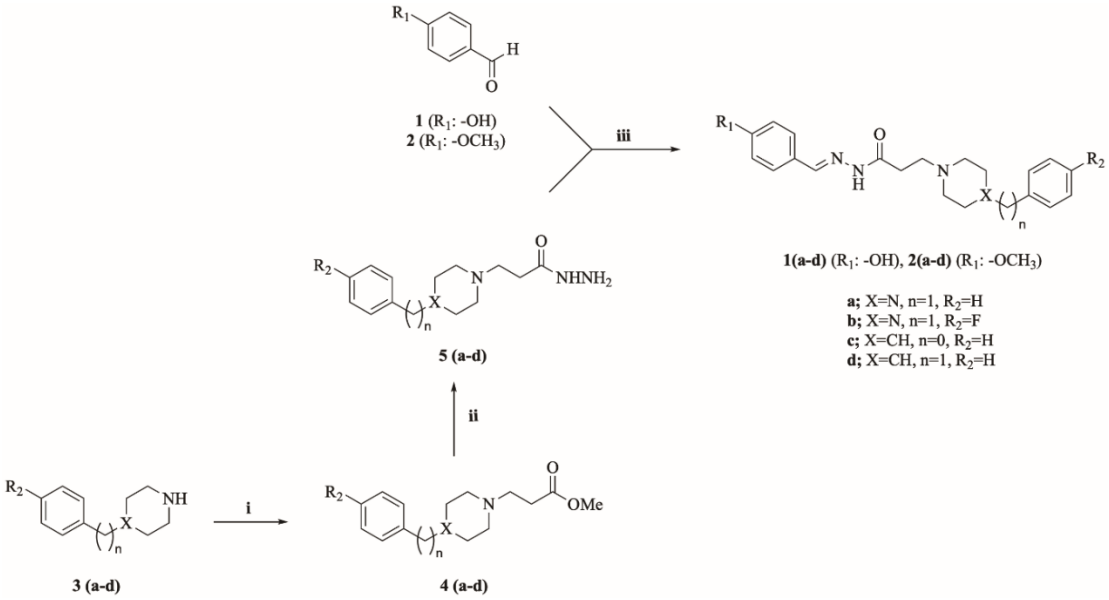

Figure 2. Synthesis of benzylidene propanehydrazide derivatives **1a-d** and **2a-d**. Reagents and conditions: **(i)** Methyl acrylate, DCM, rt, 24h; **(ii)** Hydrazine monohydrate, EtOH, reflux, 4h; **(iii)** EtOH, reflux, 4h

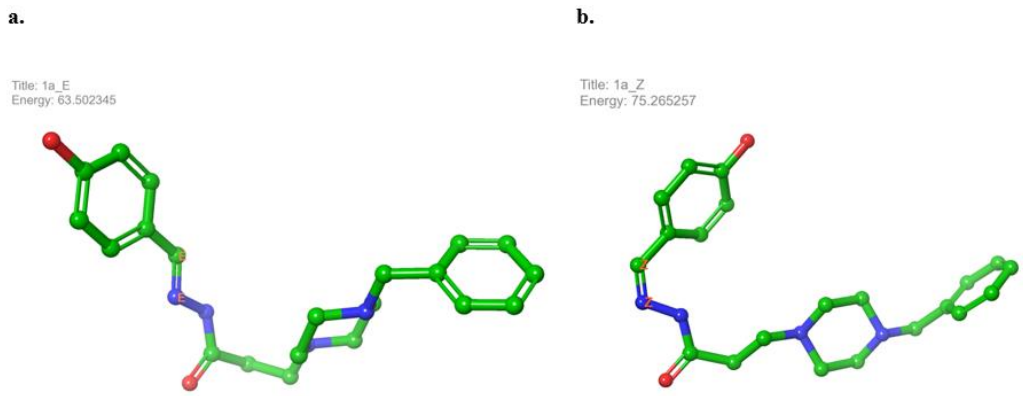

Figure 3. Calculated energies by LigPrep for *E* isomer **(a)** and *Z* isomer **(b)** of compound **1a**

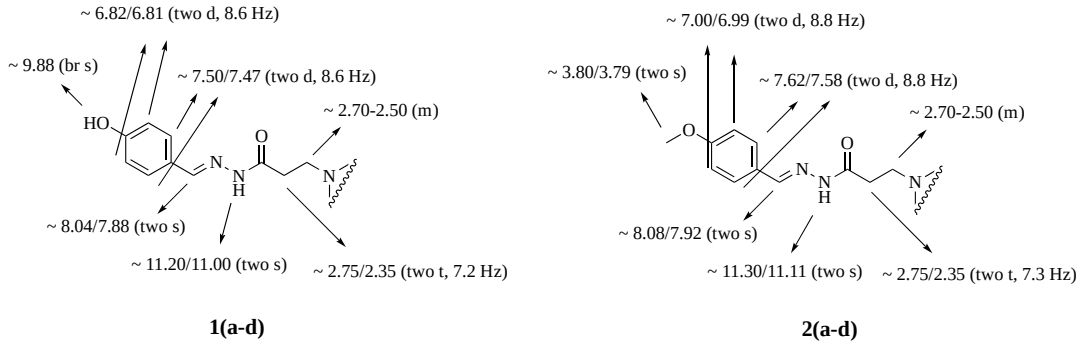

Figure 4. Common ^{1}H NMR peaks of benzylidene propanehydrazide series **1a-d** and **2a-d**

Biology

Cholinesterase Inhibition

By using modified Ellman's method, inhibition percentages of compounds on cholinesterases were evaluated at 10, 33.3 and 100 μM. Subsequently, the IC_{50} values of the final compounds were assayed and calculated. Donepezil and Galantamine were used as positive controls [38]. All the cholinesterase (ChE) inhibitory activity results were presented in Table 1. All the tested compounds, except **2c**, exhibited moderate AChE or BChE inhibitory activity. Among them, only compound **2a** (IC_{50} values for AChE = 12.83 μM and BChE = 72.78 μM) assigned as a dual ChE inhibitor. Interestingly, structure-activity relationship (SAR) results showed us that piperazine derivatives (**1a**, **1b**, **2a**, and **2b**) were preferable for AChE inhibition and piperidine derivatives (**1c**, **1d**, and **2d**) were preferable for BChE inhibition. Additionally, switching from phenol to anisole generally enhances ChE inhibition; just in **2c**, this effect plays its role contrarily.

Table 1. Cholinesterase inhibitory activity results of the synthesized compounds

Compound	***ee*AChE**				***eq*BChE**			
	%inh (10 μM)	%inh (33.3 μM)	%inh (100 μM)	IC_{50}	%inh (10 μM)	%inh (33.3 μM)	%inh (100 μM)	IC_{50}
1a	43.4 ± 0.8	72.8 ± 1.9	87.2 ± 1.1	16.72 ± 4.61	≤10	13.3 ± 3.5	33.1 ± 2.6	≥100
1b	21.4 ± 1.3	46.9 ± 0.4	74.2 ± 1.3	34.04 ± 4.61	≤10	12.5 ± 0.8	36.8 ± 1.3	≥100
1c	≤10	10.5 ± 0.7	36.8 ± 2.9	≥100	20.6 ± 2.1	41.2 ± 4.2	65.5 ± 1.4	59.56 ± 3.5
1d	≤10	29.8 ± 0.1	50.4 ± 3.2	≥100	21.0 ± 2.7	43.7 ± 0.4	66.5 ± 2.8	53.76 ± 4.2
2a	44.4 ± 1.2	73.0 ± 0.2	89.5 ± 1.4	12.83 ± 3.80	18.4 ± 1.5	45.1 ± 1.7	59.6 ± 2.6	72.78 ± 5.68
2b	23.1 ± 1.7	51.4 ± 1.4	78.4 ± 0.3	29.26 ± 4.45	≤10	25.5 ± 2.0	54.0 ± 0.2	≥100
2c	≤10	≤10	16.4 ± 0.4	≥100	26.6 ± 3.4	53.9 ± 1.3	42.2 ± 1.1	≥100
2d	≤10	24.0 ± 9.9	54.7 ± 1.1	≥100	42.9 ± 2.5	73.1 ± 1.0	89.4 ± 3.7	16.02 ± 1.2
Donepezil	98.7 ± 0.4	-	-	0.062 ± 0.002	89.9 ± 0.3	-	-	3.55 ± 0.07
Galantamine	91.9 ± 0.7	-	-	nd	29.9 ± 0.5	-	-	nd

Antioxidant Activity

Both the radical scavenging (DPPH assay) and oxygen radical absorbance (ORAC-FL assay) capabilities of the compounds were investigated. Antioxidant standarts, Gallic acid [39] and Trolox [40] were recruited for DPPH and ORAC-FL assays, respectively. Table 2 displays the results. At 100 μM, phenol derivatives **(1a-d)** showed low radical scavenging activity (between %12 and %33). However, at the same concentration, none of the anisole derivatives **(2a-d)** exhibited radical scavenging activity. Nonetheless, every molecule exhibited very high ORAC-FL values, which varied from 0.743 to 24.958 Trolox equivalents. Furthermore, phenol derivatives **(1a–d)**, in particular compound **1b** (as 24.958 trolox equivalent), have extremely significant capabilities to absorb oxygen radicals.

Metal Binding

Cu(II), Fe(II), and Zn(II) binding capabilities of the compounds were assessed by screening their absorptions in the 230-500 nm range. Any change in the metal-treated ligand's spectra relative to the spectra of the ligand alone, as determined by the UV-vis spectrophotometry method, is ascribed to complexation [41]. "Difference UV-vis spectra" were created for improved assessment. Figure 5 shows the "difference UV-vis spectra" that were obtained by subtracting the separate absorbances of the ligand and metal from the absorbance of the metal-ligand mixture. All compounds demonstrated chelation with every tested ion, though at different rates, as we saw. Interestingly, **1c** and **2d** showed significant Fe(II) and Cu(II) chelation. Additionally, **2d** showed significant Zn(II) chelation.

Table 2. Antioxidant activity results of the compounds

Compound	DPPH % inhibition (100 µM)	ORAC-FL
1a	12.15 ± 1.08	11.436 ± 0.381
1b	12.76 ± 0.88	24.958 ± 0.490
1c	22.67 ± 1.00	14.546 ± 0.535
1d	33.63 ± 1.47	2.556 ± 0.337
2a	≤10	1.153 ± 0.150
2b	≤10	1.159 ± 0.099
2c	≤10	0.692 ± 0.147
2d	≤10	0.743 ± 0.093
Gallic acid (100 µM)	92.71 ± 0.90	nd
Trolox	nd	1.000

The mean ± SD of three independent experiments. nd; not determined

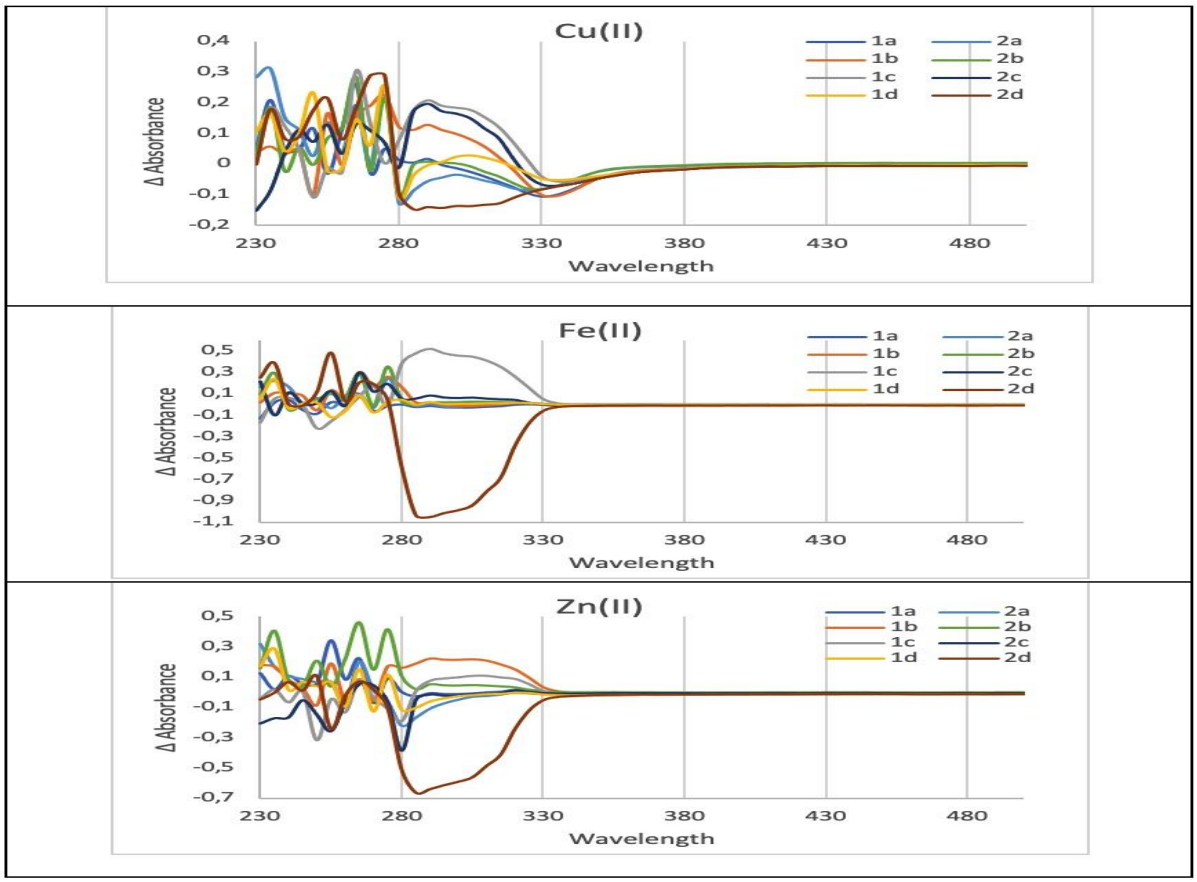

Figure 5. The UV–vis difference spectra between title compounds and metal ions

Evaluation of *in silico* physicochemical parameters

For the calculation of in-silico physicochemical properties and ADME predictions QikProp Schrodinger Suite 2023 was used [42]. The results are presented in Table 3. With QPlogBB values ranging from -1.029 to 0.149, all of the compounds were predicted to be BBB-permeable. Most of the compounds were forecasted to exhibit a CNS score of 1, falling within the range of -2 (inactive) to +2 (active). Exceptionally, **1c** and **1d**, but these compounds have not significant chlolinesterase inhibition.

All of the compounds follow Lipinski's rule of five [43]. Additionally, all the compunds, except **2d**, follow Jorgensen's rule of three [44]. QPlogS value of **2d** (-5.739) slightly over Jorgensen's but it is in the recommended range by the Qikprop manual. As a result, *in silico* ADME predictions revealed that the compounds generally have acceptable drug-likeness as well as significant BBB permeation capacity.

Table 3. Predicted physicochemical parameters of compounds

Descriptor	Compounds								Recommended value
	1a	1b	1c	1d	2a	2b	2c	2d	
MW	366.462	384.452	351.447	365.474	380.489	398.479	365.474	379.501	130 - 725
vol	1.259.398	1278.077	1237.221	1291.965	1313.241	1329.378	1280.682	1346.391	500 - 2000
n-rot	9	9	7	9	9	9	7	9	0 - 15
DHB	2.000	2.000	2.000	2.000	1.000	1.000	1.000	1.000	0 - 6
AHB	7.250	7.250	5.250	5.250	7.250	7.250	5.250	5.250	2 - 20
PSA	84.620	84.634	79.464	79.305	70.267	70.322	62.087	65.105	7 - 200
QPlogS	-2.752	-3.221	-5.006	-5.151	-3.197	-3.562	-5.203	-5.739	-6.5 - 0.5
QlogP$_{o/w}$	2.525	2.745	3.681	4.025	3.339	3.572	4.540	4.904	2.0 - 6.5
QPPCaco	39.905	37.195	151.930	151.811	132.013	131.449	687.218	500.498	<25 poor, >500 great
QlogBB	-0.537	-0.490	-0.886	-1.029	0.043	0.149	-0.102	-0.422	-3.0 - 1.2
CNS	1	1	0	-2	1	1	1	1	-2 (inactive) to +2 (active)
#metab	5	5	5	4	5	5	5	4	1 - 8
%HOA	70.385	71.129	87.548	89.554	84.453	85.783	100.000	100.000	>80% high <25% poor
VRF	0	0	0	0	0	0	0	0	-
VJR	0	0	0	0	0	0	0	1	-

MW: Molecular weight **vol:** Total solvent-accessible volume **n-rot:** Number of rotatable bonds **DHB:** Estimated number of hydrogen bond donors **AHB:** Estimated number of hydrogen bond acceptors (2.0-20.0) **PSA:** Van der Waals surface area of polar nitrogen and oxygen atoms and carbonyl carbon atoms **QPlogS:** Predicted aqueous solubility **QlogPo/w:** Predicted octanol/water partition coefficient **QPCaco:** Predicted apparent Caco-2 cell permeability **QlogBB:** Predicted brain/blood partition coefficient **CNS:** Predicted central nervous system activity **#metab:** Number of likely metabolic reactions.**%HOA:**Predicted human oral absorption percent **VRF:** Number of violations of Lipinski's rule of five (The rules are: MW < 500, logP <5, DHB ≤ 5, AHB ≤ 10) **VJR:** Number of violations of Jorgensen's rule of three (QPlogS > -5.7, QP PCaco > 22 nm/s, # Primary Metabolites < 7)

In this study, we designed and synthesized novel *N'*-(benzylidene)propanehydrazide derivatives. Subsequently, their anti-cholinesterase, antioxidant, and metal-chelating abilities were evaluated with the aim of obtaining new hit compounds. Based on the ChE inhibitory activity results, all the tested compounds, except **2c**, exhibited moderate AChE or BChE inhibitory activity. Especially, **2a** (IC_{50} values for AChE = 12.83 μM and BChE = 72.78 μM) and **2d** (IC_{50} value for BChE = 16.02 μM) were determined as the most potent inhibitors for AChE and BChE, respectively. Additionally, **2a** (1.153) and **2d** (0.743) exhibited similar antioxidant activity to trolox. Unfortunately, the antioxidant capacities of the cholinesterase inhibitory active compounds were relatively lower than the inactive ones. Moreover, metal-binding studies indicated that all the compounds were chelators for copper, iron, and zinc ions. Especially, the active compound **2d** showed significant Zn(II) chelation. Besides, compounds demonstrated appreciable blood-brain barrier (BBB) permeation capacity along with acceptable lead-like properties according to in-silico predictions. Overall, **2a** and **2d** could be considered as a starting point for new structural modifications in the MTDL design against AD.

ACKNOWLEDGMENTS

The author is grateful to Prof. Dr. Deniz S. Doğruer for her support and advice during this research.

AUTHOR CONTRIBUTIONS

Concept: B.K.; Design: B.K.; Control: B.K.; Sources: B.K.; Materials: B.K.; Data Collection and/or Processing: B.K.; Analysis and/or Interpretation: B.K.; Literature Review: B.K.; Manuscript Writing: B.K.; Critical Review: B.K.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 853-866, 2024
Doi: 10.33483/jfpau.1448197

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PHENOLIC AND ANTIOXIDANT PROFILE: FTIR AND LC-MS ANALYSES OF *SERAPIAS ORIENTALIS*

FENOLİK VE ANTİOKSİDAN PROFİLİ: SERAPIAS ORIENTALIS'İN FTIR VE LC-MS ANALİZLERİ

Erdi Can AYTAR[1,2]*, Yasemin Özdener KÖMPE[1]

[1]Ondokuz Mayıs University, Faculty of Sciences, Department of Biology, 55100, Samsun, Türkiye
[2]Usak University, Faculty of Agriculture, Department of Horticulture, 64200, Usak, Türkiye

ABSTRACT

Objective: *The objective of this study is to characterize the aerial parts of S. orientalis using Fourier Transform Infrared (FTIR) spectroscopy, evaluate the phenolic content and antioxidant activity of seeds, stems, and flowers, and conduct quantitative analysis of phenolic compounds using LC-MS/MS.*
Material and Method: *Fourier Transform Infrared (FTIR) spectroscopy was employed to characterize the aerial parts of S. orientalis. The analysis focused on identifying various functional groups such as -OH vibrations associated with polysaccharides, C-H vibrations from lipids and lignin compounds, and C=O vibrations related to cellulose derivatives. The total phenolic, flavonoid, flavanol, tannin, and proanthocyanidin contents of S. orientalis seeds, stems, and flowers were evaluated using standard analytical methods. DPPH radical scavenging activity was determined to assess antioxidant potential, with IC_{50} values calculated for each plant part. Quantitative analysis of phenolic compounds in the plant extract was conducted using LC-MS/MS. The abundance of various phenolic acids including P-coumaric acid, trans-ferulic acid, caffeic acid, and vanillic acid was determined. Additionally, other phenolic compounds such as gallic acid, chlorogenic acid, salicylic acid, (+) taxifolin, rutin hydrate, ellagic acid, quercetin dihydrate, and apigenin were also detected and quantified.*
Result and Discussion: *The evaluation of phenolic content showed differences among different plant parts, with flowers exhibiting the highest total phenolic and proanthocyanidin content. Seeds demonstrated superior DPPH radical scavenging activity. Quantitative analysis of phenolic compounds using LC-MS/MS highlighted the abundance of various phenolic acids and other phenolic compounds in S. orientalis. These findings underscore the potential of S. orientalis as a valuable source of natural antioxidants. Overall, the results suggest that S. orientalis possesses significant phenolic diversity and antioxidant activity, which could contribute to its potential applications in various industries, including pharmaceuticals and nutraceuticals.*
Keywords: *FTIR, LC-MS analysis, plant secondary metabolites, quantitative analysis*

ÖZ

Amaç: *Bu çalışmanın amacı, S. orientalis'in topraküstü kısımlarını Fourier Dönüşümü Kızılötesi (FTIR) spektroskopisi kullanarak karakterize etmek, tohumların, sapların ve çiçeklerin fenolik içeriğini ve antioksidan aktivitesini değerlendirmek ve LC-MS/MS kullanarak fenolik bileşiklerin nicel analizini yapmaktır.*

* **Corresponding Author / Sorumlu Yazar:** Erdi Can Aytar
e-mail / e-posta: erdicanaytar@gmail.com, **Phone / Tel.:** +905072291015

Submitted / Gönderilme : 06.03.2024
Accepted / Kabul : 07.06.2024
Published / Yayınlanma : 10.09.2024

Gereç ve Yöntem: *S. orientalis'in topraküstü kısımlarını karakterize etmek için Fourier Dönüşümü Kızılötesi (FTIR) spektroskopisi kullanıldı. Analiz, polisakkaritlerle ilişkilendirilen -OH titreşimleri, lipitlerden ve lignin bileşiklerinden kaynaklı C-H titreşimleri ve selüloz türevleriyle ilişkilendirilen C=O titreşimleri gibi çeşitli fonksiyonel grupları tanımlamaya odaklandı.S. orientalis tohumlarının, saplarının ve çiçeklerinin toplam fenolik, flavonoid, flavanol, tanin ve proantosiyanidin içeriği standart analitik yöntemler kullanılarak değerlendirildi. Antioksidan potansiyeli değerlendirmek için DPPH radikal temizleme aktivitesi belirlendi ve her bitki kısmı için IC_{50} değerleri hesaplandı. Bitki ekstraktındaki fenolik bileşiklerin nicel analizi LC-MS/MS kullanılarak gerçekleştirildi. P-kumarik asit, trans-ferulik asit, kafeik asit ve vanilinik asit gibi çeşitli fenolik asitlerin bol miktarda bulunduğu belirlendi. Ayrıca, galik asit, klorojenik asit, salisilik asit, (+) taksifolin, rutin hidrat, ellajik asit, kuersetin dihidrat ve apigenin gibi diğer fenolik bileşikler de tespit edildi ve nicel olarak belirlendi.*

Sonuç ve Tartışma: *Fenolik içeriğin değerlendirilmesi, farklı bitki parçaları arasında farklılıklar gösterdi, çiçekler en yüksek toplam fenolik ve proantosiyanidin içeriğini sergiledi. Tohumlar üstün DPPH radikal temizleme aktivitesi gösterdi.LC-MS/MS kullanarak fenolik bileşiklerin nicel analizi, S. orientalis'te çeşitli fenolik asitlerin ve diğer fenolik bileşiklerin bol miktarda bulunduğunu vurgulanmaktadır. Genel olarak, sonuçlar S. orientalis'in önemli bir fenolik çeşitliliğe ve antioksidan aktiviteye sahip olduğunu göstermektedir, bu da onun farmasötik ve nutrasötikler dahil çeşitli endüstrilerde potansiyel uygulamalarına katkıda bulunabileceğini düşündürmektedir.*

Anahtar Kelimeler: *Bitki ikincil metabolitleri, FTIR, LC-MS analizi, nicel analiz*

INTRODUCTION

Since the early 21st century, there has been significant interest in studying the origin of oxidation processes triggered by free radicals and the broader role of antioxidants. This fascination arises from the fact that free radicals, despite being neutral, are highly reactive and unstable molecules with notable impacts on human biological systems. Notably, even crucial lipid derivatives such as aldehydes can pose health risks, especially as they can naturally form due to thermal processes during food processing [1]. The potency of radicals' hinges on the presence of an unpaired electron within an atom, driving them to incessantly seek bonding with other atoms or molecules due to the deficiency of outer-shell electrons. Despite the existence of antioxidant defense mechanisms, human cells remain susceptible to damage, which can hasten the aging process and contribute significantly to the onset of various disease [2,3]. Extensive oxidative modifications to biological macromolecules, including lipids, proteins, and DNA, can culminate in tissue damage [4]. Understanding and preventing these processes are crucial for attaining a better quality of life. Oxidative stress emerges from an imbalance between prooxidant and antioxidant species responsible for maintaining cellular equilibrium. Insufficiencies in antioxidant defenses or breakdowns in preserving the cellular redox balance system result in the excessive production of reactive oxygen species, leading to oxidative stress and diverse alterations in biomolecules that foster disease development [5]. Previously known as the 'disruption of the prooxidant-antioxidant balance, 'oxidative stress has been redefined as the 'disruption of redox signaling and control and/or molecular damage' [6].

In recent years, the adverse effects associated with synthetic antioxidants, including butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, and citric acid, have raised concerns due to their demonstrated toxic and mutagenic effects [7]. Due to the long-term toxic effects associated with synthetic antioxidants, there has been a significant increase in demand for alternatives to replace their use in the food industry. This is especially important given the potential adverse effects of prolonged use, including carcinogenicity [8,9]. Phenolic compounds have become synonymous with health benefits associated with the consumption of high amounts of fruits and vegetables. These beneficial effects are at least partially attributed to their antioxidant activities [10,11]. These compounds can be a determining factor in the antioxidant potential of foods and, therefore, hold significance as natural sources of antioxidants [3].

Maceration is a commonly employed traditional extraction method for obtaining phenolic compounds from plants. In this method, plant samples are typically processed using solvents such as ethanol or methanol [12]. Traditional extraction relies on the principle that the target chemicals dissolve

in solvents with similar structures. In maceration extraction, techniques like shaking and agitation in an incubator are frequently employed to increase the contact surface area between samples and the solvent, thus enhancing the extraction rate and yield. Additionally, temperature control is crucial to enhance the effectiveness of the extraction process [13]. The advantages of this method include its ease of application, cost-effectiveness, and suitability for use on a wide variety of plant samples. Furthermore, being a tradition-based approach is indicative of its long-standing use for obtaining plant compounds [14].

Serapias orientalis (Greuter) Bauman & Künkele is a plant that thrives in grasslands with high water tables in the Middle and Eastern Black Sea regions, moist forest edges, and hazelnut orchards. The *S. orientalis* plant can reach a height of 15-30 cm and has thick, light green roots. Its flowers are red. This plant species is rarely found in moist meadows and open pine forests and typically grows in alkaline or calcareous soils. There is no study available regarding the antioxidant activity of *S. orientalis*. There is no information on the presence of antioxidants in this species. Therefore, the is study aims to evaluate the total phenolic, flavonoid, flavanol, proanthocyanidin, and tannin contents of extracts obtained from the seeds and *ex vitro*-produced stems and flower parts of the *S. orientalis* plant using the maceration method with methanol. Additionally, the study seeks to determine their antioxidant activities. Furthermore, characterization of the characteristic peak regions of the aerial parts of the plant will be conducted through FTIR analysis, and the identification of phenolic compounds will be carried out using the LC-MS technique.

MATERIAL AND METHOD

Plant Material and Extraction

In this study, mature flowering specimens of *S. orientalis* (2 years old) were collected during the flowering season in May for the aerial parts (stem and flower), while the seeds were collected after maturation in July (Ondokuz Mayıs Unıversity Campus, Samsun, Turkey). Subsequently, flowers, stems (leaves were considered as part of the stem), and seeds were dried in an oven at 40°C. The dried samples were ground into a fine powder and stored at +4°C until further analysis. For the maceration method, 0.5 g of the powder was extracted using 80% methanol at 35°C for 24 hours [15].

FTIR Analysis of *S. orientalis* in The Aerial Part

The FTIR spectra of *S. orientalis* aerial part were obtained by using the PERKIN ELMER Spectrum TWO FTIR Spectrometer equipped with a diamond attenuated total reflectance (ATR) accessory and a $LiTaO_3$ detector. Spectral measurements were conducted in the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1}. The samples were directly analyzed without any prior treatment. The Perkin Elmer Spectrum Two FTIR software was employed to determine peak frequencies.

Total Phenolic Content

In this study, equal volumes of the sample and diluted Folin-Ciocalteu reagent were mixed. After incubating at room temperature for 3 minutes, 1 ml of 2% sodium carbonate solution was added. The mixture was then left to incubate in the dark at room temperature for 1 hour, followed by measuring the absorbance at 760 nm using a UV spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram of dried extract (mg GAE/g extract). All measurements were performed in triplicate [16] .

Total Favonoid Content

This method was assessed using the $AlCl_3$ method with minor adjustments, following the procedure by [17]. Extracts were mixed with distilled water, followed by $NaNO_2$ (5%) addition and a standing period. Subsequently, $AlCl_3$ (10%) was added, and the solution underwent incubation. NaOH (1M) was then introduced, and the solution was left at room temperature. Absorbance was measured using a UV spectrophotometer, and total flavonoid content was quantified as quercetin equivalent (QE) per gram of dried extract (mg QE/g). All measurements were performed in triplicate.

Total Flavanols Content

The total flavonoid content was determined using the aluminum chloride method [18]. Briefly, 1 ml of the extracts was mixed with $AlCl_3$. Subsequently, 3 ml of sodium acetate solution was added. The mixture was then kept at room temperature in a dark environment for 30 minutes. After the incubation period, the absorbance of the sample was measured at 415 nm using quercetin as the standard. The total flavanols was expressed as quercetin equivalents (mg QE/g).

Total Tannin Content

Total tannin content was assessed using the Folin-Ciocalteu reagent as described in [19]. A calibration curve was prepared using different concentrations of gallic acid in methanol. Samples were mixed with diluted Folin–Ciocalteu reagent in water and an aqueous sodium carbonate solution. After incubation in darkness at room temperature, absorbance was measured at 760 nm. Total tannin content was quantified as gallic acid equivalent (GAE) per gram of dried extract (mg GAE/g). All measurements were performed in triplicate.

Total Proanthocyanin Content

Total proanthocyanidins content was determined using the butanol-acid assay [20] Diluted phenolic extract was mixed with n-butanol/HCl reagent, followed by the addition of ferric ammonium sulfate in HCl. After boiling and cooling, the absorbance of the solutions was measured at 550 nm. Total proanthocyanidins content was expressed as catechin equivalent (CAE) per gram of dried extract. All measurements were performed in triplicate.

DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Assay

The DPPH assay was employed to evaluate the free radical scavenging potential of the extracts, with modifications based on [21]. Extracts at various concentrations were mixed with a methanol solution of DPPH radical, and incubated in the dark at room temperature, and absorbance was measured at 517 nm using a UV spectrometer against a blank. Ascorbic acid served as a reference standard.

Determination of Phenolic Compound Contents by LC-MS Analysis

Phenolic standard stock solutions were prepared at a concentration of 5000 ppm in 100% methanol (hypergrade, Merck, Darmstadt, Germany). Each molecule's stock solution was then prepared at a concentration of 1000 ppm. Calibration curves were constructed using a diluted mixture with concentrations of 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 ppm. Standard phenolic compound concentrations were determined by extrapolation from these calibration curves, which were generated using linear regression equations based on peak areas in the extracts (R^2 = 0.99). All dilutions were carried out using 50% methanol.

For the analysis, a Zorbax SB-C18 column (2.1 mm x 50 mm x 1.8 µm) was employed, maintained at a temperature of 35°C. A binary mobile phase consisting of water (solvent A) containing 0.05% formic acid + 5 mM ammonium formate, and methanol (solvent B) was used. These mobile phases were applied at a flow rate of 0.5 ml/min, resulting in a total runtime of 13 minutes. Phenolic compounds were analyzed using a methanol gradient, with a five µL injection volume for all samples.ESI ionization was conducted with the following settings: Nebulizer gas (nitrogen) temperature at 350°C, gas flow rate of 10 ml/min, nebulizer gas pressure at 45 psi, sheath gas temperature at 350°C, sheath gas flow rate of 9 ml/min, the capillary voltage at 4000 V (+, -), and nozzle voltage at 500 V (+, -). Detection and quantification were performed using Agilent G3793AA Mass Hunter Optimizer software. This software was utilized for both the identification and quantification of the selected phenolic compounds.

RESULT AND DISCUSSION

ATR- FTIR Profile Aerial Part

The FTIR peak details of the aerial parts of *S. orientalis* are illustrated in Figure 1. Based on these findings, the peak at 3291 cm^{-1} corresponds to the -OH vibration associated with polysaccharides

[22]. Peaks ranging from 2919 to 2845 cm^{-1} are attributed to the C-H vibrations originating from lipids and lignin compounds [23]. Peaks at 1738 cm^{-1} are indicative of the C=O vibrations related to cellulose derivatives [24]. Peaks at 1612 cm^{-1} are associated with the amid 1 vibrations of proteins. The presence of peaks at 1411 cm^{-1} is attributed to the C=C and C-OH vibrations stemming from glycosylated phenols and uronic acid [25,26]. Peaks at 1369 and 1312 cm^{-1} represent the asymmetric CH_2 bond vibrations of cellulose and lignin [27]. Peaks at 1218 cm^{-1} signify the planar -OH vibration characteristic of cellulose and lignin [28]. Peaks at 1139 cm^{-1} are associated with the C-H vibrations of phenolic acids [29]. Peaks at 1101 cm^{-1} are linked to the C-H vibration of lignin [30]. Peaks at 1020 cm^{-1} correspond to the primary alcohol -OH vibration present in polysaccharides, lignin, and cellulose, while peaks at 837 cm^{-1} indicate out-of-plane C-H vibrations observed in aromatic lignin, terpenes, and alkenes [31]. Specifically, peaks at 1139 cm^{-1} are associated with the C-H vibrations of phenolic acids, and the peaks at 837 cm^{-1} indicate out-of-plane C-H vibrations observed in aromatic lignin, terpenes, and alkenes. These points can potentially provide preliminary information, particularly about phenolic components and, consequently, antioxidant content. Such information could serve as a foundation for understanding and identifying potential antioxidants in the *S. orientalis*.

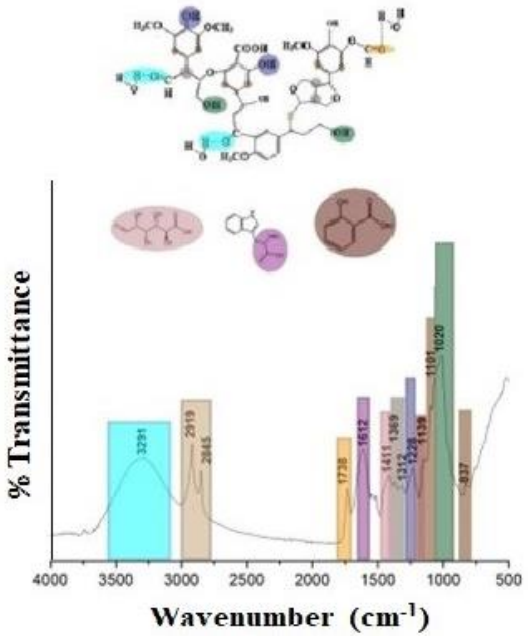


Figure 1. FTIR peaks of *S.orientalis* aerial part

Variation in the Content of Some Groups of Phenolic Compounds and Antioxidant Activity of *S. orientalis*

Based on the results obtained, the total phenolic content of seeds, stems, and flowers were quantified as follows: 100.14±1.58, 56.58±5.10, and 122.33±4.02 mg GAE/g, respectively, using the gallic acid standardization method. Similarly, the total flavonoid content, determined using the quercetin standardization method, was estimated as 177.84±8.75, 67.11±14.73, and 226.02±65.73 mg QE/g crude extract for seeds, stems, and flowers, respectively. Flavonol content, also determined using the quercetin standardization method, yielded values of 177.84±8.75, 67.11±14.73, and 226.02±65.73 mg QE/g crude extract for seeds, stems, and flowers, respectively.

Furthermore, the total tannin content, assessed via the gallic acid standardization method, was estimated as 2.36±0.16, 3.92±0.44, and 3.45±0.46 mg GAE/g crude extract for seeds, stems, and flowers, respectively. Finally, the total proanthocyanidin content, determined using the catechin standardization method, was found to be 133.42±39.82, 126.52±5.73, and 171.97±8.59 mg CAE/g crude extract for seeds, stems, and flowers, respectively.

These findings highlight the richness of phenolic compounds across different parts of the plant, with detailed results presented in Table 1. Additionally, Table 1 includes the DPPH radical scavenging activity of *S. orientalis* seeds, stems, and flowers, along with the presence of the well-known natural antioxidant, ascorbic acid. The results reveal significant antioxidant properties in seeds, stems, and flowers, with IC_{50} values of 54.37±14.73, 71.77±6.46, and 75.48±7.07, respectively.

In summary, seeds exhibit the highest DPPH activity, reflecting their potent radical scavenging effect. The flower part shows the highest total flavonoid, total phenolic, and total proanthocyanidin content, while seeds have the highest total flavanol content, and stems exhibit the highest total tannin content.

Table 1. Bioactive components and antioxidant activity of *S. orientalis* seed, stem and flower

Plant Name	DPPH (IC_{50} mg/ml)	Total Flavonol Compound (mg QE/g extract)	Total Flavonoid Compound (mg QE/g extract)	Total Phenolic Compound (mg GAE/g extract)	Total Proanthocyanidin content (mg CAE/g extract)	Total Tanen Content (mg GAE/g extract)
S. orientalis-Seed	54.37 ± 14.73	27.21 ± 3.72	177.84 ± 8.75	100.14 ± 1.18	133.42 ± 39.82	2.36 ±0.16
S. orientalis-Stem	71.77 ± 6.46	7.98 ± 3.66	67.11 ± 3.57	56.58 ± 5.10	126.52 ± 5.73	3.92±0.44
S. orientalis-Flower	75.48 ± 7.07	20.66 ± 6.85	226.02 ± 65.73	122.33 ± 4.02	171.97 ± 8.59	3.45 ±0.46

Phenolic Compound Contents by LC-MS analysis

The LC-MS/MS analysis was employed for the quantitative determination of total phenolic compounds in the aerial parts of *S. orientalis*. The sample was subjected to quantitative analysis of twenty-one distinct compounds, and the findings are presented in Table 2. As anticipated, the extract was observed to contain trace amounts of phenolic acids such as epigallocatechin-3-gallate, syringic acid, (-) epicatechin, coumarin, trans-resveratrol, hesperidin, and myricetin.

As indicated in Table 2, substantial quantities of p-coumaric acid (9.29 mg/g), trans-ferulic acid (3.84 mg/g), caffeic acid (2.548 mg/g), and vanillic acid (1.401 mg/g) were identified. Furthermore, gallic acid, chlorogenic acid, salicylic acid, (+) taxifolin, rutin hydrate, ellagic acid, quercetin dihydrate, apigenin, were also detected in the sample. Comprehensive compound structures were illustrated using the ChemDraw program.

Table 2. The LC-MS analysis of *S. orientalis* aerial parts and their molecular structures

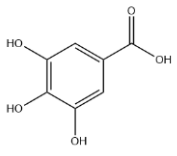
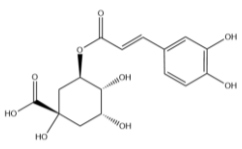
Phenolic Compound	Molecular Structure	*S. orientalis* Aerial Part of Methanol Extract (mg/g)
Gallic acid		0.081 ± 0.0003
Chlorogenic acid		0.193 ± 0.0002

Table 2 (*continue*). The LC-MS analysis of *S. orientalis* aerial parts and their molecular structures

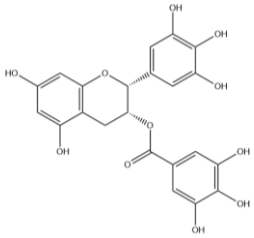
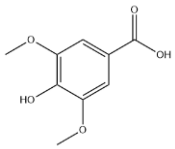
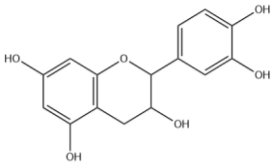
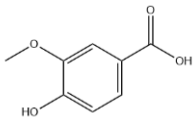
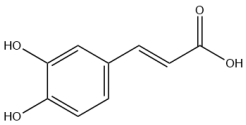
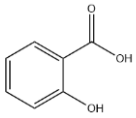
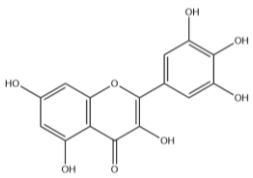
Phenolic Compound	Molecular Structure	*S. orientalis* Aerial Part of Methanol Extract (mg/g)
Epigallocatechın-3-gallate		≤ 0.02
Syringic acid		≤ 0.02
(-) epicatechin		≤ 0.1
Vanillic acid		1.401 ± 0.0979
Caffeic acid		2.548 ±0.0370
Salicylic acid		0.025 ± 0.0010
Myricetin		≤ 0.02

Table 2 (*continue*). The LC-MS analysis of *S. orientalis* aerial parts and their molecular structures

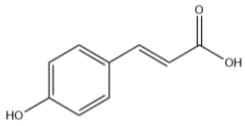
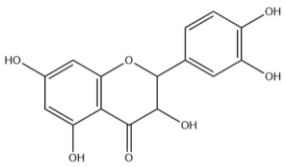
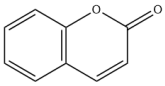
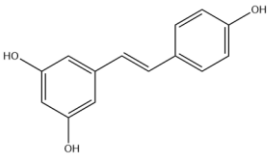
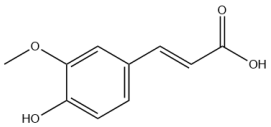
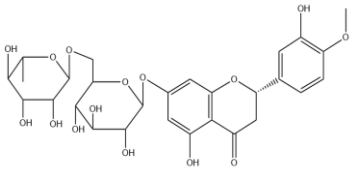
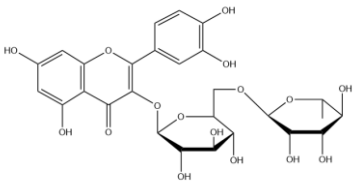
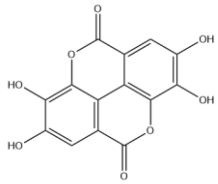
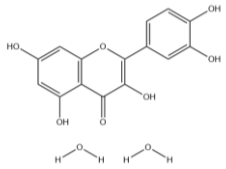
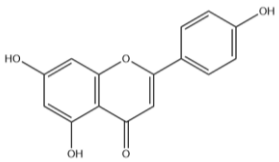
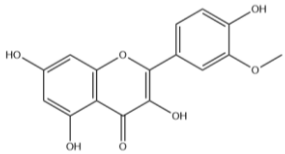
Phenolic Compound	Molecular Structure	*S. orientalis* Aerial Part of Methanol Extract (mg/g)
P-Coumaric acid		9.29 ± 0.0414
(+) Taxifolin		0.934 ± 0.0431
Coumarin		≤0.002
Trans- resveratrol		≤0.002
Trans-ferulic acid		3.84 ± 0.0873
Hesperidin		≤0.005
Rutin hydrate		0.096 ± 0.0074

Table 2 (*continue*). The LC-MS analysis of *S. orientalis* aerial parts and their molecular structures

Phenolic Compound	Molecular Structure	*S. orientalis* Aerial Part of Methanol Extract (mg/g)
Ellagic acid		0.661±0.0760
Quercetin dihydrate		0.495 ± 0.0039
Apigenin		0.031± 0.0002
Isorhamnetin		0.063 ± 0.0020

The excessive oxidation and reduction of cellular components can be harmful; hence, maintaining redox balance is of vital importance [32]. Plants, generally possessing widespread antioxidant capabilities, delay or prevent cell damage through effective antioxidants at low concentrations [33]. The diversity of antioxidants in plants includes water-soluble compounds such as ascorbate, glutathione, and phenols, as well as lipid-soluble ones like tocopherols, tocotrienols, and carotenoids [33,34].

Recent emphasis on the antioxidant potential of primary metabolites, polysaccharides, and glycoconjugates derived from natural sources highlights that plant species from various organs exhibit exceptional antioxidant potential [35]. The Orchidaceae, widespread worldwide and discovering approximately 200 new species annually, is particularly valuable for its diverse potential applications, including anti-inflammatory, anticancer, antimicrobial, antiviral, and immune-boosting properties[36]. While extensive research is available on tropical orchids, studies on terrestrial orchids are notably limited. In previous studies with orchids, Debnath and Kumari (2023) examined the accumulation of plant secondary metabolites in *Pholidota articulata* Lindl. and found that the accumulation was influenced by the polarity of different solvents used and various solvents (methanol, acetone, and chloroform) were effective in metabolite extraction. In this context, methanol was identified as the most effective solvent, providing the best results. In *P. articulata*, it was observed that phenols were the predominant secondary metabolites, followed by alkaloids, flavonoids, and tannins. Additionally, after treatment with 4 mg/L chitosan, the plants exhibited the highest accumulation of TPC (Total Phenolic Content) (81.73±0.32 mg/g dry weight), TFC (Total Flavonoid Content) (30.21±0.04 mg/g dry weight),

and TTC (Total Tannin Content) (33.10±0.10 mg/g dry weight) in leaf tissues [37]. However, when compared to *S. orientalis*, all parts of *S. orientalis* had higher phenolic content, but the results were reversed in terms of tannins. Furthermore, Hasnu et al. (2022) found that the methanolic leaf extract of *Vanilla borneensis* Rolfe exhibited a significant amount of phenolic content at 50 µg/ml and its flavonoid content was noted to be 73.87 µg/ml quercetin equivalent [38]. These results suggest that *V. borneensis* possesses high antioxidant activity, especially when compared to commercially cultivated Vanilla species. It is noteworthy to mention that species like *V. planifolia* Andrews, which are among the most cultivated, also have a remarkable total phenolic content. Additionally, natural *V. planifolia* extract has been found to have high DPPH radical scavenging activity and reduced power activity [39]. Another study determined that the dichloromethane extract of *Tridactyl tridentata* (Harv.) Schltr. roots exhibited a DPPH activity with an IC_{50} value of 0.02 mg/ml. However, it was observed that the ethanol extract showed low activity [40]. Similarly, a study conducted using methanol extracts of the wild *Rhynchostele rossii* (Lindly) plant found that the highest antioxidant activity was in the root extracts of the plant, with an IC_{50} value of 53.64±0.82 µg/ml [41]. In the study conducted by Ertürk et al. (2023), the antioxidant activity of *Serapias vomeracea* (Burm.f.) Briq., a member of the *Serapias* genus, was determined using the DPPH method [42]. The IC_{50} value obtained in the study was reported to be higher than that of the aerial part of *S. orientalis* (IC_{50}: 13.33). Consequently, it has been observed that tropical orchid species have more effective antioxidant properties compared to *S. orientalis*. However, this study also highlights that the *S. orientalis* plant possesses significant antioxidant potential and points to the potential use of different plant organs.

One advantage of attenuated total reflection Fourier-transform infrared spectroscopy is that it requires minimal sample preparation and shortens the analysis time by obtaining spectra on a wide range of samples, including powders, liquids, and pastes [43,44] In previous studies, amid I, II, and III peaks were prominent, and aromatic rings, geminal methyl, and ether linkages were commonly found in callus and leaf extracts. These observed peaks suggested the presence of flavanones and terpenoids. Terpenoids may also play a role in the reduction of metal ions, which can occur through the oxidation of aldehyde groups to carboxylic acids [45,46]. Similar observations were made in the case of *Carica papaya* Linn, callus culture [47]. In another study, low-intensity absorption bands were observed due to OH groups originating from gallic acid, quercetin, rutin, and tannic acid. These groups play a significant role in antidiabetic, antioxidant, and antibacterial activities. Therefore, FTIR has been used to determine different phytochemical components in plant samples [48,49]. According to the obtained FTIR spectral results, various functional groups such as carboxyl, hydroxyl, lipids, alcohols, amides, and phenolic compounds were found in the aerial parts of the *S. orientalis* plant.

According to the LC-MS results of *S. orientalis*, the revealed phenolic compounds exhibit strong antioxidant activities. Particularly, key components such as p-coumaric acid (9.29 mg/g), trans-ferulic acid (3.84 mg/g), caffeic acid (2.54 mg/g), and vanillic acid (1.401 mg/g) stand out. Previous studies have identified antioxidant activities in extracts of *Sasa quelpaertensis* Nakai plant, containing compounds such as p-coumaric acid and myricetin. The amount of p-coumaric acid was determined to be 3.92 µg per 1 mg of extract [50]. On the other hand, phenolic compounds like ferulic acid, salicylic acid, and p-coumaric acid were detected in all tested extracts of upland and wetland rice (*Oryza sativa* L.) samples from Malaysia. Especially, in the Bario variety of upland rice, ferulic acid (10.31 mg/100g) and p-coumaric acid (1.10 mg/100g) were found at higher levels [51]. In comparison to the above-ground parts of *S. orientalis*, p-coumaric acid is present at approximately 9 times higher levels. In another study related to orchids, the above-ground parts of *Dactylorhiza osmanica* (Kl) Soo var. *osmanica* were analyzed by RP-HPLC, revealing the presence of phenolic compounds such as benzoic acid (289.123 mg/l), protocatechuic acid (1006 mg/l), p-hydroxybenzoic acid (1472 mg/l), vanillin (0.694 mg/l), and p-coumaric acid (3133 mg/L) [52]. According to the UPLC-PDA results of *Malaxis acuminata*, prominent components such as syringic acid, p-coumaric acid, rutin, and quercetin were identified. Analysis of the aerial parts of *M. acuminata* revealed the presence of rutin at a concentration of 3.78 ± 0.051 µg/mg. In the leaf part of M. acuminata, phenolic compounds including p-coumaric acid (3.20 ± 0.031 µg/mg), rutin (1.12 ± 0.058 µg/mg), and quercetin (0.40 ± 0.002 µg/mg) were detected. Similarly, the pseudobulb of *M. acuminata* exhibited the presence of phenolic compounds such as p-coumaric acid (3.35 ± 0.06µg/mg), rutin (1.10 ± 0.084 µg/mg), and quercetin (0.24 ± 0.003µg/mg) [53].

The highest concentration of ferulic acid (432.68 µg/g DW) was detected in the conjugate phenolic leaf extracts of the Phalaenopsis hybrid Younghome Golden Leopard "Peachy," while the highest concentrations of sinapic acid (2232.81 µg/g) and p-coumaric acid (767.81 µg/g) were found in the root extracts of the Sogo Yukidian "V3" and Sakura Hime hybrids, respectively. Previously, phenolic acids such as ferulic, sinapic, p-coumaric, and ellagic acids have been reported to possess strong antioxidant activity [54] studies conducted on orchids, deficiencies regarding these compounds are observed, particularly indicating limited literature on research concerning black orchids. Furthermore, the limitations of these studies are clearly evident in our literature review. In conclusion, LC-MS analysis of *S. orientalis* demonstrates the presence of phenolic compounds and their strong antioxidant activities. Key components such as p-coumaric acid, Trans-ferulic acid, caffeic acid, and vanillic acid are present in high concentrations. These components have been observed to have positive effects on the antioxidant potential of plants, consistent with other studies.

Conclusion

In conclusion, our study has definitively demonstrated the rich phenolic content and potent antioxidant properties of *S. orientalis*. These findings underscore the potential significance of this plant as a valuable resource for health-related applications. Particularly, the high concentrations of phenolic compounds such as p-coumaric acid, trans-ferulic acid, caffeic acid, and vanillic acid significantly contribute to the plant's antioxidant activity. Therefore, it can be concluded that *S. orientalis* holds great potential for use in traditional medicine, as well as in the food and pharmaceutical industries. Future research efforts should aim to further investigate the effects of these compounds in greater detail to better understand their contributions to human health.

ACKNOWLEDGEMENTS

This study was prepared from Erdi Can Aytar's doctoral thesis “Germination of Some Orchid Species from Seed and Determination of Their Phytochemical Properties”. This work was supported by Ondokuz Mayis University Scientific Research Projects Coordination. (Project Number: PYO.FEN.1904.22.012).

AUTHOR CONTRIBUTIONS

Concept: E.A., Y.O.K.; Design: E.A., Y.O.K.; Control: E.A., Y.O.K.; Sources: E.A., Y.O.K.; Materials: E.A., Y.O.K.; Data Collection and/or Processing: E.A.; Analysis and/or Interpretation: E.A.; Literature Review: E.A.; Manuscript Writing: E.A.; Critical Review: E.A., Y.O.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 867-875, 2024
Doi: 10.33483/jfpau.1465360

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ENHANCING SOLUBILITY AND DEVELOPING AN ITRACONAZOLE-BETA-CYCLODEXTRIN COMPLEX FOR ANTIFUNGAL THERAPY IN ORALLY DISINTEGRATING TABLETS

AĞIZDA DAĞILAN TABLETLERDE ANTİFUNGAL TEDAVİ İÇİN ÇÖZÜNÜRLÜĞÜN ARTIRILMASI VE İTRAKONAZOL-BETA-SİKLODEKSTRİN KOMPLEKSİNİN GELİŞTİRİLMESİ

Tansel COMOGLU[1]*

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06560, Ankara, Türkiye

ABSTRACT

Objective: *This study aimed to create an orally disintegrating tablet (ODT) formulation using an itraconazole (ITZ)-beta-cyclodextrin (β-CD) complex to enhance itraconazole's solubility, a drug with limited solubility. β-CD was chosen for its compatibility with ITZ.*
Material and Method: *The study prepared equimolar mixtures of ITZ and β-CD through kneading, assessing their solubility and dissolution rates. The inclusion complexes significantly increased ITZ's solubility. This complex was used to develop directly compressed ODTs with a lower ITZ content (25 mg), incorporating D-Mannitol as a bulking agent, sweetener, and to enhance mouthfeel, facilitating rapid disintegration and drug release.*
Result and Discussion: *ODT formulations containing the ITZ-β-CD complex showed a significantly higher dissolution rate of ITZ compared to formulations with pure ITZ. This enhancement in dissolution is expected to significantly improve ITZ's bioavailability, suggesting a potential for reducing ITZ dosage and minimizing adverse effects.*
Keywords: *β-cyclodextrin, complexation, itraconazole, kneading method, orally disintegrating tablet*

ÖZ

Amaç: *Bu çalışma, sınırlı çözünürlüğe sahip itrakonazolün (ITZ) hızlı ve tam çözünürlüğünü sağlamak üzere ITZ-beta-siklodekstrin (β-CD) kompleksi kullanarak oral olarak dağılan bir tablet (ODT) formülasyonu geliştirmeyi hedeflemiştir. ITZ ile uyumluluğu nedeniyle β-CD tercih edilmiştir.*
Gereç ve Yöntem: *İtrakonazol ve β-CD'nin ekimolar karışımları yoğurma yöntemiyle hazırlanmış, çözünürlük ve çözünme oranları değerlendirilmiştir. İnklüzyon kompleksleri sayesinde ITZ'nin çözünürlüğünde önemli bir artış sağlanmıştır. Bu kompleksler kullanılarak, daha düşük ITZ içeriği (25 mg) ile doğrudan basılan ODT'ler geliştirilmiştir. D-Mannitol, hacim arttırıcı, tatlandırıcı olarak kullanılmış ve ağızda hızlı dağılma sağlamıştır.*
Sonuç ve Tartışma: *ITZ-β-CD kompleksini içeren ODT formülasyonları, saf ITZ içerenlere göre daha yüksek çözünmüş ilaç miktarı sergilemiştir. Bu, itrakonazolün çözünürlüğünde ve biyoyararlanımında önemli bir artış sağlamış, ITZ dozajının azaltılması ve yan etkilerin minimize edilmesi potansiyelini ortaya koymuştur.*

* **Corresponding Author / Sorumlu Yazar:** Tansel Çomoğlu
e-mail / e-posta: comoglu@pharmacy.ankara.edu.tr, **Phone / Tel.:** +903122033164

Submitted / Gönderilme : 04.04.2024
Accepted / Kabul : 10.06.2024
Published / Yayınlanma : 10.09.2024

Anahtar Kelimeler: *Ağızda hızlı çözünen tablet, β-siklodekstrin, itrakonazol, kompleksleştirme, örme yöntemi*

INTRODUCTION

Oral itraconazole (ITZ) is a potent triazole antifungal agent widely used for the treatment of a broad range of fungal infections. Despite its broad antifungal spectrum and clinical efficacy, ITZ's clinical utility is significantly hampered by its poor aqueous solubility and low oral bioavailability. These limitations not only affect the drug's absorption and therapeutic effectiveness but also contribute to variability in patient responses and necessitate the development of alternative formulations to enhance its delivery and performance [1].

The complexation of ITZ with beta-cyclodextrin (β-CD) presents a promising approach to overcome these challenges. β-CD, a cyclic oligosaccharide, is known for its ability to enhance the solubility and stability of poorly soluble drugs by forming inclusion complexes. This interaction can significantly improve ITZ's solubility, leading to enhanced bioavailability, and potentially, improved therapeutic outcomes [2,3].

Orally disintegrating tablets (ODTs) offer a novel and patient-friendly dosage form, designed to disintegrate rapidly upon contact with saliva, allowing for the administration of the drug without the need for water. This feature makes ODTs particularly beneficial for patients who experience difficulty swallowing conventional tablets or capsules, such as pediatric, geriatric, and bedridden patients, or those with dysphagia. Furthermore, the rapid disintegration and dissolution of ODTs in the oral cavity can expedite drug absorption and onset of action, which is crucial for conditions requiring prompt antifungal intervention [4].

The development of ITZ and β-CD complex containing ODTs aims to combine the enhanced solubility provided by the complexation with the convenience and patient compliance offered by the ODT formulation. This innovative approach not only addresses the limitations associated with ITZ's physicochemical properties but also aligns with the growing demand for more accessible and easier-to-administer medications.

Such a formulation could significantly impact the management of fungal infections, offering a more effective and user-friendly therapeutic option for a wide range of patients, including those with special needs regarding drug administration. By improving the bioavailability and patient experience, ITZ and β-CD complex containing ODTs hold the potential to enhance adherence to antifungal therapy, thereby optimizing clinical outcomes and contributing to the more effective management of fungal diseases.

MATERIAL AND METHOD

Materials

Itraconazole (It was gifted by Nobel İlaç, Turkey), β-cyclodextrin (Roquette, France), D-Mannitol (Roquette, France), Low-substituted Hydroxypropylcellulose (Shin-Etsu, Japan), Ac-Di-Sol (Colorcon, UK), Mg stearate (Ataman Kimya, Türkiye), Aerosil (Degussa, Germany) Mint flavor (Ataman Kimya, Türkiye).

Methods

Measuring the Solubility of ITZ in Water

To examine the solubility of ITZ in water, an accurately weighed amount of ITZ is added to a predetermined volume of water, ensuring the drug's potential saturation. This mixture is then agitated continuously at a constant temperature, at 37°C to mimic body conditions, for 24 hours to reach equilibrium. After sufficient mixing, the solution is filtered or centrifuged to remove undissolved particles. The concentration of ITZ in the resultant clear solution is then quantified spectrophotometrically at a wavelength of 262 nm using a Shimadzu UV-1800 device [5].

Preparation of ITZ-β-CD Complex

Equimolar ITZ-β-CD solid systems were prepared by kneading equimolar physical mixtures with the minimum volume of an ethanol-water (50/50 v/v) solution. By using this method, complexes in three distinct molar ratios of ITZ to β-CD-1:1, 1:2, and 1:3 (w/w) were produced. The mixtures were kneaded for a duration of 20 minutes [6,7].

Determination of Solubilities of Pure ITZ and ITZ- βCD Inclusion Complexes

Excessive quantities of pure ITZ, its inclusion complexes with ITZ-β-CD, were added into distilled water. These mixtures were subjected to sonication for one hour at ambient temperature, followed by agitation for 24 hours at the same temperature. The concentration of ITZ present in these solutions was measured using spectrophotometry at a wavelength of 262 nm [7].

Evaluation of the Micromeritic Characteristics of Powders

The flow characteristics of powder blends are essential for the quality of ODTs. For each formulation, the powder mixture's properties, including the angle of repose, bulk density, and tapped density, were assessed. The angle of repose (θ) was determined using the fixed funnel technique and calculated with Equation 1. Where, θ is angle of repose, h is height of the cone, and r is radius of the cone base.

$$\text{Tan } \theta = h/r \quad \text{(Equation 1.)}$$

The powder was thoroughly mixed, and around 2 grams of this finely blended powder was transferred into a funnel. The setup was adjusted so the funnel's lower tip lightly touched the apex of the powder mound. Positioned over a flat surface, the powder was allowed to flow freely through the funnel. The diameter of the resulting powder heap was measured for subsequent calculations based on the previously mentioned equation [8]. To measure bulk density, 2 grams of the well-mixed powder from each formulation was placed into a 10 ml graduated cylinder, and the volume of the powder was recorded [9]. The tapped density was determined by tapping the cylinder on a firm surface every 2 seconds, continuing the process until there was no further change in the powder's volume. The final volume was then recorded. The Carr's (Compressibility) index, indicative of the powder's compressibility, was calculated using the values obtained for bulk and tapped densities, applying Equation 2.

$$\text{Compressibility index} = (\text{Tapped Density} - \text{Bulk density})/\text{Tapped Density} \times 100 \quad \text{(Equation 2)}$$

Following the pre-compression evaluations, the powder mixture for each formulation was compacted utilizing a tablet machine (Korsch) equipped with a 12 mm punch.

Preparation of ODTs

To address the issue of ITZ's solubility, ODTs were formulated featuring a 1:2 molar ratio of inclusion complex and pure drug, created through direct compression techniques [7]. D-Mannitol, is a sugar alcohol used widely in pharmaceutical formulations, including ODTs, as a bulking agent, sweetener, and to enhance the mouthfeel of the final product. It is especially valuable in ODTs for its ability to promote rapid disintegration in the oral cavity, thereby facilitating faster drug release and absorption. Low-substituted Hydroxypropylcellulose (L-HPC) is employed in ODTs primarily as a disintegrant and binder, contributing to rapid tablet disintegration without compromising tablet cohesion. Magnesium stearate (Mg stearate) serves as a lubricant, ensuring the smooth ejection of tablets from the press and preventing sticking, though it's used sparingly to avoid impacting the tablet's dissolution profile. Aerosil, a colloidal form of silicon dioxide, acts as a glidant to improve powder flow during tablet compression and can also help in stabilizing blends by preventing segregation. Ac-Di-Sol, or croscarmellose sodium, is a superdisintegrant that facilitates rapid tablet break-up upon contact with saliva, ensuring quick release of the active pharmaceutical ingredient for absorption. Mint flavor is added to mask any unpleasant tastes of the active ingredients, enhancing patient compliance by improving the overall palatability of the ODTs [10]. Each of the selected excipients plays a crucial role in optimizing the formulation for effectiveness, manufacturability, and patient acceptability. The

components of the ODT formulations are detailed in Table 2.

Post-Compression Parameters of ODTs

The manufactured tablets underwent evaluations for post-compression attributes such as weight variation, hardness, and friability. To assess weight variation, twenty tablets from each batch were measured on an analytical scale (Sartorius BL 210 S). Tablet strength was gauged through tests of hardness and friability. The hardness for six tablets was determined using a Monsanto hardness tester, while the friability for ten tablets was assessed with a Roche friabilator, operating for 4 minutes at a speed of 25 rotations per minute [11].

Disintegration Time Measurement

The disintegration time was determined using a USP disintegration testing apparatus. This device includes a basket rack assembly with six vertically aligned open-ended tubes. Each tube's bottom is fitted with a number 10-mesh stainless steel wire screen. For the disintegration test, a single tablet from each formulation was placed into the apparatus's basket, with the temperature adjusted to 37 ± 2°C. The duration until complete disintegration occurred was then meticulously documented [9].

Measurement of Wetting Time and Water Absorption Ratio

For this assessment, one tablet from each formulation was positioned on a piece of circular tissue paper, which had been folded twice, inside a Petri dish with a 10 cm diameter. A solution of 0.5% (w/v) phenol red in 10 ml of water was prepared and poured into the Petri dish. The time taken for the tablet to become completely wet was recorded with a stopwatch. To evaluate the water absorption ratio, the procedure was replicated without the addition of phenol red. The water absorption ratio (R) was then calculated using Equation 3. [7].

$$R = Wa\text{-}Wb/Wb\times100 \qquad \text{(Equation 3.)}$$

Wa represents weight of tablet after wetting, while Wb represents weight of tablet before wetting.

Measurement of Dispersion Time

A tablet was chosen at random from each formulation to determine the time it took for complete dispersion. This was done by adding 10 ml of water to a glass beaker and then introducing the tablet into the water. The time until the tablet was fully dispersed was timed with a stopwatch. This procedure was performed three times for each formulation to ensure accuracy and the results were documented accordingly [12].

Drug Content Analysis

To prepare the sample solution, 10 tablets from each batch were randomly chosen and pulverized into a fine powder using a pestle and mortar. An amount of the powder equivalent to 25 mg of ITZ was then dissolved in 100 ml of 0.1N HCl and subjected to sonication for 30 minutes. Following sonication, the solution was filtered, and the drug content was measured using a UV- visible spectrophotometer at a wavelength of 262 nm [12].

Dissolution Testing of ODTs

The dissolution behavior of ITZ ODTs and the ODT formulation containing ITZ-β-CD (1:2 molar ratio) complex were evaluated using a Type II dissolution apparatus. The procedure was performed over a duration of 45 minutes, utilizing 900 ml of distilled water maintained at 37±0.5°C with a stirring speed of 50 rpm. To assess the in vitro release profile, 5 ml samples were withdrawn at predetermined time intervals (0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 minutes) and immediately replaced with an equivalent volume of fresh medium. The collected samples were then subjected to analysis using a UV-visible spectrophotometer set to 262 nm [1].

Evaluation of Stability

To evaluate the stability of both active ingredient and F1 and F2 formulations, the studies were

conducted in accordance with International Council for Harmonisation (ICH) guidelines. Samples were meticulously wrapped in aluminum foil and placed in a humidity-controlled chamber. The conditions within the chamber were maintained at 40±2°C and 75±5% relative humidity for a duration of one month [9].

RESULT AND DISCUSSION

Solubility of ITZ in Water

Table 1 presents the solubility values for pure ITZ and the prepared the ITZ-β-CD inclusion complexes in pH 6.8 buffer solution. Based on the obtained results, the highest solubility of ITZ (5.22±0.19 mg/ml) was achieved with the ITZ-β-CD complex prepared at a molar ratio of 1:2. Gökbulut and Özdemir, reported in their studies that on the preparation of cyclodextrin complexes with ITZ, prepared complexes of ITZ with HP-βCD and RAMEB at 1:1 and 1:2 ratios, first through physical mixtures, then employing kneading and co-precipitation methods. When solubility determinations were conducted in a pH 1.2 environment, they found that the solubility of ITZ, originally at 4.5 µg/ml, was highest in complexes prepared at a 1:2 molar ratio of IT:CD using the kneading method. Additionally, they reported that the formation of the ITZ-β-CD complex exhibits a BS-type solubility profile. In BS-type phase diagrams, the formation of complexes with limited solubility between a solute and solvent is depicted, indicating that the complex reaches its maximum solubility at a specific concentration. Beyond this point, any additional solute precipitates out, indicating a saturated solution. Consequently, it can be stated that the CD complexes of ITZ effectively increased the solubility of pure ITZ in pH 6.8 buffer. This enhancement in solubility can be attributed to the CD's ability to interact with both hydrophilic and hydrophobic structures, thereby improving the solubility of ITZ, which has poor solubility, However, it was determined that the highest solubility is achieved with a 1:2 molar ratio of ITZ:β-CD, beyond which the solubility of the complex decreases [3].

Pre-compression Parameters

The evaluated formulation blends, comprising both drug, drug-β-CD complex and excipients, were subject to various tests to determine their flow properties and suitability for direct compression. These tests included measurements of the angle of repose, bulk density, tapped density, and compressibility index. The bulk density was observed to range from 0.35 to 0.52 g/ml, while the tapped density varied between 0.28 and 0.47 g/ml. The angle of repose values fell within the range of 23.10 to 29.31. Additionally, the powder blends across all formulations demonstrated a compressibility index ranging from 11.35 to 12.47, as detailed in Table 3. These findings indicate that the powder blends exhibit ideal characteristics for manufacturing via the direct compression method [13].

Post-compression Parameters

ODTs containg both pure ITZ and ITZ-β-CD complex (1.2 molar ratio) were meticulously prepared and the composition of ODTs were given in Table 2. These formulations were crafted utilizing the direct compression technique, a method noted for its efficiency and precision. Subsequent to their preparation, these tablets underwent rigorous evaluation through a series of post-compression tests aimed at assessing various parameters critical to their performance and stability. These parameters included tablet thickness, hardness, friability, weight variation, drug content, wetting time, and disintegration time, with the results collated in Table 4 for comprehensive analysis [13].

Table 1. Solubility results of ITZ-β-CD inclusion complexes in water

Formulation code (ITZ:β-CD)	Molar ratio	Solubility in water (mg/ml)
C1	1:1	3.06±0.19
C2	1:2	5.22±0.23
C3	1:3	3.69±0.41
Pure ITZ	-	0.47±0.26

Table 2. Composition of ODTS

Formulation	Ingredients (mg)	
	F1	F2
ITZ	25	-
C2 (ITZ-β-CD inclusion complex equiavalent to 25 mg ITZ)	-	105
D-Mannitol	250	170
L-HPC	6	6
Ac-Di-Sol	6	6
Mg stearate	6	6
Aerosil	6	6
Mint flavor	1	1

Table 3. Pre-compression parameters of the formulations

Parameters (n=3±SD)	F1	F2
Bulk Density (g/ml)	0.52±0.13	0.35±0.24
Tapped Density (g/ml)	0.47±0.28	0.28±0.41
Hausner's Ratio	1.27±0.59	1.19±0.36
Angle of Repose	29.31±0.44	23.10±0.64
Compressibility Index (%)	12.47±0.62	11.35±0.39

Table 4. Post-compression parameters of the formulations

Parameters (n=3±SD)	F1	F2
Hardness (kg/cm^2)	4.83±0.32	4.94±0.38
Thickness (mm)	3.20±0.11	3.21±0.21
Weight variation	300.01±014	299.49±0.15
Fraibility (w/w%)	0.37±0.52	0.39±0.43
Disintegration time (sec)	>3min	50
Wetting time (sec)	>3min	56
Drug content (%)	99.98±0.51	100.02±0.15

The tablets exhibited hardness values ranging from 4.83 to 4.94 kg/cm², comfortably within the acceptable range of 5-8 kg/cm². This indicated that the tablets possess commendable mechanical strength, essential for enduring the physical and mechanical stresses encountered during handling and transportation. Furthermore, the thickness of these tablets varied between 3.20 to 3.21 mm, aligning with the acceptance criterion of ± 5%, showcasing uniformity in size and shape across all formulations [7].

In terms of friability, a parameter indicative of the tablet's resistance to crumbling under stress, values were recorded at less than 1% for all formulations, satisfying Pharmacopoeial standards. Specifically, the loss in weight percentage due to friability ranged from 0.37 to 0.39, well beneath the permissible limits set by official guidelines [7].

The wetting time, a measure of the time required for a tablet to become sufficiently wet to disintegrate, found to be 56 sec for F2 formulation prepared using the complexation technique. The wetting time of ITZ ODTs (F1) exceeding 3 minutes can be attributed to a key factor, primarily rooted in the inherent properties of ITZ. ITZ, classified as a Biopharmaceutics Classification System (BCS) Class II drug, is known for its poor water solubility, which is a significant challenge when developing a rapidly disintegrating oral dosage form. The drug's hydrophobic nature directly impacts the wetting and subsequent disintegration of the tablets, as the low solubility hinders water's ability to penetrate the tablet matrix efficiently [14].

Disintegration time, which is the time taken for the tablet to disperse into smaller particles, was determined to be 50 seconds for F2 formulation. This value is significantly lower than the acceptable standard of 3 minutes, underscoring the rapid disintegration capability of the tablet, a hallmark of

effective orally disintegrating formulations. In the F1 formulation containing ITZ, the disintegration results were again found to be more than 3 minutes. As explained above, the delay in disintegration can be attributed to ITZ being a BCS Class II drug, characterized by its low solubility active ingredient [14].

Dissolution studies further illuminated the efficiency of these formulations, with a particular focus on the cumulative percentage of drug release over time, as depicted in Figure 1. This analysis revealed that formulations which contained ITZ-β-CD complex, respectively, demonstrated a remarkably fast drug release rate, achieving %89.03 drug release within 30 minutes. This performance starkly contrasted with ITZ ODT formulations featuring β-CD which is a complexation agent. The notably enhanced dissolution rate observed in orally ODT formulation containing the ITZ-β-CD complex, in comparison to ODTs with free ITZ, can be attributed to the molecular encapsulation mechanism facilitated by β-CD. This cyclodextrin is known for its ability to form inclusion complexes with hydrophobic molecules like ITZ, significantly improving their aqueous solubility. The process involves the hydrophobic core of beta-cyclodextrin encapsulating the ITZ molecule, effectively masking its hydrophobicity and thus enhancing its solubility in water. This inclusion complex formation is crucial for ITZ, a BCS Class II drug characterized by its low solubility and high permeability, as it directly influences the drug's bioavailability by improving its dissolution profile. Moreover, the enhanced dissolution rate of ITZ when complexed with β-CD in ODT formulations can be further explained by the improved wettability and dispersibility of the complex within the gastrointestinal fluid. The presence of β-CD reduces the interfacial tension between the hydrophobic drug and the aqueous medium, facilitating quicker water penetration and, consequently, rapid disintegration and dissolution of the ODT. Additionally, the structural properties of β-CD, which include a toroidal shape with a hydrophobic cavity and a hydrophilic outer surface, contribute to its effectiveness as a solubility enhancer. This unique structure allows it to interact favorably with both the drug molecule and the aqueous environment, promoting the dissolution of poorly soluble drugs [15].

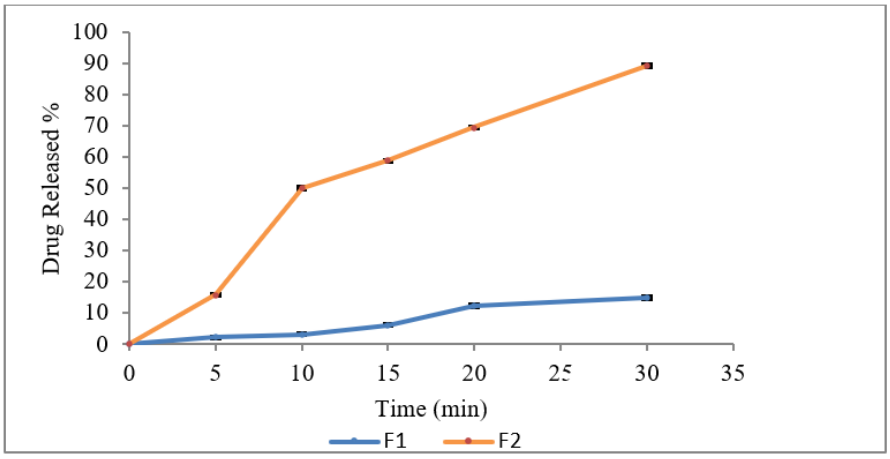

Figure 1. Drug release (%) of formulations versus time (min) (n=3±SD)

Additionally, the significant improvement in the dissolution rate of ODT formulations containing the ITZ-β-CD complex can be further enhanced by the incorporation of formulation excipients such as L-HPC, D-Mannitol, and Ac-Di-Sol. Each of these excipients plays a critical role in optimizing the formulation for rapid disintegration and dissolution, thereby complementing the solubility enhancement provided by the complex.

L-HPC acts as a disintegrant that facilitates water uptake and swelling of the tablet, leading to rapid disintegration. Its effectiveness is particularly notable in ODT formulations where the immediate release of the active pharmaceutical ingredient (API) is crucial. The mechanism by which L-HPC operates involves absorbing water rapidly upon contact with saliva, expanding, and creating porosity within the tablet matrix, which promotes quick disintegration.

D-Mannitol, a polyol with excellent taste-masking properties, serves as a bulking agent and also

enhances the palatability of ODTs. Its inclusion in the formulation contributes to the overall sensory acceptance of the dosage form. Furthermore, D-Mannitol has a high aqueous solubility, which aids in the dissolution process by creating a more favorable environment for the disintegration and dissolution of the drug-cyclodextrin complex.

Ac-Di-Sol, a superdisintegrant, significantly accelerates the disintegration of tablets by drawing water into the tablet matrix through capillary action and swelling. This rapid expansion exerts a disintegrative force within the tablet, leading to its breakdown. The use of Ac-Di-Sol in ODT formulations is particularly advantageous for achieving the quick release of the API, which is essential for ensuring rapid onset of action [4].

The synergistic effect of these excipients with the ITZ-β-CD complex significantly enhances the dissolution rate of ITZ from ODT formulations. By improving the wettability, porosity, and overall disintegration behavior of the tablet, these formulation aids ensure that the solubility enhancement achieved through the inclusion of β-CD is effectively translated into superior bioavailability and therapeutic efficacy.

The stability of pure ITZ and the F1 and F2 formulations was rigorously assessed under the challenging storage conditions of 40±2°C and 75±5% relative humidity, mimicking the harsh environments that might be faced in tropical regions or during uncontrolled transport, over a one-month period. Initial measurements showed ITZ concentrations of 84.35%, 90.29%, and 95.13% in the pure ITZ, F1, and F2 formulations, respectively, indicating the drug's potency at the beginning of the study. Despite the demanding storage conditions, the formulations demonstrated remarkable stability after one month. This study is essential for maintaining the therapeutic effectiveness and safety of the medication throughout its shelf life, ensuring the reliability of these formulations under various global climatic conditions [16].

AUTHOR CONTRIBUTIONS

Concept: T.C.; Design: T.C.; Control: T.C.; Sources: T.C.; Materials: T.C.; Data Collection and/or Processing: T.C.; Analysis and/or Interpretation: T.C.; Literature Review: T.C.; Manuscript Writing: T.C.; Critical Review: T.C.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 876-889, 2024
Doi: 10.33483/jfpau.1348367

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SIMPLE AND SENSITIVE SPECTROPHOTOMETRIC ASSAYS FOR THE DETERMINATION OF TERBINAFINE HCL ANTIFUNGAL DRUG IN PHARMACEUTICALS

FARMASÖTİKLERDE TERBİNAFİN HCL ANTİFUNGAL ETKEN MADDESİNİN BELİRLENMESİ İÇİN BASİT VE HASSAS SPEKTROFOTOMETRİK TAYİNLER

Nagib QARAH[1]*, Ezzouhra EL-MAAIDEN[2], Kanakapura BASAVAIAH[3]

[1]Hodeidah University, Faculty of Education- Zabid, Department of Chemistry, 3114, Hodeidah, Yemen
[2]University of Hassan 1[er], Laboratory of Biochemistry and Neurosciences, Department of Chemistry, 26000, Settat, Morocco
[3]University of Mysore, Department of Chemistry, 570 006, Mysuru, India

ABSTRACT

Objective: *Two highly sensitive, accurate, inexpensive, and simple spectrophotometric assays were developed and validated for the determination of an anti-fungal drug, Terbinafine HCl (TBH), in pure drug and tablets using potassium permanganate (PP) and disodium 2-(1,3-dioxo-2,3-dihydro-1H-inden-2-yl)quinoline-6,8-disulfonate (DSOQ).*
Material and Method: *In the present study, Sebifin and Terbiforce 250 mg tablets were used as pharmaceuticals, potassium permanganate $KMnO_4$ and disodium 2-(1,3-dioxo-2,3-dihydro-1H-inden-2-yl)quinoline-6,8-disulfonate in water were used as reagents, and DR 3900 spectrophotometer equipped with 1cm matched quartz cells was used for absorbance measurements.*
Result and Discussion: *The amount of terbinafine hydrochloride reacting with permanganate and disodium 2-(1,3-dioxo-2,3-dihydro-1H-inden-2-yl)quinoline-6,8-disulfonate in an acidic medium has been determined. The colored reaction products in both cases were measured at the maximum absorptions of 540 nm and 440 nm, respectively. The absorbance measured in each assay as a function of TBH concentration was related to TBH concentrations. Different experimental and variable conditions of assays were done carefully, accurately studied, and optimized. The validation of two assays also was done by following the current guidelines of the International Conference on Harmonization (ICH). Beer's law for the two methods is obeyed over the concentration ranges 1-15 µg/ml (Correlation coefficient = 0.9983) and 1-18 µg/ml (Correlation coefficient = 0.9989) for methods PP and DSOQ, respectively. Molar absorptivity, limits of detection, and quantification (LOD & LOQ) values were (1.38×10^4 l/ mol cm, 0.92 & 2.78 µg/ml) for PP assay, and (1.73×10^4 l/ mol cm, 0.09 & 0.27 µg/ml) for DSOQ assay, respectively. The two assays were successfully applied for the determination of TBH in commercial tablets with reliable and satisfactory results, and hence the proposed assays can be applied in pharmaceutical laboratories of quality control.*
Keywords: *Ion-pair, $KMnO_4$, spectrophotometry, terbinafine HCl*

ÖZ

Amaç: *Bir anti-fungal ilaç olan Terbinafin HCl'nin (TBH) saf ilaç ve tabletlerde potasyum permanganat (PP) ve disodyum 2-(1,3-diokso-2,3-dihidro-1H-inden-2-il)kinolin-6,8-izülfonat (DSOQ) kullanılarak tayini için oldukça hassas, doğru, ucuz ve basit iki spektrofotometrik test*

* **Corresponding Author / Sorumlu Yazar:** Nagib Qarah
e-mail / e-posta: nagib2015qarah@gmail.com, **Phone / Tel.:** +967734296789

Submitted / Gönderilme : 24.09.2023
Accepted / Kabul : 13.06.2024
Published / Yayınlanma : 10.09.2024

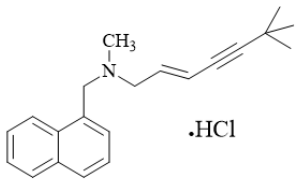
geliştirilmiş ve valide edilmiştir.
Gereç ve Yöntem: *Bu çalışmada, ilaç olarak Sebifin ve Terbiforce 250 mg tabletler, reaktif olarak potasyum permanganat $KMnO_4$ ve disodyum 2-(1,3-diokso-2,3-dihidro-1H-inden-2-il)kinolin-6,8-izülfonat ve absorbans ölçümleri için 1 cm eşleştirilmiş kuvars hücrelerle donatılmış DR 3900 spektrofotometre kullanılmıştır.*
Sonuç ve Tartışma: *Asidik bir ortamda permanganat ve disodyum 2-(1,3-diokso-2,3-dihidro-1H-inden-2-il)kinolin-6,8-disülfonat ile reaksiyona giren terbinafin hidroklorür miktarı belirlenmiştir. Her iki durumda da renkli reaksiyon ürünleri sırasıyla 540 nm ve 440 nm maksimum absorpsiyonlarında ölçülmüştür. TBH konsantrasyonunun bir fonksiyonu olarak her bir deneyde ölçülen absorbans TBH konsantrasyonları ile ilişkilendirilmiştir. Deneylerin farklı deneysel ve değişken koşulları dikkatlice yapıldı, doğru bir şekilde incelendi ve optimize edildi. İki tahlilin validasyonu da Uluslararası Uyumlaştırma Konferansı'nın (ICH) güncel kılavuzları takip edilerek yapılmıştır. İki yöntem için Beer yasasına, PP ve DSOQ yöntemleri için sırasıyla 1-15 µg/ml (Korelasyon katsayısı = 0.9983) ve 1-18 µg/ml (Korelasyon katsayısı = 0.9989) konsantrasyon aralıklarında uyulmuştur. Molar absorptivite, tespit ve miktar belirleme sınırları (LOD ve LOQ) değerleri PP testi için sırasıyla (1.38×104 l/mol cm, 0.92 ve 2.78 µg/ml) ve DSOQ testi için (1.73×104 l/mol cm, 0.09 ve 0.27 µg/ml) idi. Bu iki analiz, ticari tabletlerde TBH tayini için güvenilir ve tatmin edici sonuçlarla başarıyla uygulanmıştır ve bu nedenle önerilen analizler kalite kontrol farmasötik laboratuvarlarında uygulanabilir.*
Anahtar Kelimeler: *İyon çifti, $KMnO_4$, spektrofotometri, terbinafin HCI*

INTRODUCTION

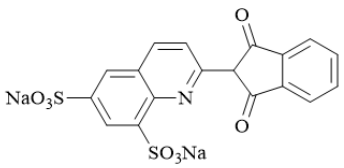
Terbinafine hydrochloride (TBH) (Figure 1) antifungals drug belong to the allylamine class. According to IUPAC, terbinafine hydrochloride TBH drug is known as [(2E)-6,6-dimethylhept-2-en-4-yn-1-yl](methyl)[(naphthalene-1-yl)methyl]amine hydrochloride [1]. The drug has a molecular weight corresponding to its molecular formula of $C_{21}H_{26}ClN$ is 327.9 g mol^{-1}. With good efficacy, terbinafine hydrochloride is used as a short-term treatment for various skin infections and fungi, especially, for the treatment of onychomycosis [2]. Onychomycosis is the most prevalent nail disorder attributed to yeasts; non-dermatophytes molds, and dermatophytes. Terbinafine hydrochloride has been approved medication for onychomycosis treatment by the Food and Drug Administration (FDA) of the US [3]. The drug is used in different therapeutic doses; as tablet formulations for fingernail and toenail fungus treatment, cream formulations for jock itch, athlete's foot, and other similar skin infections treatment, and other formulations of the drug as needed with therapeutic doses ranging from 1 to 5% [4].

The anti-fungal drug is official in United States Pharmacopeia (USP) [5], British Pharmacopeia (BP) [6], and European Pharmacopeia (EP) [7]. The USP recommended an HPLC method with UV detection at 280 nm. The BP and EP recommended a titrimetric assay in which the TBH drug is dissolved in ethanol (96%), and 5 ml of HCl (0.01M) is added followed by potentiometric titration with 0.1M NaOH.

Apart from the pharmacopoeial methods, in pharmaceuticals, the TBH drug has been determined by different methods, including titrimetry using non-aqueous solvent [8], Electrochemical [9-12], UV-spectrophotometry [8,13-16], spectrofluorimetry [17], capillary electrophoresis [18,19], and HPLC [20-32].



Terbinafine hydrochloride (TBH)



disodium 2-(1,3-dioxo-2,3-dihydro-1H-inden-2-yl) quinoline-6,8-disulfonate (DSOQ)

Figure 1. Chemical structures of TBH and DSOQ

Many of the reported methods [8-32] need expensive and sophisticated instruments, which are

not available in most quality control laboratories, as well as, involve tedious multiple extraction steps and are time-consuming. They also pose the problem of maintenance. Hence, they are not within the reach of most quality control laboratories and small-scale pharmaceutical industries, especially in the Asian sub-continent.

The official titrimetric methods [6,7] are cumbersome and require a large quantity of drug (250 mg) for each trial. The non-aqueous titrimetric method reported by Cardoso et al. [8] requires a perfect anhydrous medium and a large quantity of mercuric acetate. The presence of the amine group in the molecule has led to the development of a few visible spectrophotometric assays based on ion-pair complex, and redox reactions. Methyl orange [33], orange-G, molybdenum(V) thiocyanate, and alizarin red S [34], bromophenol blue bromothymol blue, and bromocresol green [35], alizarin red S [36], bromocresol purple [37], have been used as ion-pair reagents where the ion-pair complexes with the drug were formed, extracted into an organic solvent and measured, but few reported methods [33-37] suffer from elevated temperature for reactions to occur and poor sensitivity, with a narrow dynamic of linear ranges. Also, permanganate in alkaline medium [38], cerium (IV)-Chromotrope 2R [39] and $KBrO_3$-KBr mixture-methylene blue/methyl red [40], have been used as redox reagents, but also that reported methods suffer from some disadvantages such as elevated temperature for reactions to occur [38-40], yields considerable blank absorbance [38], employ multiple reagents and time-consuming [39,40].

To avoid one or more disadvantage in the reported spectrophotometric methods, the proposed methods, which are inexpensive, mild experimental conditions, simple, rapid, sensitive, and accurate assays were developed and validated for the terbinafine HCl antifungal drug in bulk form of drug as well as in tablets using PP and DSOQ reagents. The first proposed assays relied on the oxidation of TBH by PP in an H_2SO_4 medium, which results in the fading of PP solution. The Beer's law is obeyed between the concentration of TBH and the decrease value of absorbance, and the content of TBH can be indirectly measured by measuring the decrease value of absorbance at 540 nm (PP method). The second assay relied on the reaction of opposite charges ions, TBH as a cation and DSOQ as an anion, to form an ion-pair complex in acidic buffer conditions and measured at 440 nm. The proposed methods' results, under the optimum conditions, were found to be sensitive and simple compared to the most currently available methods for TBH.

MATERIAL AND METHOD

Pure TBH Drug, Dosage Forms, and Reagents Used

All reagents and other chemicals used in the present assays were of analytical reagent grade. The aqueous solutions required were prepared in double distilled water. Pure Terbinafine hydrochloride (TBH, 99.9%), was obtained from Cipla Ltd. (Maharashtra India). Permanganate PP (Merck, Mumbai, India), and DSOQ (Loba Chemie, Mumbai, India), the concentration of PP and DSOQ reagents were for each 0.06% (w/v) in water, respectively. A solution of PP (0.1%) was prepared and standardized according to a described procedure in a textbook of quantitative inorganic analysis, the standard solution was then diluted appropriately with water to a concentration of 0.06% and kept in a dark bottle [41], preparing all solutions freshly each day. Sebifin and Terbiforce tablets used (Glaxo Smith/ Lifestar Pharmaceuticals Ltd., India) contain 250 mg TBH of each tablet purchased from the local market.

Potassium biphthalate buffer (KHP) solution at pH 3.4, which was used in the present study was prepared by placing 50 ml of KHP solution (0.2 M) in a 200 ml volumetric flask and adding the specified volume of the hydrochloric acid (0.2 M) solution, 15.7 ml, then dilute to 200 ml with distilled water [42]. Buffer solutions covering a wide range of pH (1.4 to 5.4) were freshly prepared using (0.2M KCl – 0.2M HCl)) system for buffers in the pH range of 1.4 – 2.2, (0.2M potassium biphthalate, 0.2 M HCl) system for buffers in the pH range of 2.6 – 3.8, and (0.2M biphthalate , 0.2M NaOH) system for buffers in the pH range 4.2 – 5.4. all buffer solutions systems were prepared according to procedures described by the official methods [42].

Preparation of Standard Terbinafine HCl Drug Solutions

A 1 mg/ml (1000 μg/ml) stock standard drug solution was prepared accurately. Stock TBH

Solutions were prepared in H_2SO_4 (0.1 M) For reaction with PP, and in HCl (0.1 M) for reaction with DSOQ. A working concentration of assays using PP (20 µg/ml) and DSOQ (40 µg/ml), were prepared using a stock solution by accurately stepwise diluted with the corresponding solvents.

General Procedures

PP Method

One ml each of H_2SO_4 (1 M) followed by PP solution (0.06% w/v) was transferred into the two comparison flasks (10 ml). Then different volumes of 20 µg/ml TBH solution (0.0-7.5) ml equivalent to (0.0–15) µg/ml were added into one of the two a set of 10 ml comparison flasks. The flasks were diluted with distilled water to the mark, and standing for 5 min at room temperature (30±1 ºC). After the standing time for 5 min, the absorbance (A1) of the blank solution ($KMnO_4$+H_2SO_4) and the absorbance (A2) of the determination solution ($KMnO_4$+H_2SO_4+TBH) are measured at 540 nm against water. The △A(A1-A2) is calculated.

DSOQ Method

In the DSOQ method, aliquots (0.25-4.5 ml) of 40 µg/ml TBH solution were accurately transferred, into a set of 125 ml separating funnels, and the volume of each funnel was brought to 4.5 ml using (0.1 M) HCl. Six milliliters each of KHP buffer pH 3.4, and DSOQ (0.06% w/v) solutions to each funnel separately were added and the volume of each funnel was brought to 20 ml with distilled water. After the shaking time for 3 min, the organic layers were separated using dichloromethane and dried using anhydrous Na_2SO_4. The resulting absorbance of solutions was measured at 440 nm vs. the reagent blank prepared simultaneously.

The calibration graph in each case was plotted, and using the regression equation, the concentration of unknown was computed.

The Procedure Applied for Formulations

Twenty tablets (250 mg for each) of TBH were weighed and powdered and mixed well. A portion of the tablet powder equivalent to a hundred mg of TBH was transferred to a volumetric flask (100 ml). The quantity was treated with solvent and shaken for 20 min. solvents were sulphuric acid for the PP assay and hydrochloric acid for the DSOQ assay. The volume of resulting solutions was brought to mark with the corresponding solvent and then filtered. The resulting tablet extracts were stepwise diluted with the corresponding solvent to get 20 µg/ml for the PP assay and 40 µg/ml for the DSOQ assay. Five milliliters and a 2.5-milliliter aliquot of that concentrations respectively, were subjected accurately to analysis in five replicates (n=5) as described in PP and DSOQ procedures. Also, the fine powder of the tablet was subjected to analysis by reference method [5] for comparison.

Procedure for Excipients and Additives

Ten mg of placebo blank, which consists of magnesium stearate (15 mg), sodium citrate (15 mg), starch (20 mg), acacia (15 mg), talc (20 mg), and methylcellulose (10 mg), was extracted uniformly as described under procedure for the assay of TBH in formulations. After that, 2 milliliters of extract were analyzed by following the general procedures. To 10 milligrams of the placebo, 10 milligrams of a pure drug (synthetic mixture) were added and mixed well. The content was transferred accurately to a 100 ml volumetric flask and followed the procedure steps for formulations.

RESULT AND DISCUSSION

Chemistry and Pathway of Reactions / Absorption Spectrum

Here (Figure 2), in the first, TBH was treated with an excess known concentration of PP in an acid condition, and after appropriate standing time, the unreacted PP was determined by measuring its decreasing absorbance value at 540 nm. The amount of permanganate reacted corresponds to the drug amount which served as the basis of assay. Based on the proposed assay, in the range of 420-720 nm the absorption spectrums of the determination solutions ($KMnO_4$+H_2SO_4+TBH) and the permanganate

solutions ($KMnO_4$+H_2SO_4) were plotted, and the maximum absorption wavelength of each was found at 540 nm as shown in the Figure 3(a). So, 540 nm was chosen for all measurements.

In the second method, TBH reacted with the DSOQ reagent solution in an acidic condition (pH = 3.40), to form an ion-pair complex (Figure 2). The ion-pair product was extracted using dichloromethane and then measured at 440 nm. The absorption spectrum of blank and determination solutions were also plotted, and the maximum absorption wavelength of 440 nm was selected for all measurements. The absorbance of the reagent blank was not high as shown in Figure 3(b).

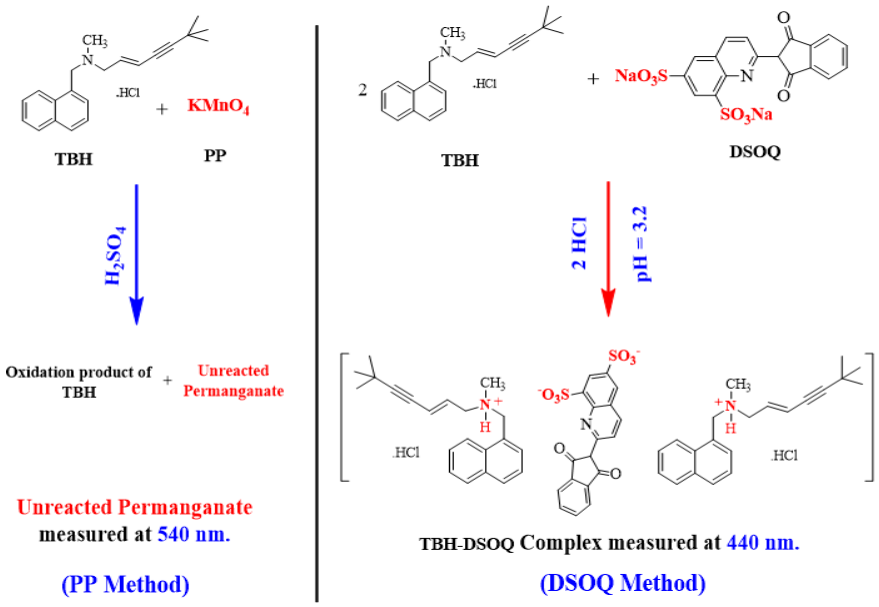


Figure 2. The probable reaction pathways

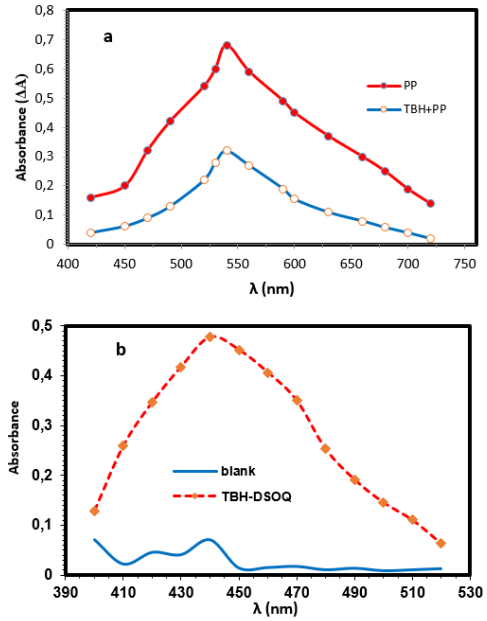


Figure 3. Absorption spectra of **(a)** (—●—) PP [PP= 0.06% (w/v), H_2SO_4=1 M] against water, (—○—) the reaction product of TBH and PP against water [TBH= 9 µg/ml, PP= 0.06% (w/v), H_2SO_4=1 M]. **(b)** the reaction product of TBH and DSOQ [TBH= 9 µg/ml, DSOQ = 0.06% (w/v), pH=3.4] against the blank

Optimization and Method Development of Experimental Variables

Several variables were investigated for spectrophotometric methods, such as PP, DSOQ, acid concentrations, reaction time, pH, and volumes of buffer. The investigated variables were optimized by varying one experimental variable at a time and keeping the other variables constant.

PP Method

Effect of Volume of Sulfuric Acid

The reaction between fixed concentrations of TBH and permanganate was carried out with used different volumes of 1 M H_2SO_4 in the range (0.5-2.5 ml). It was found to be optimum, complete, and quantitative with corresponds to the highest absorbance reading when using one ml of sulfuric acid (Figure 4).

Effect of PP Reagent

To study this effect, Different volumes of PP were examined to determine the optimum volume of permanganate that gives the highest absorbance at wavelength 540 nm in acidic conditions while keeping the other reagent concentrations constant. Various volumes of PP were token in the range of 0.5 to 3.5 ml as shown in Figure 4. A 1.5 ml of PP (0.06% (w/v)) gives the maximum absorbance. However, 1.5 ml of PP was selected in the final solution as the optimum reagent volume.

Effect of Reaction Time and Stability

Here, the reaction mixture between TBH drug and PP was subjected to different standing times in the range from 5 to 25 min (Figure 4). It was found to be completed in 5 min, and the absorbance was found to be constant up to 25 min thereafter.

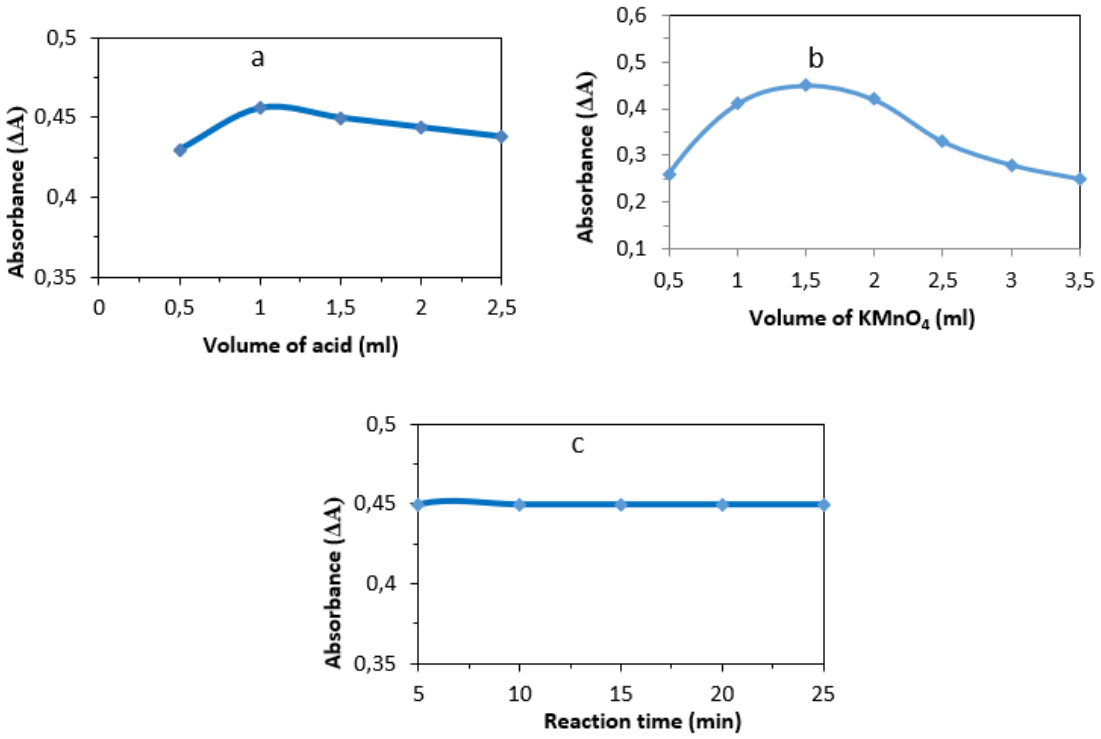


Figure 4. The effects of different parameters on the developed methods: volume of H_2SO_4 (TBH: 9 µg/ml) **(a)**, reaction time and stability (TBH: 9 µg/ml) **(b)**, volume of PP (TBH: 9 µg/ml) (**c**), pH of buffer (TBH: 10 µg/ml)

DSOQ Method

Effect of Buffer Solutions

The pKa value of TBH is 8.86 and says the TBH drug is the strongest basic, this means, the reaction at acidic pH has proceeded because the amino group of TBH easily becomes in the protonated form as well as the acid dye DSOQ becomes an anionic form. So, the reaction of TBH with DSOQ is only performed in an acidic medium.

To investigate the effect of pH on the absorbance of ion-pair formed in the aqueous layer, the procedure using DSOQ was adjusted to the pH in the range of 1.4 – 5.4. The resulting absorbance of that ion-pair increases with increases in the pH from 1.4 to 2.9, then the absorbance remains almost constant at pH 3.9, after that the absorbance was decreased. So, the pH value of 3.4 was chosen as to optimum value (Figure 5). The volume of biphthalate buffer at pH 3.4 was investigated in the range of 2 to 10 ml (Figure 5), and 6 ml of buffer solution was optimum.

Effect of DSOQ Reagent

The ion-pair complex formation of drug and DSOQ solutions was examined using 2-10 ml volumes of DSOQ dye (0.06% (w/v)), which was added to a constant concentration of TBH (10 µg/ml). Six ml of DSOQ solution in the aqueous phase (20 ml) adequately for optimum complex formation (Figure 5).

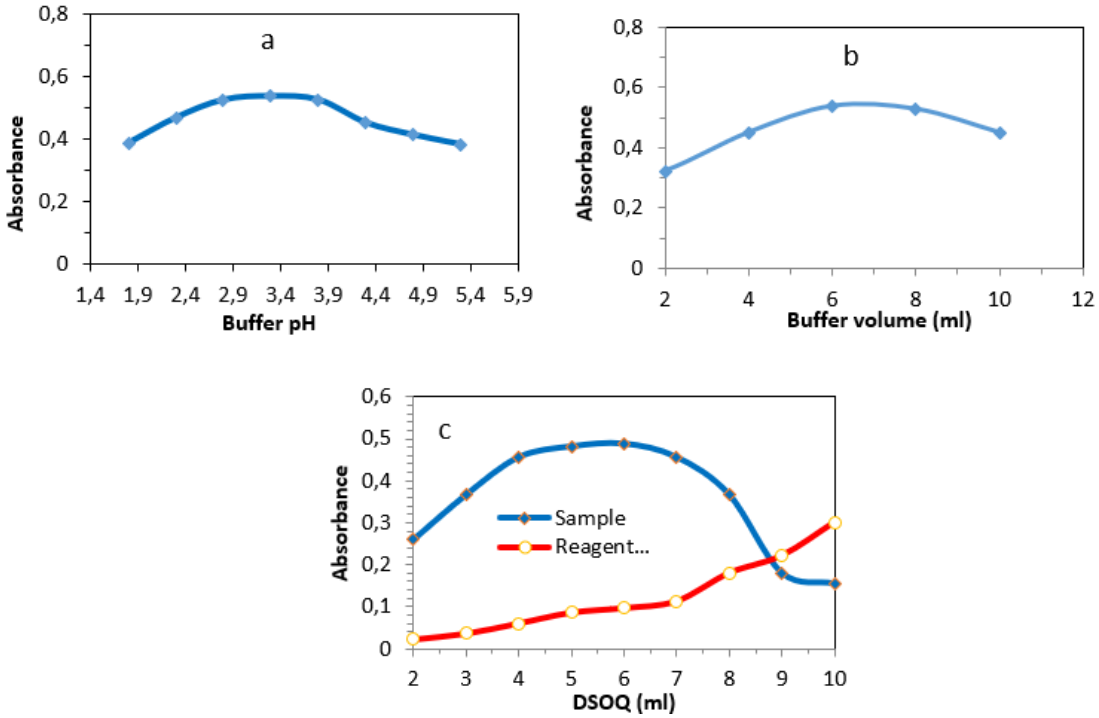


Figure 5. pH of buffer (TBH: 10 µg/ml) **(a)**, the volume of buffer (TBH: 10 µg/ml) **(b)**, the volume of DSOQ (TBH: 10 µg/ml) **(c)**

Method Validation

Range and Sensitivity

According to the optimum conditions for spectrophotometric methods using PP and DSOQ reagents, a linear resulting range between concentration and absorbance at λ_{max} was observed in 1-15 µg/ml for PP assay and 1-18 µg/ml for DSOQ assay, and Beer's law is obeyed inversely (Figure 6). Regression parameters values: intercept (a), slope (b), and the correlation coefficient (r) of each assay were calculated by linear regression (method of the least squares), and the other parameters of sensitivity

such as linear ranges, Sandell's sensitivity, molar absorptivity, LOD and LOQ values for each method are compiled in Table 1, which all generally indicating highly sensitive of the proposed methods.

The LOD and LOQ values were calculated using the formulae:

$$\text{LOD } (\mu g\ ml^{-1}) = \frac{3s}{b} \quad \text{and} \quad \text{LOQ } (\mu g\ ml^{-1}) = \frac{10s}{b}$$

where 's' is the standard deviation of the replicate absorbance values of the reagent blank, and 'b' is the slope of the calibration curve.

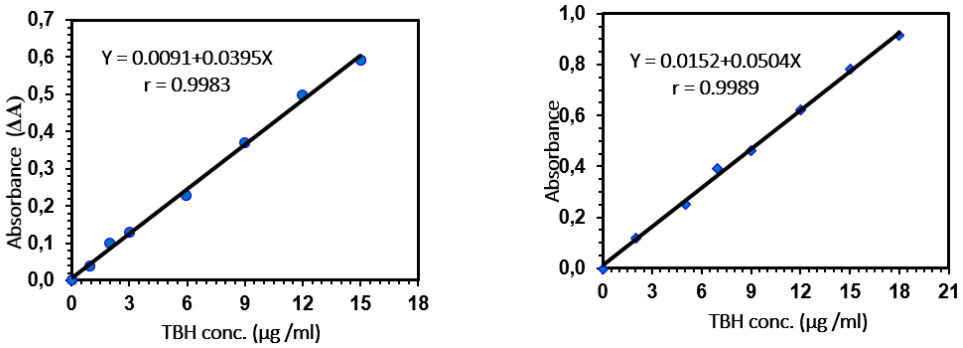


Figure 6. The calibration curves for PP (●) and DSOQ (■) assays

Table 1. Sensitivity and regression parameters were obtained from calibration curves for the determination of the TBH drug by spectrophotometric assays

Parameters	PP method	DSOQ method
λ_{max}, nm	540	440
Color stability	25 min	45 min
Linear range (µg/ml)	1–15	1–18
Molar absorptivity (l/mol cm)	1.38×10^4	1.73×10^4
Sandell sensitivity (µg/cm^2)	0.0238	0.0189
Intercept (a) ± (STD_a)*	0.0091 ± 0.0989	0.0152 ± 0.0998
Slope (b) ± (STD_b)*	0.0395 ± 0.00757	0.0504 ± 0.0052
LOD/LOQ (µg/ml)	0.13/0.39	0.09/0.27
Correlation coefficient (r)	0.9983	0.9989

*STD: standard deviation

Precision and Accuracy

The proposed PP and DSOQ assays were validated for each intra-day (within-day) and inter-day (between-day) precision. The precision and accuracy were evaluated by subjecting the pure TBH standard solution at three concentration levels (2,4,6 µg/ml using PP and 4,8,12 µg/ml using DSOQ) to within a day for intra-day variation analyzed, and on five successive days for inter-day variation analyzed, on seven replicates for each, taking into account the preparation of all chemical solutions daily to ensure accurate results. The precision was expressed as relative standard deviation (RSD). The %RSD (precision) for the intra-day assay ranged from 0.54–1.73 %, and 0.41–1.66 % for the inter-day assay, which should be satisfactory to the determination of the TBH in the different sample matrices used. The accuracy was calculated using the formula, Bias% = (found-taken/found x 100) and expressed as relative error percent (%RE). For three levels of drug, the accuracy varied from 0.67 to 1.5 % for intra-day and

0.84% to 2.25% for inter-day assays. These results are summarized in Table 2 and meet the requirement for precision, and the results indicate that the proposed methods are sufficiently precise and accurate.

Table 2. Intra-day and inter-day accuracy and precision (Reproducibility and intermediate precision)

Assay		Intra-day precision (n=7)			Inter-day precision (n=5)		
	Taken µg/ml	[a]Found µg/ml	RSD%	RE%	[a]Found µg/ml	RSD%	RE %
PP	2	2.02	0.91	1.0	2.03	0.83	1.50
	4	3.97	1.61	0.75	4.04	1.37	1.00
	6	6.04	0.54	0.67	6.05	0.41	0.84
DSOQ	4	4.06	1.23	1.50	3.99	1.53	2.00
	8	8.07	1.73	0.88	7.93	0.81	2.25
	12	12.08	1.07	0.67	12.19	1.66	1.58

[a] Mean value of 7 determinations

Robustness and Ruggedness

The optimized experimental variables were slightly altered in each method and the impact of these alterations was investigated on the performance of the proposed assays as a part of a robustness study. Experimental variables slightly altered were: the volume of acid, reaction time (PP method), buffer pH, and dye volume (DSOQ method). Assays were repeated and performed by only one analyst on the three different instruments in the laboratory and also by three analysts using the same instrument to understand the ruggedness of the methods. The results expressed as intermediate precision in RSD%, were encouraging assuring the utility of the proposed methods in routine use. The results are shown in Table 3.

Table 3. Evaluation of robustness and ruggedness for proposed methods

Method	TBH taken µg/ml	Robustness (RSD%)[a]				Ruggedness (RSD%)[a]	
		Parameters altered				Inter-analysts	Inter-instruments
		Acid volume (ml)	Reaction time (min)	Buffer pH	DSOQ volume (ml)		
PP	3	0.63	1.18	-	-	1.01	1.27
	6	0.75	0.92	-	-	1.53	1.19
	9	1.21	1.06	-	-	0.74	1.47
DSOQ	4	-	-	1.33	0.87	1.84	1.23
	8	-	-	1.55	1.35	1.45	1.65
	12	-	-	1.21	1.46	1.77	1.78

[a] Mean value of three determinations

Selectivity

This was investigated by studying and evaluating the impact of the additives and excipients present in the tablets by applying the proposed methods on the placebo blank and synthetic mixture. The results of the measurements showed that they were equal to those obtained from the blank. When applied the proposed methods to synthetic mixture analysis yielded recoveries of row TBH drug ranging from 98.46±0.76 to 103.6±1.85. The results saying non-interference from the additives and excipients of tablets such as sodium citrate, talc, starch, stearate, methylcellulose, etc. in the assays.

Application to Tablets Analysis

The developed methods were applied to the determination of terbiforce and sebifin tablet brands, each containing terbinafine HCl. The results for both assays presented in Table 4 say the excellent agreement with its label claim and with those obtained using the reference assay (USP) [5]. Statistically, for accuracy using the student's t-test, and for precision using F-test (variance ratio), the results revealed no significance when performing the developed methods and the reference method.

Table 4. Statistical analysis of obtained results by the proposed methods in tablets and comparison of it with the official method

Tablets		PP method	DSOQ method	Official method
Terbiforce 250 mg	Found[a] (% of label claim ± SD)	99.54 ± 0.95	99.89 ± 0.7	100.5±0.54
	t-test	1.97	1.54	
	F-test	3.09	1.68	
Sebifin 250 mg	Found[a] (% of label claim ± SD)	99.17 ± 1.63	99.77 ± 1.44	98.92 ± 1.29
	t-test	0.27	0.98	
	F-test	1.60	1.25	

[*]Mean found concentrations of n=5 determinations, tabulated t-value= 2.77 and F-value= 6.39, with 95% confidence level

Accuracy

To realize the accuracy of the present assays using PP and DSOQ reagents, the standard addition procedure (recovery study), was followed. A pure TBH drug was spiked well with tablet brands powder at three different levels, then the total amount was determined by proposed methods in the triplicate determinations. The results of the standard addition procedure, that present in Table 5 reveal that the co-formulated substances in the tablets do not affect the proposed methods.

Table 5. Recovery experiment of proposed methods via standard-addition method

Methodology	Formulations	Taken (μg/ml)	Added (μg/ml)	Found (μg/ml)	[a]Recovered % ± SD
PP	Terbiforce	2.99	1.5	4.550	101.3 ± 1.63
		2.99	3.0	5.960	99.51 ± 0.81
		2.99	4.5	7.440	99.37 ± 0.71
	Sebifin	2.98	1.5	4.440	99.15 ± 1.07
		2.98	3.0	6.110	102.1±1.150
		2.98	4.5	7.410	99.02 ± 0.63
DSOQ	Terbiforce	5.95	3.0	3.890	98.29 ± 0.61
		5.95	5.0	12.07	101.12 ± 1.07
		5.95	9.0	15.12	100.99 ± 1.06
	Sebifin	5.15	3.0	9.090	99.05 ± 1.42
		5.15	6.0	12.25	100.93 ± 1.52
		5.15	9.0	15.20	100.33 ± 1.18

[a]Mean value of three determinations

Table 6. Comparison of the proposed methods with the reported methods

Reported methods	Reagents	Range (μg/ml)	LOD/LOQ (μg/ml)	Remarks
Method reported 33	MO	6-17	Not available	Narrow range
Method reported 34	ARS	5-55	Not available	Narrow linear ranges, less sensitive
	Mo (v)/SCN	5-75		
	OG	10-80		
Method reported 35	BPB	2-25	0.23/0.71	Higher LOD and LOQ values
	BTB	2-25	0.28/0.84	
	BCG	2-25	0.54/1.62	
Method 36	ARS	2.5-60	0.22/0.66	Narrow linear ranges, less sensitive
Method 37	BCP	1-10	0.25/0.75	Higher LOD and LOQ values
Method 38	PP	2-16	0.65/1.96	heated at 85°c for 30minutes is required, High blank value
Method 39	Ce(IV) Ce(IV)-C2R	1-7 1-7	1.06/3.53 0.93/3.11	Heating step is required
Method 40	$KBrO_3$-KBr-MEB $KBrO_3$-KBr-MER	1-3 2.5-5	0.3/0.95 0.15/0.46	Heating step is required, high standing time used
Presented method	PP	1-15	0.92/2.78	**Highly sensitive, uses aqueous medium to critical pH adjustment**
Presented method	DSOQ	1-18	0.09/0.27	**Widest linear dynamic range (18-fold), lower LOD and LOQ values**

Conclusion

Terbinafine HCl despite its therapeutic importance in the treatment of fungi, the spectrophotometric methods that dealt with this drug are very few, including two methods by the author. The author presents this new research as a continuation of previous work to develop simple, high-accuracy, and rapid methods to cover the need for such low-cost methods for use in pharmaceutical quality control and clinical laboratories. The proposed spectrophotometric methods are simple without requiring any stringent experimental variable encountered in the published methods. The proposed methods are reasonably sensitive with a wide linear dynamic range as shown in Table 6. They can be useful in routine analysis and quality control assays of TBH in formulations. However, when compared to the HLPC method, which is widely used in pharmaceutical quantity control laboratories, the proposed methods lack selectivity, sensitivity, and speed.

ACKNOWLEDGEMENTS

The author (NASQ) wishes to express his thanks to Hodeidah University, Republic of Yemen for supporting research.

AUTHOR CONTRIBUTIONS

Concept: N.Q., E.E.; Design: N.Q., E.E.; Control: N.Q., E.E.; Sources: N.Q., K.B.; Materials: N.Q., K.B.; Data Collection and/or Processing: N.Q.; Analysis and/or Interpretation: N.Q.; Literature Review: N.Q., K.B.; Manuscript Writing: N.Q., E.E.; Critical Review: N.Q., E.E., K.B.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 890-902, 2024
Doi: 10.33483/jfpau.1439430

ORIGINAL ARTICLE / ÖZGÜN MAKALE

UNVEILING THERAPEUTIC TARGETS THROUGH PATHWAY ANALYSIS AND IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN ULCERATIVE COLITIS

ÜLSERATİF KOLİTTE YOLAK ANALİZİ VE FARKLI İFADE EDİLEN GENLERİN BELİRLENMESİ YOLUYLA TERAPÖTİK HEDEFLERİN AÇIĞA ÇIKARILMASI

Omnia Amir Osman ABDELRAZIG[1]*, Fadilah FADILAH[2,3], Linda ERLINA[2,3], Badriul HEGAR[4]

[1]Master Program in Biomedical Sciences, Faculty of Medicine, University of Indonesia, DKI Jakarta, 10430, Indonesia
[2]Department of Medical Chemistry, Faculty of Medicine, University of Indonesia, DKI Jakarta, 10430, Indonesia
[3]Bioinformatics Core Facilities, Indonesian Medical Education and Research Institute, Faculty of Medicine, University of Indonesia, DKI Jakarta, 10430, Indonesia
[4]Department of Child Health, Faculty of Medicine Universitas Indonesia, Dr. Cipto Mangunkusumo Hospital DKI Jakarta, 10430, Indonesia

ABSTRACT

Objective: *This study utilizes integrated bioinformatics to investigate Differentially Expressed Genes (DEGs) and pathways related to ulcerative colitis (UC).*
Material and Method: *Differentially Expressed Genes were identified from UC patients' colonic mucosal samples and controls using GSE13367 and GSE134025 datasets. Differentially Expressed Genes selection utilized GEO2R and Venn diagrams, followed by functional annotation, pathway analysis, PPI determination via the STRING database, and GO/KEGG enrichment analysis using Metascape.*
Result and Discussion: *Analysis unveiled 197 DEGs, with 76 up-regulated and 121 down-regulated genes. Up-regulated genes were enriched in humoral immune response, peptidoglycan binding, and NADPH oxidase complex, while down-regulated genes were linked to inorganic anion transport, transmitter-gated ion channel activity, and integral plasma membrane components. In the PPI network, up-regulated DEGs formed a dense network (75 nodes, 190 edges), indicating significant interactions, whereas down-regulated DEGs formed a less dense network (114 nodes, 63 edges). Five hub genes (CXCR4, CXCL13, CXCL1, MMP3) were identified among the 197 DEGs. These findings provide new insights into UC's causes and offer promise for more effective therapeutic approaches.*
Keywords: *Bioinformatic analysis, gene expression, inflammatory bowel disease, pathway enrichment analysis, protein interactions*

ÖZ

Amaç: *Bu çalışma, ülseratif kolit (ÜK) ile ilişkili DEG'leri ve yolları araştırmak için entegre biyoinformatik kullanır.*
Gereç ve Yöntem: *DEG'ler, GSE13367 ve GSE134025 veri kümelerini kullanarak ÜK hastalarının*

* **Corresponding Author / Sorumlu Yazar:** Omnia Abdelrazig
e-mail / e-posta: omniyajay@gmail.com, **Phone / Tel.:** +6288223720952

Submitted / Gönderilme : 19.02.2024
Accepted / Kabul : 15.06.2024
Published / Yayınlanma : 10.09.2024

kolonik mukozal örneklerinden ve kontrollerden belirlendi. DEG seçimi için GEO2R ve Venn diyagramları kullanıldı, ardından fonksiyonel anotasyon ve yol analizi yapıldı. Protein-protein etkileşimleri (PPI'ler) STRING veritabanı kullanılarak belirlendi ve Metascape, Gen Ontolojisi (GO) ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) zenginleştirme analizi için kullanıldı.

Sonuç ve Tartışma: *Analiz, 197 DEG ortaya koydu, bunların 76'sı yukarı regüle edilmiş ve 121'i aşağı regüle edilmiş genlerdi. Yukarı regüle edilmiş genler, humoral immün yanıt, peptidoglikan bağlanma ve NADPH oksidaz kompleksi gibi süreçlerde zenginleşmişti. Aşağı regüle edilmiş genler, inorganik anyon taşıma, alıcı-gated iyon kanal aktivitesi ve integral plazma membran bileşenleri ile ilişkilendirildi. PPI ağındaki yukarı regüle edilmiş DEG'ler, 75 düğüm ve 190 kenarla yoğun bir ağ oluşturdu, önemli etkileşimleri gösterirken, aşağı regüle edilmiş DEG'ler, 114 düğüm ve 63 kenarla daha az yoğun bir ağ oluşturdu. 197 DEG arasında beş merkezi gen (CXCR4, CXCL13, CXCL1, MMP3) tanımlandı. Bu bulgular, ÜK'nin nedenleri hakkında yeni içgörüler sunmakta ve daha etkili tedavi yaklaşımları için umut vaat etmektedir.*

Anahtar Kelimeler: *Biyoinformatik analizi, gen ifadesi, inflamatuar bağırsak hastalığı, yol zenginleştirme analizi, protein etkileşimleri*

INTRODUCTION

Ulcerative colitis (UC) is a chronic subtype of inflammatory bowel disease characterized by extensive inflammation of the colonic mucosa. Several factors can influence the development of ulcerative colitis, including lifestyle choices such as stress, drug usage, diets high in sugar and fat, and smoking. Genetic factors also play a role in susceptibility to UC [1]. Additionally, immune regulatory disorders, as well as the continuous stimulation of antigens by commensal enteric bacteria, fungi, and viruses, can contribute to chronic inflammation in individuals with genetic abnormalities and impaired mucosal barrier function. The primary clinical symptoms of UC include abdominal discomfort and diarrhea with blood and mucus [2].

Identifying differentially expressed genes (DEGs) in the colonic mucosa that exhibit altered expression patterns in UC patients compared to healthy individuals can provide valuable insights into the disease. Advances in gene chip technology have generated substantial amounts of data on gene expression profiles [3]. To gain a deeper understanding of the pathophysiology of ulcerative colitis, this study used an integrated bioinformatics analysis to examine the DEGs associated with UC and their related pathways. This approach offers a comprehensive view of the molecular mechanisms involved in the disease, facilitating further research and potential therapeutic interventions [4].

MATERIAL AND METHOD

DEGs Identification from Datasets GSE13367 and GSE134025

DEGs (Differentially Expressed Genes) were identified in normal samples regarding Ulcerative Colitis (UC) using GEO2R, a collaborative digital tool available at http:// www.ncbi.nlm.nih.gov/geo/geo2r [5]. This software adopts a powerful algorithm to help identify DEGs of multiple experimental conditions by pairing datasets from the Gene Expression Omnibus (GEO) series. Data on gene expression profiles including GSE13367 and data from the GSE134025 were downloaded from the GEO database. DEGs were derived from samples of their colonic mucosa as well as healthy controls. These datasets consisted of endoscopically collected mucosal colonic biopsies emanating from ulcerative colitis patients and healthy volunteers. The GSE13367 model has been subjected to gene expression analysis using the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The GSE13367 dataset consisted of 54 mucosal colonic biopsy samples in which 34 samples belonged to ulcerative colitis patients and 20 samples were taken from healthy controls. Gene expression analysis in the case of GSE134025 dataset was specifically performed with the help of GPL6947 Illumina Human HT-12 V3.0 expression bead chips. The GSE134025 dataset focused on the expression of genes in six samples, with three from ulcerative colitis patients and three from healthy subjects.

To identify DEGs, the variably expressed genes in each of the two datasets (GSE13367 and GSE134025) were initially analyzed individually with the criteria of a Fold Change (FC) greater than 1

and a p-value less than 0.05. Subsequently, the overlapping DEGs were determined by combining the two datasets, and this analysis was facilitated using the venny tool. This comprehensive approach allowed for the identification of common DEGs across the two datasets, providing valuable insights into gene expression changes associated with ulcerative colitis [6].

Analysis of the Functional and Pathway Associated with the DEGs

This research used the Metascape website which provides a comprehensive platform that seamlessly combines various tools and resources for biological analysis. It offers functional enrichment analysis, interaction analysis, genetic annotation, and the ability to search for specific memberships within datasets. This integration encompasses over 40 different knowledge databases [7]. Notably, we also used the Kyoto Encyclopedia of Genes and Genomes (KEGG) which serves as a repository within Metascape for highlighting the broad impacts and functions of biological systems [8]. Additionally, Gene Ontology (GO) was used to identify high-quality functional gene annotations across biological processes (BP), molecular functions (MF), and cellular components.

In our analysis, we configured Metascape with specific screening criteria, including a minimum overlap threshold of three and a minimum enrichment threshold of 1.5 (with a significance level of $p < 0.01$). These settings were applied to identify and elucidate the functions of Differentially Expressed Genes (DEGs).

PPI And Hub Genes Identification

The PPI network for the 197 DEGs was constructed, and 0.4 was the minimum required interaction score through the STRING database. Meaning that the PPI network active interaction sources were detected through text mining, co-expression, neighborhood, gene fusion, experiments, and databases among others. The Cytohubba ranking algorithm was applied in determining the hub genes of the 197 DEGs. Using the MCC algorithm and the cytoHubba Cytoscape plug-in, the top 5 hub genes were then identified. The functions of hub genes were predicted using Metascape with the screening settings of Min overlap of three and Minimum Enrichment of 1.5. which are Statistics considered significant at $p \geq 0.01$.

RESULT AND DISCUSSION

DEGs Identification

To refine the gene list, common genes from both datasets were filtered, and their representation was visualized using Venn diagrams (refer to Figure 1). 21.956 genes were found to be differentially expressed (DEGs). 9.990 DEGs were identified in GSE13367 meanwhile 18.487 DEGs were identified in GSE134025. Among these DEGs, 6521 were found to be differentially expressed, based on certain criteria $P<0.05$ and $logFC \geq 1$, we found 197 genes upregulated and downregulated significantly with 76 genes showing up-regulated and 121 genes showing down-regulated. Figure 2 illustrates the patterns of DEG gene expression in the two datasets, each containing two sets of sample data. It's noted that the majority of the up and downregulated genes were identified in GSE13367 (Figure 2a).

GO and KEGG Enrichment of the 197 DEGs

As per the Gene Ontology (GO) biological functional analysis, specifically depicted in Figure 3a, the 76 up-regulated genes exhibited significant enrichment in several key areas. In terms of biological processes, these genes were primarily associated with the regulation of antimicrobial humoral immune response mediated by antimicrobial peptides. In the realm of molecular functions, they were notably linked to peptidoglycan binding. Additionally, concerning cellular components, the up-regulated genes were enriched in the NADPH oxidase complex. Furthermore, the KEGG pathway analysis unveiled the predominant pathways associated with these 76 up-regulated genes. Notably, they were abundant in pathways related to viral protein interaction with cytokines and cytokine receptors, as well as pathways associated with amoebiasis. On the other hand, the 121 down-regulated genes exhibited distinct patterns of enrichment according to GO analysis (depicted in Figure 3b). These genes were prominently associated with functions related to transmitter-gated ion channel activity, inorganic anion

transmembrane transport, and plasma membrane integration, indicating a potential impact on cellular processes and membrane functions. Lastly, the KEGG analysis highlighted the primary functions associated with the 121 down-regulated genes. These genes were notably involved in the production and breakdown of keton substarnces indicating alterations in metabolic pathways associated with their downregulation.

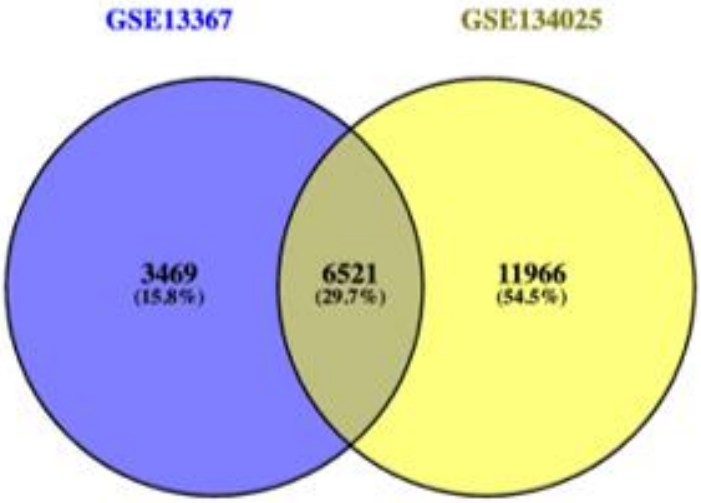
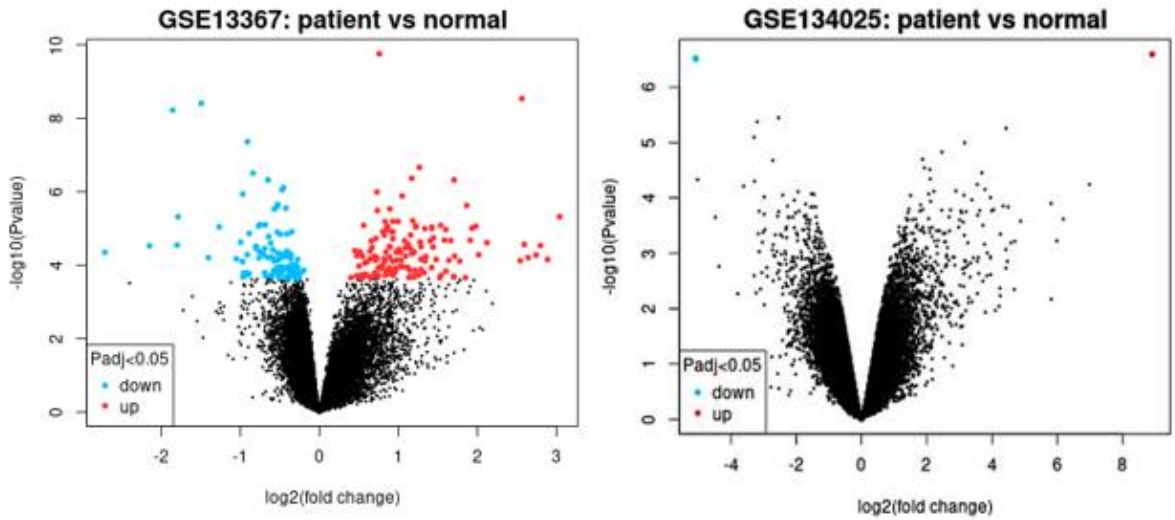
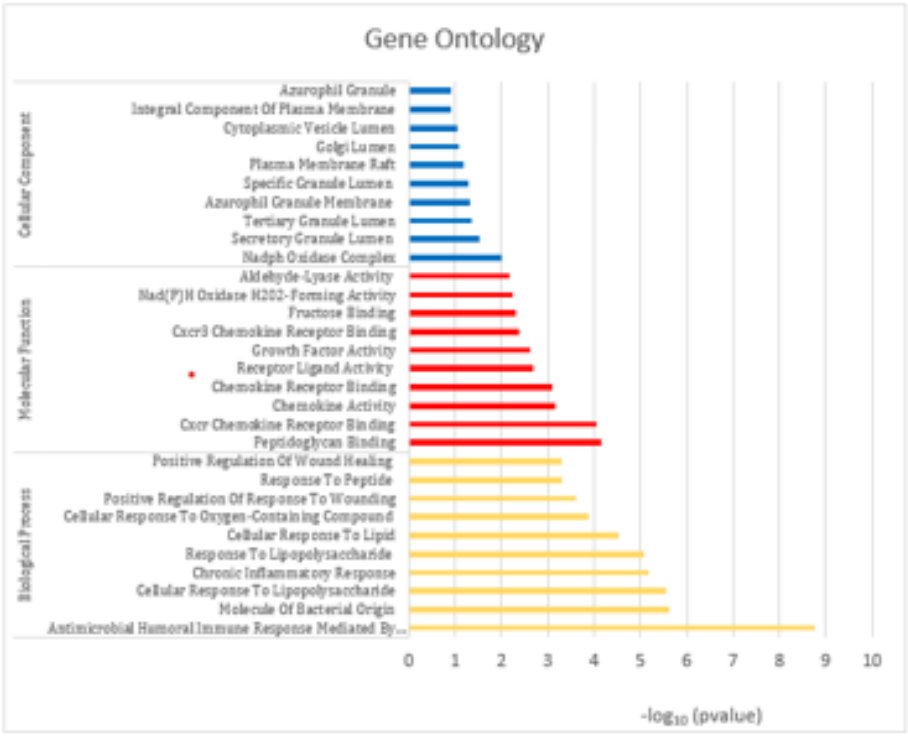


Figure 1. Venn diagram were used to visually represent the overlap of differentially expressed genes (DEGs) between the two Gene Expression Omnibus (GEO) datasets

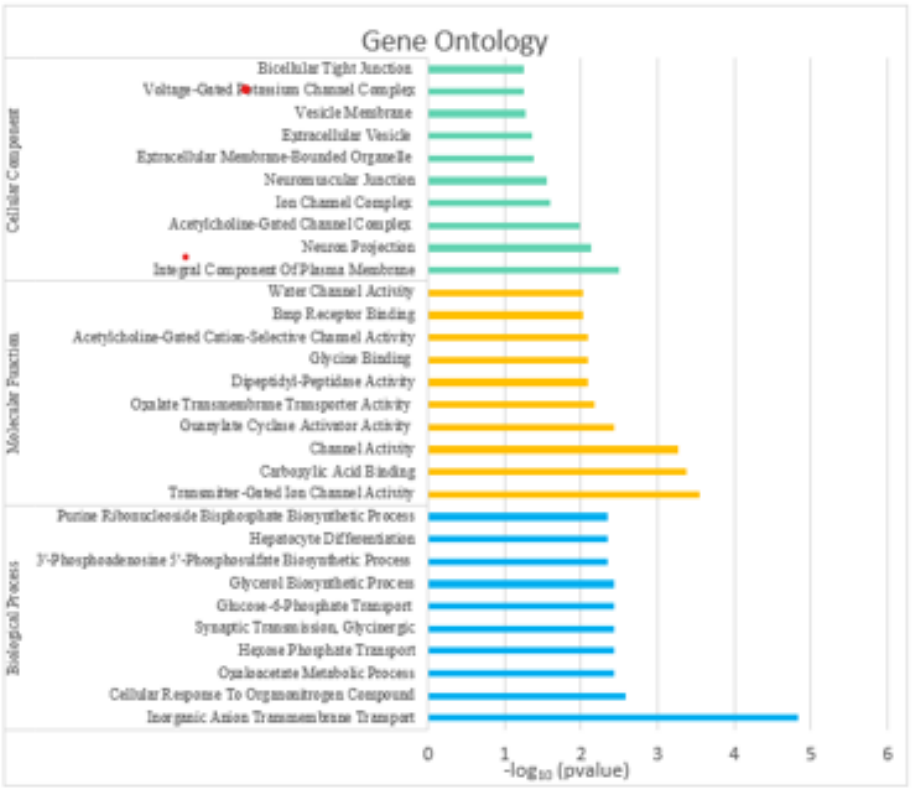


(a) (b)

Figure 2. The volcano plots were drawn to present the differentially expressed genes for UC over control comparison. The DEGs from GSE13367 and GSE134025 datasets have been presented individually in (a-b). These plots depict the down-regulated genes with the blue points, the similarly up-regulated genes are indicated by red points and black points indicate the same expressed genes that showed no significant differences in expression



(a)



(b)

Figure 3. Analysis of functional enrichment in DEGs. The bar graphs illustrate the top 10 results of the up-regulated gene (a) and down-regulated gene enrichment analysis (b)

PPI Hub Genes Identification

The Protein-Protein Interaction (PPI) was done for network analysis of Differentially Expressed Genes (DEGs) using the STRING database, and the results are as shown in Figure 4a and 4b. The 76 up-regulated DEGs-formed PPI network contained 75 nodes and 190 edges, resulting in an average node degree of 5.07 and local clustering coefficient of 0.443. Taken together, these metrics point to a statistically significant enrichment in Protein-Protein Interactions (PPIs) amongst these up-regulated genes, with a p-value of 1.0e-16.

On the other, PPI network with 121 DEGs down-regulated compromised of 114 nodes with an average node degree of 1.11 and local clustering value of 0.258 in 63 edges. This network, like the up-regulated genes, also displayed a highly enriched PPI interaction with a p-value of 3.22e-09. Notably, the top five genes within the PPI network were identified using the MCC algorithm and the cytoHubba Cytoscape plug-in (Figure 5). These genes include CXCL11, CXCR4, CXCL13, CXCL1, and MMP3, suggesting their potential importance in the context of the analyzed DEGs and their associated biological processes.

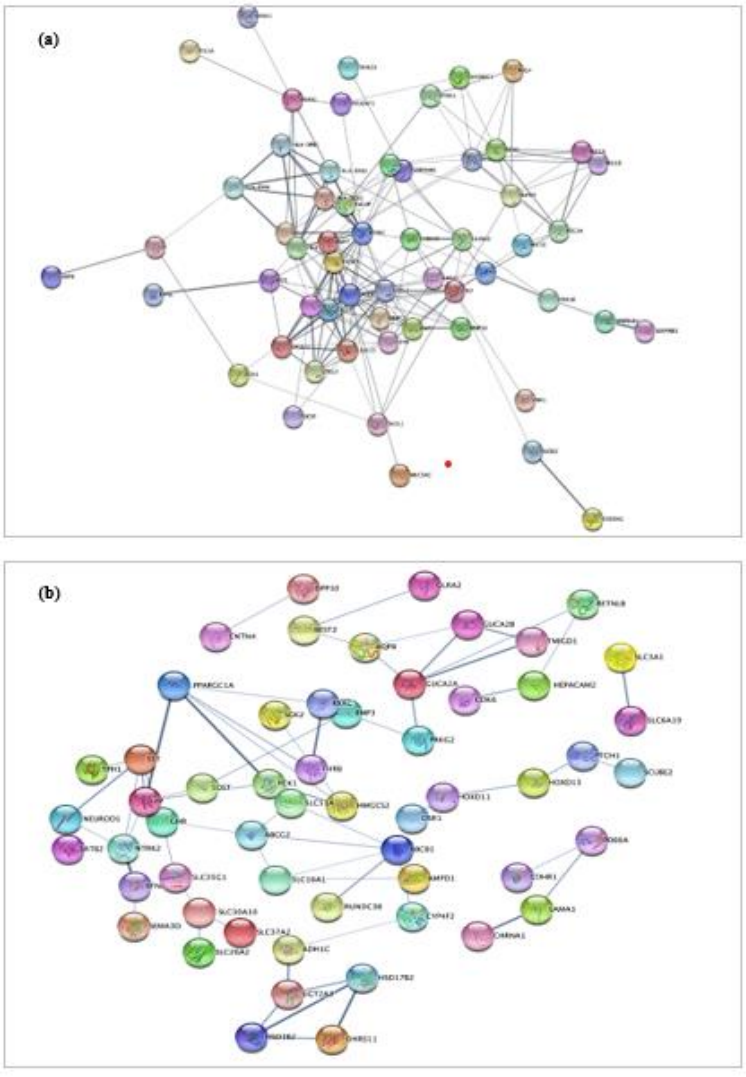


Figure 4. Cytoscape was used to create networks illustrating the protein-protein interactions (PPI) for the 76 up-regulated genes (a) and the 121 down-regulated genes (b). These networks visually portray the relationships and interactions among genes, where each gene is represented as a node. The connections or links between genes are illustrated as edges, and the size and color of each gene node indicate the extent of its interaction within the network

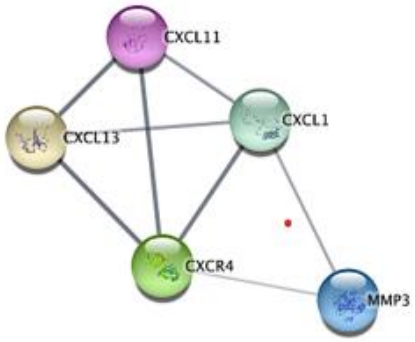


Figure 5. Cytoscape protein-protein interaction (PPI) networks were generated for the five identified hub genes through the MCC algorithm implemented in cytoHubba

Analysis of Hub Genes

The hub genes were subjected to functional enrichment analysis, yielding the following outcomes (as shown in Figure 6): Based on biological processes, the five hub genes were predominantly involved in the positive regulation of cell-cell adhesion mediated by integrin and the signaling pathways of chemokine ligand 12. Regarding molecular functions, they were notably associated with CXCR chemokine receptor binding, suggesting their role in binding to CXCR chemokine receptors. In terms of cellular components, the hub genes exhibited enrichment in the tertiary granule lumen, highlighting their presence in specific cellular compartments. Furthermore, when examining KEGG pathways, these five hub genes were predominantly enriched in pathways related to viral protein interaction with cytokines and cytokine receptors, suggesting their involvement in signaling pathways associated with viral interactions. These findings collectively provide insights into the functional roles of the identified hub genes within the context of the analyzed DEGs and their potential contributions to inflammatory and immune-related processes.

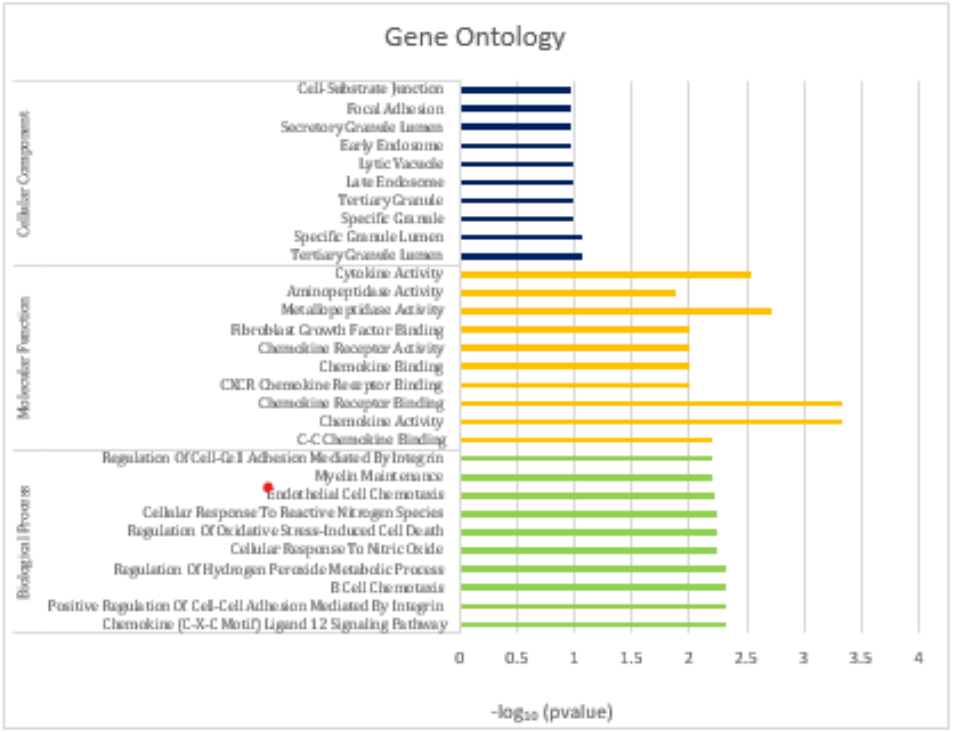


Figure 6. Evaluation of the functional enrichment of the five hub genes

Ulcerative colitis is a prevalent genetic inflammatory disorder of the colon characterized by symptoms such as bleeding, extensive fragility, and erosions on the colonic wall. This form of inflammatory bowel disease (IBD) is the most common worldwide, distinguished by its specific impact on the mucosa and submucosa of the colon. Typically, the disease initiates in the rectum and progresses inward [9]. Ulcerative colitis ranks among the most frequently occurring IBDs, with an incidence rate of twenty cases per 100,000 individuals in the United States. Both males and females are affected in nearly equal numbers [10]. Its prevalence is higher in populations in North America and Northern Europe compared to those in Asia. Given that gene expression patterns are frequently utilized to investigate ulcerative colitis, bioinformatics analysis serves as a valuable tool to uncover the pathophysiological mechanisms underlying UC [11].

In total, 197 differentially expressed genes (DEGs) were identified in this study among which 121 down-regulated genes and 76 up-regulated genes. From the Gene Ontology (GO) analysis, these DEGs were functionally categorized mainly to be associated with antimicrobial humoral immune response mediated through regulation of antimicrobial peptides. Additionally, they were linked to biological processes such as inorganic anion transmembrane transport. In terms of molecular function, the DEGs were associated with peptidoglycan binding and transmitter-gated ion channel activity. Regarding cellular components, they were enriched in the NADPH oxidase complex and integral components of the plasma membrane.

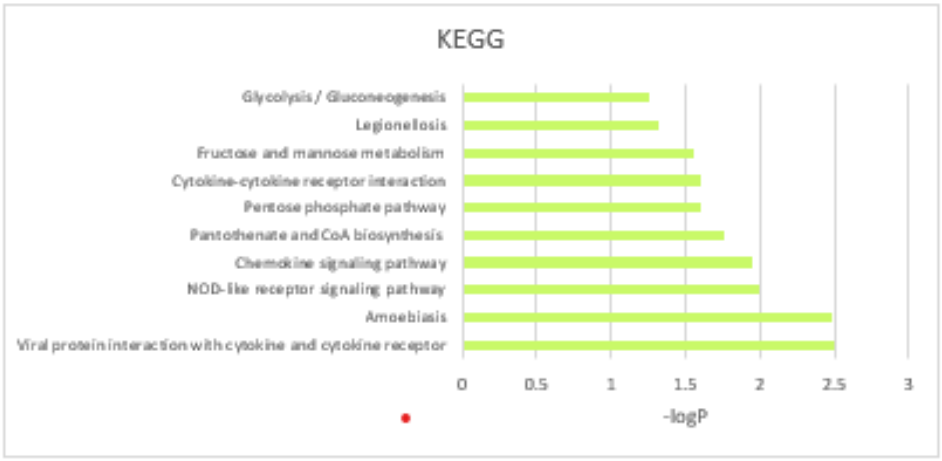
In addition, the KEGG pathway analysis showed that the DEGs that were upregulated were notably enriched in pathways associated with the interaction of viral proteins with cytokines and cytokine receptors, as well as in pathways related to amoebiasis. This aligns with the observation that the downregulated genes had extensive involvement in the synthesis and degradation of ketone bodies, as corroborated by KEGG pathway analysis. The both regulated DEGs had a very extensive involvement of glycolysis or gluconeogenesis (as demenosterated in Figure 7). Overall, this study sheds light on the molecular mechanisms underlying ulcerative colitis, providing valuable insights into the regulation of immune responses and metabolic pathways associated with this condition.

Among the 121 genes in the Protein-Protein Interaction (PPI) network of Differentially Expressed Genes (DEGs), five genes (CXCL11, CXCR4, CXCL13, CXCL1, MMP3) displayed a notable level of interaction, indicating their centrality in the network. In patients with ulcerative colitis (UC), all five of these hub genes exhibited increased expression levels. According to Gene Ontology (GO) analysis, these five genes were particularly enriched in the tertiary granule lumen as a cellular component, CXCR chemokine receptor binding as a molecular function, and the enhancement of cell-cell adhesion mediated by integrin as a biological process. Furthermore, when examining their involvement in signaling pathways, the five hub genes were predominantly associated with the TNF signaling pathway, viral protein interaction with cytokines and cytokine receptors, as demonstrated in KEGG pathway analysis (as demenosterated in Figure 8).

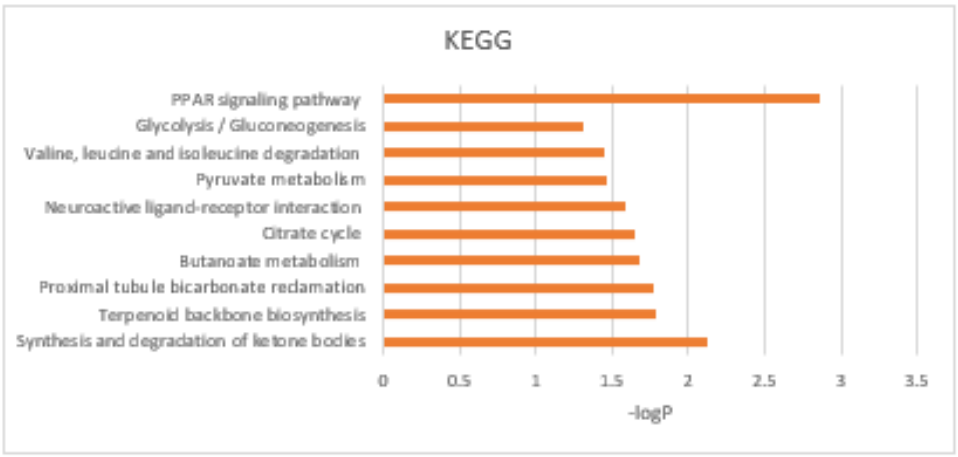
Ulcerative colitis (UC) is the idiopathic and recurrent inflammation of the mucosa of the intestines while its exact etiology is yet to be known. It usually initiates with the rectum and may go on to affect the entire colon. This engages multifaceted and dynamic web of cells and cytokines adjusting the immune response and inflammatory cascade within UC pathogenesis. Cytokine receptors on cell surfaces play a crucial role by binding precisely to cytokines and transmitting their signals, allowing cells to respond to signals from nearby or distant locations in the body [12]. The DEGs and hub genes identified in this study potentially contribute to the development of UC by participating in these intricate mechanisms, shedding light on the molecular pathways underlying the disease and providing a basis for further investigation into therapeutic interventions.

Table 1 presents the hub gene symbols, acronyms, and their corresponding functions. CXC motif chemokine ligand 1 (CXCL1) belongs to one of the four subfamilies comprising around 50 chemotactic cytokines [18]. This subgroup is distinguishable by the presence of a pair of disulfide bridges formed through the homologous CXC motif. CXCL1 plays a crucial role in the development of inflammatory bowel disease. Both ulcerative colitis and Crohn's disease patients have been found to have elevated blood levels of CXCL1, with ulcerative colitis patients having higher levels [16]. CXCL1 shows promise as a potential biomarker for ulcerative colitis and has been identified as a hub gene in ulcerative colitis through gene expression analyses. In the context of inflammatory bowel disease, CXCL1 contributes to

the chemotaxis of neutrophils and their infiltration into sites of inflammatory responses. It's important to note that in inflammatory bowel disease, CXCL1 is not the sole chemoattractant for neutrophils [19].



(a)



(b)

Figure 7. KEGG pathway analysis of functional enrichment in DEGs. The bar graphs illustrate the top 10 results of the up-regulated gene (a) and down-regulated gene enrichment analysis (b)

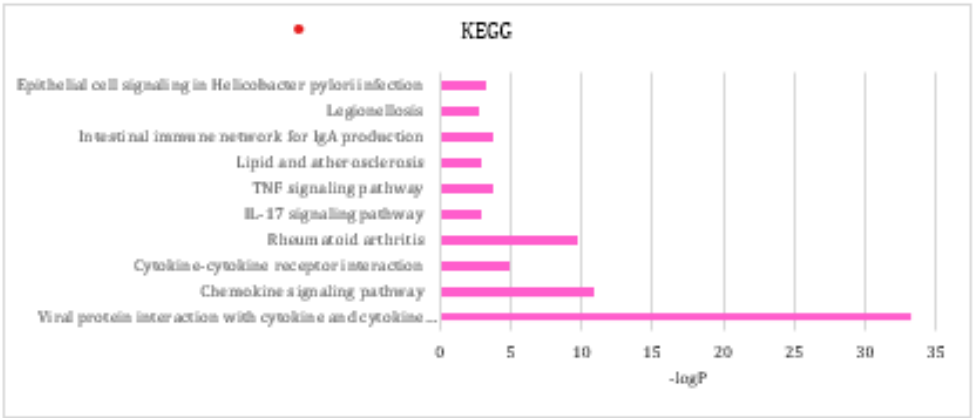


Figure 8. KEGG pathway analysis of 5 hub genes. The bar chart depict the top ten findings for the five hub genes

Table 1. Five hub genes and their functions

Gene symbol	Description	Function
CXCL11	chemokine ligand 11	A well-known controller of T-cell entry into tumors is CXCL11 [13].
CXCR4	C-X-C chemokine receptor type 4	CXCR4 produces a protein that penetrates outer cell layers, including those in the brain, spinal cord, and white blood cells [14].
CXCL13	C-X-C Motif Chemokine Ligand 13	(CXC chemokine ligand 13) has been proved to be generated persistently by stromal cells in lymphoid follicles of human lymph nodes and It serves as a potent attractant for naïve B cells in laboratory settings. This chemokine was also named B lymphocyte chemoattractant (BLC) and was initially discovered in mice [15].
CXCL1	C-X-C Motif Chemokine Ligand 1	Melanomagrowth-stimulating activity/growth-regulated protein, or CXCL1, is chemokine that is crucial for inflammation, angiogenesis, carcinogenesis, and wound healing [16].
MMP3	Stromelysin-1or as Transin-1	Extracellular matrix (ECM) components including matrix proteins, growth factors, proteases,surface receptors, and adhesion molecules can all be broken down by MMP-3 [17].

On the other hand, CXCL13 was initially identified in B cell follicle stromal cells. CXCL13 plays a pivotal role in the pathogenesis of various inflammatory diseases, including autoimmunity. It is constitutively expressed in secondary lymphoid tissues and promotes lymphoid neogenesis when expressed [20]. The presence of CXCL13 in human inflammatory diseases such as ulcerative colitis, chronic gastritis caused by Helicobacter pylori, and chronic inflammation associated with human lymphoid neogenesis suggests a potentially harmful role for CXCL13 [21].

In ulcerative colitis, the ELR-chemokine CXCL13 exhibits increased CXCL13 mRNA expression in intestinal tissues and interacts with CXCR5 as its receptor. These molecular insights provide valuable information about the involvement of CXCL1 and CXCL13 in the pathogenesis of ulcerative colitis and other inflammatory conditions [22].

Chemokines, essential for various biological processes including growth and homeostasis, exert significant effects on central nervous system cells and endothelial cells involved in both angiogenesis and angiostasis. Among these chemokines, CXCL11 stands out as the most potent CXCR3 agonist based on chemotaxis experiments. CXCL11 primarily induces T cell transepithelial migration and leads to receptor down-regulation[23]. Interestingly, CXCL10 and CXCL11 practiced enormous mRNA valorisation in of ulcerative colitis (UC) as well as colorectal cancer (CRC). Moreover, miR-34a-5p and miR-203a-5p appear to be potential regulators' miRNAs for CXCL10 and CXCL11. In the context of UC, interactions between CXCL10 and CXCL11 and cytokine receptors can activate the JAK-STAT signaling cascade. Furthermore, it has been observed that in both UC and CRC, CXCL10 and CXCL11 are positively correlated with the tissue infiltration of proinflammatory M1 macrophages [24].

CXCR4 (Chemokine (C-X-C motif) Receptor 4 represents a specific subclass of G protein-coupled receptors (GPCRs) that bears resemblance to an amino acid rhodopsin. Notebly It was shown that in the peripheral blood of UC patients the number of immature plasma cells increases significantly versus healthy donors and further reveals severe CXCR4 overexpression [25].

A study by Mina T. shows that these particular IgG plasma cells mediate the exacerbation of mucosal inflammation, functioning as one of the key elements in the pathogenesis of UC. This is through CXCR4 infiltration in inflamed mucosa to initiate "pathogenic" intestinal CD14 macrophages by means of IgG-ICFrC signaling. These findings provide valuable insights into the complex interplay of chemokines, receptors, and immune cells in the context of ulcerative colitis pathogenesis [26].

The extracellular matrix (ECM) is an important structural component of tissues normally degraded by proteins of the matrix metalloproteinase (MMP) family. MMPs have two roles to play firstly that in pathological circumstances as in arthritis and metastasis, and those in beneficial physiological activities like embryonic development, reproduction, and remodeling of tissues [27]. Many MMPs are initially released as inactive proproteins, which can be activated by extracellular proteinases. In the context of mucosal degeneration induced by the pokeweed mitogen, pharmaceutical inhibition of MMP-3 has been shown to effectively halt this degenerative process [28]. This highlights the significance of MMPs in tissue homeostasis and their potential as therapeutic targets in certain pathological conditions.

ACKNOWLEDGEMENTS

We express our gratitude to the University of Indonesia for providing the UI Great Scholarship 2022, as well as for the bioinformatics core facilities and all laboratories at the Indonesia Medical Educational Research Institute (IMERI) within the University of Indonesia.

AUTHOR CONTRIBUTIONS

Concept: O.A.O.A., L.E., F.F.; Design: O.A.O.A., L.E.; Supervision: F.F., B.H.; Resources: F.F., B.H.; Materials: L.E., F.F., B.H.; Data Collection and/or Processing: O.A.O.A., L.E.; Analysis and/or Interpretation: O.A.O.A., L.E.; Literature Search: O.A.O.A., L.E.; Writing: O.A.O.A.; Critical Reviews: L.E., F.E., B.H.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 903-911, 2024
Doi: 10.33483/jfpau.1452000

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EFFECT OF MATURITY ON PHYTOCHEMICAL CONSTITUENT, ANTIOXIDANT ACTIVITY, AND NUTRIENT COMPOSITION OF *MUNTINGIA CALABURA* FRUITS CULTIVATED IN INDONESIA

MATURİTENİN, ENDONEZYA'DA YETİŞTİRİLEN MUNTINGIA CALABURA MEYVELERİNİN FİTOKİMYASAL BİLEŞENLERİ, ANTİOKSİDAN AKTİVİTESİ VE BESİN KOMPOZİSYONU ÜZERİNDEKİ ETKİSİ

Hendrawan Hasanuddin MURNI[1], Nur KHAIRI[2], Alfat FADRI[2], Wahyuddin WAHYUDDIN[3], Andi Nur AISYAH[2], Amriani SAPRA[2], Maulita INDRISARI[4], Lukman MUSLIMIN[1]*

[1]Department of Pharmaceutical Chemistry, Almarisah Madani University, 90242, Makassar, Indonesia
[2]Department of Pharmaceutical Technology, Almarisah Madani University, 90242, Makassar, Indonesia
[3]Department of Biological Pharmacy, Almarisah Madani University, 90242, Makassar, Indonesia
[4]Department of Pharmacology, Almarisah Madani University, 90242, Makassar, Indonesia

ABSTRACT

Objective: *Cultivation location and maturity levels could affect Muntingia calabura's bioactive compounds and biological activities. The present investigation evaluated two different maturity stages (young and ripened) of Indonesian M. calabura on their phytochemical constituents (total phenolic [TP] and total flavonoid [TF]), antioxidant activity, and nutrition composition.*
Material and Method: *The TP and TF were measured using the Folin-Ciocalteau reagent and ammonium chloride ($AlCl_3$). Antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS). Nutrition composition: total soluble solids (TSS) were determined by the gravimetric method; soluble sugars used anthrone-sulfuric acid colorimetric assays; and vitamin C established 2,6-dichloroindophenol (DCIP) titration.*
Result and Discussion: *The ripened fruit presented the most potent antioxidant activity. DPPH and ABTS IC_{50} values were 28.38 ± 0.84 µg/ml and 29.92 ± 3.05 µg/ml, respectively. In contrast, the young fruit exhibited the highest TP (56.85 ± 1.08 mg/g GAE) and TF (8.45 ± 0.65 mg QE). Our findings additionally suggested that ripened fruit was a good source of nutrients, such as soluble sugar (SS; 12.34 ± 0.76%) and vitamin C (21.88 ± 2.73 mg/g).*
Keywords: *Antioxidant, bioactive compound, fruit, maturation, proximate*

ÖZ

Amaç: *Yetiştirme yeri ve olgunluk seviyeleri, Muntingia calabura'nın biyoaktif bileşenlerini ve biyolojik aktivitelerini etkileyebilir. Bu çalışmada, Endonezya'da yetiştirilen M. calabura'nın iki farklı olgunluk aşaması (genç ve olgun) fitokimyasal bileşenleri (toplam fenolik [TP] ve toplam flavonoid [TF]), antioksidan aktivitesi ve besin kompozisyonu açısından değerlendirildi.*
Gereç ve Yöntem: *TP ve TF, Folin-Ciocalteau reaktifi ve amonyum klorür ($AlCl_3$) kullanılarak*

* **Corresponding Author / Sorumlu Yazar:** Lukman Muslimin
e-mail / e-posta: lukman_m01@yahoo.co.id / lukman@univeral.ac.id, **Phone / Tel.:** +628971561010

Submitted / Gönderilme : 15.03.2024
Accepted / Kabul : 20.06.2024
Published / Yayınlanma : 10.09.2024

ölçüldü. Antioksidan aktivitesi, 2,2-difenil-1-pikrilhidrazil (DPPH) ve 2,2′-azino-bis(3-etilbenzotiyazolin-6-sülfonik) asit (ABTS) kullanılarak değerlendirildi. Besin kompozisyonu: toplam çözünür katılar (TSS) gravimetrik yöntemle; çözünür şekerler, antron-sülfürik asit kolorimetrik testleri ile; ve vitamin C, 2,6-dikloroindofenol (DCIP) titrasyonu ile belirlendi.

Sonuç ve Tartışma: *Olgunlaşmış meyve, en güçlü antioksidan aktiviteyi gösterdi. DPPH ve ABTS IC_{50} değerleri sırasıyla 28.38 ± 0.84 µg/ml ve 29.92 ± 3.05 µg/ml idi. Buna karşılık, genç meyve en yüksek TP (56.85 ± 1.08 mg/g GAE) ve TF (8.45 ± 0.65 mg QE) değerlerini gösterdi. Bulgularımız ayrıca, olgunlaşmış meyvenin çözünür şeker (SS; %12.34 ± 0.76) ve vitamin C (21.88 ± 2.73 mg/g) gibi besinlerin iyi bir kaynağı olduğunu öne sürdü.*

Anahtar Kelimeler: *Antioksidan, biyoaktif bileşen, meyve, vejetasyon süresi*

INTRODUCTION

Free radicals are one of the harmful products of the body's metabolic processes, especially respiration. The body naturally produces endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione-S-transferase (GST) to get rid of free radicals [1,2]. However, uncontrolled free radicals will cause several metabolic syndrome diseases such as inflammatory disease [3], diabetes [4], cardiovascular disease [5], cancer [6], neurodegenerative diseases [7], and Alzheimer's [8]. To neutralize these radicals, additional antioxidants are needed from external sources, such as consuming vegetables and fruits (e.g., cherry fruit) (*Muntingia calabura* L.).

As a member of the Muntingiaceae family, *M. calabura* can be found in countries with tropical and sub-tropical climates, such as Indonesia, Thailand, Malaysia, and the Philippines. In both urban and rural areas, the tree grows wild in gardens and yards [9]. Despite its widespread presence, the community has not used it effectively. The children prefer the *M. calabura* fruit because it is sweet and sour. Ripe fruits are red, whereas young ones are green.

According to Muslimin et al. (2020), *M. calabura* fruit has a very high antioxidant activity of 3.27 mg AAE/g (ascorbic acid equivalent per gram). It has high levels of mineral essentials such as potassium (K), calcium (Ca), and iron (Fe). Furthermore, the fruit is also rich in moisture content [10]. High antioxidant activity is associated with a high vitamin C content of 171.36 mg/100 g and a high carotene content of 1576.97 µg/100 g [11].

The level of fruit ripeness greatly influences the nutritional content of fruit. Ripe fruit contains high levels of phenolics and flavonoids [12]. Meanwhile, young fruit generally has higher levels of tannins and alkaloids, and the taste becomes astringent and bitter [13]. The ripe fruit also has a higher carbohydrate content, making it delicious. This study compared the levels of total phenolic (TP) and total flavonoids (TF) contained in the young and ripe fruits of *M. calabura*. Antioxidant activity was also determined using two test methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Antioxidants are closely related to vitamin C content and are determined using titration. Furthermore, total soluble solid (TSS) and soluble sugar (SS) levels were also analyzed.

MATERIAL AND METHOD

Plant Material

The fruits of *M. calabura* were harvested at two different development stages (i.e., young and ripened) from Sudiang, Makassar, and South Sulawesi in December 2022 (rainy season). Fruit samples from each maturation stage were sorted based on their morphology and color. The young fruit samples were green, while the ripened ones were red. The sample was cleaned under running water to remove dirt and then washed again with aquadest.

Phenolic and Flavonoid Extraction

Sample extraction was carried out in a dark room based on the method described by Mokhtar et al. [12]. A total of 200 g of fruit were extracted sequentially using methanol and ethyl acetate (0.05% v/v hydrochloric acid/solvent [10:90]), followed by simultaneous sonication for 30 minutes. Both extracts were combined, filtered, and evaporated.

Determination of Total Phenolic

A method from Pakki et al. (2020), the Folin-Ciocalteu method, was adopted to determine the total phenolic (TP) [14]. About 0.1 ml of each extract (10 mg/ml) was added to 1 ml of 50% Folin-Ciocalteu and 1 ml of 7.5% sodium carbonate. After the allowed 15 minutes in a dark room, the mixture was filtered, and water was added to bring the total volume to 10 ml. The optical density (OD) was recorded using a spectrophotometer (Agilent 8453, USA) at λ 730 nm. This experiment was performed in triplicate.

Determination of Total Flavonoids

Total flavonoid (TF) content was measured using aluminum chloride ($AlCl_3$) and was slightly modified from what is described by Mokhtar et al. [12]. An amount of 0.1 ml of each extract was transferred to a 5 ml volumetric flash, and 0.1 ml of $AlCl_3$ (10%) and 0.1 ml of $NaNO_2$ were added. After a 5 minutes incubation, 0.5 ml of NaOH (1 M) was added to it. The OD was recorded using a spectrophotometer (Agilent 8453, USA) at λ 510 nm. This experiment was performed in triplicate.

DPPH Test

The free radical-scavenging ability of the fruit of *M. calabura* was tested by bleaching the stable radical DPPH. The DPPH method was adopted from the method of Mokhtar et al. [12]. Briefly, 0.25 ml of the tested sample was added to 1 ml of DPPH (0.1 mmol) in 5 ml of volumetric flash and added to ethanol. The mixture was incubated for 30 minutes in the dark room. After incubation, the OD was measured at λ 515 nm using a spectrophotometer (Agilent 8453, USA). Vitamin C was used as the reference standard, and the tested sample was replaced with ethanol for the control. This experiment was performed in triplicate.

ABTS Test

The ABTS antioxidant activity was generated according to Wołosiak et al. [15]. An equal volume of substrate solution (ABTS, 7 mM) and oxidant (potassium persulfate, 2.45 mM) was made to react overnight in the dark. An amount of 1 ml of each sample was mixed with 3 ml of ABTS solution, stirred, and incubated for 10 minutes. The OD was measured at λ 734 nm using a spectrophotometer (Agilent 8453, USA). Vitamin C was used as the positive control. This experiment was performed in triplicate.

Total Soluble Solids

The TSS of the fruit of *M. calabura* was used in a membrane filter with a 0.4 µm pore size (Merck, Germany). The fruits were crushed using a blender (Philips), and the liquids were centrifuged at 14.500 rpm for 2 minutes. The filter was collected and passed through Millipore 0.4 µm. The number of dissolved substances was compared with the sample weight to calculate TSS.

Soluble Sugars

Soluble sugar in the fruit was measured using the anthrone colorimetric method, with slight modifications [16]. The fruits were crushed using a blender (Philips), then the liquids were centrifuged at 14.500 rpm for 2 minutes and dried. In a beaker, 0.25 ml of each sample was added to 50 µl of NaOH (2 mol/l) and boiled at 90°C for 5 minutes until room temperature reached. Briefly, 1 ml of transparent layer was mixed with 2.5 ml of anthrone and boiled at 90°C for 10 minutes. The corresponding OD values were measured at λ 620 nm using a spectrophotometer (Agilent 8453, USA). D-glucose was used to create a standard curve.

Vitamin C

The vitamin C in the fruit was analyzed using 2,6-dichlorophenolindophenol (DCPIP). An aliquot of 10 ml of each sample was titrated with 0.2% (w/v) DCPIP until the pink color appeared. Metaphosphoric (w/v) acid (5%) was used as a solvent to prevent degradation during the titration.

Statistic

The data were expressed as mean ± standard of deviation (± SD) and statistically evaluated by unpaired T-test at $p < 0.05$ level of significance. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 20 software.

RESULT AND DISCUSSION

People are advised to consume lots of vegetables and fruit to maintain their health because these foods are rich in general metabolite compounds, especially polyphenols, flavonoids, and terpenoids, which have pharmacological properties [17]. The secondary metabolites are generally efficacious as antioxidants, anticancer agents, antihyperglycemic agents, and cofactors. Therefore, it is essential to know the levels of polyphenols and flavonoids in fruits to predict their biological activity. One of the many factors influencing the compound content of a fruit is the level of ripeness [18]. This study compared the TP and TF content of young and ripe *M. calabura* fruits.

Determination of TP levels use gallic acid as a reference standard, while TF levels are compared with quercetin. The TP and TF levels are expressed as gallic acid equivalents (GAE) and quercetin equivalents (QE). A standard curve was obtained for the TP levels using the equation $y = 0.0053x + 0.1677$, yielding an R^2 value of 0.9987. In TF levels, a standard curve was obtained using the regression equation $y = 0.0193x + 0.132$, with an R^2 value of 0.9987 (Figure 1). The R^2 value shows the linearity of a curve; the closer the value to 1, the straighter the line equation is. R^2 is a figure of merit demonstrating the linearity of calibration curves in method validations [19].

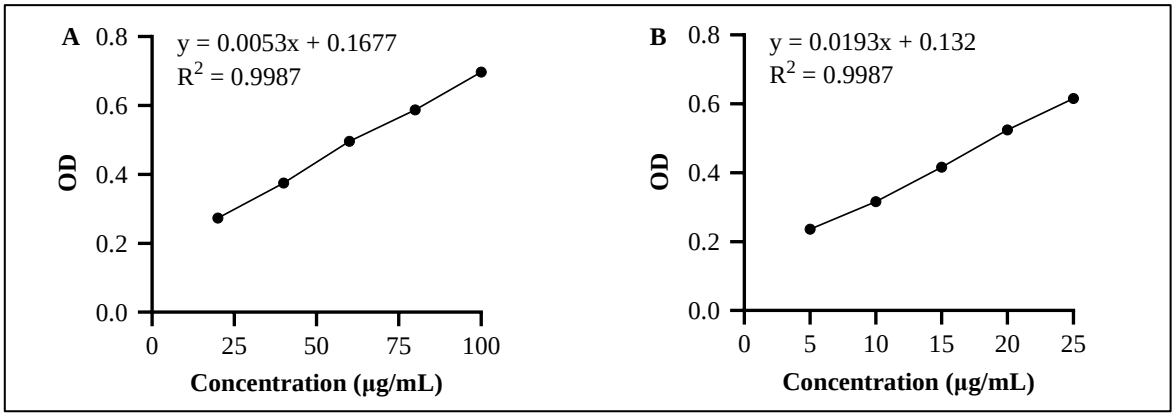

Figure 1. Phytochemical content on TP and TF in the fruit of *M. calabura*. Linear regression of phenolic (A) and flavonoid (B) determination

Our results showed that *M. calabura* fruits' TP and TF depend on their maturity stage. Figure 2 shows that the TP content of young fruits (56.85 ± 1.08 mg/g GAE) was higher than that of ripened fruits (42.29 ± 4.09 mg/g GAE). A similar result was also seen in TF levels; young fruits (6.99 ± 0.80 mg/g QE) had higher levels than ripened fruits (4.80 ± 0.52 mg/g QE). Pereira et al. (2018) reported that ripened *M. calabura* fruits collected from Campinas-SP, Brazil, contained TP of 6.88 ± 0.02 mg GAE/g [20]. Young fruits generally contain many chemical compounds. Fruit ripening necessitates the decomposition of these compounds. In addition, young fruits have high levels of certain active compounds, particularly the tannin group, which decrease as the fruits mature to protect them from predators [21].

Mokhtar et al. (2021) found that young pumpkin fruits have a 3.3-time higher polyphenol content and a 2.8-time higher flavonoid content than ripened ones [12]. The major volatile compounds, such as terpenes β-farnesene and dendrolasin, are concentrated in the ripened fruit of *M. calabura.* Meanwhile, also found in ripened fruits were gallic acid was 5325 µg/g dried weight (DW), and cyanidin-3-O-glucoside was 171 µg/g DW [20].

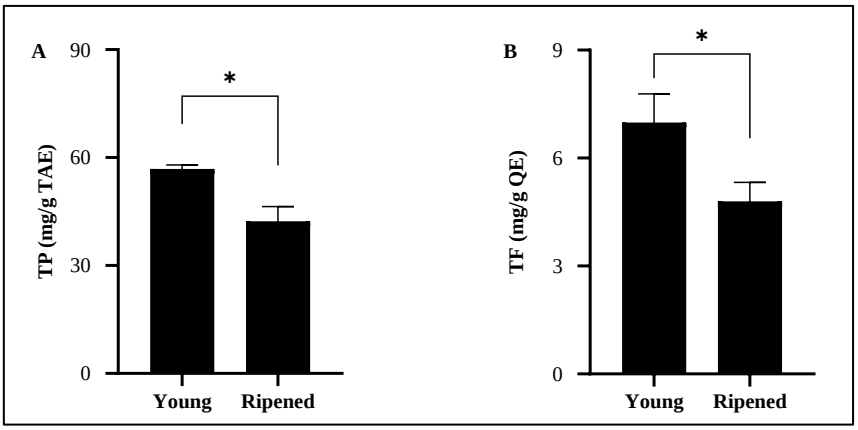

Figure 2. Total phenolic (A) and flavonoids (B) of the fruit of *M. calabura* at 2 stages of ripeness (n=3). *Characters represent significant differences between young and ripened group at $p < 0.05$ by unpaired T-test

One of the primary functions of fruits is to provide antioxidants. Many studies have proven that fruits are good sources of natural antioxidants for the body, which help prevent metabolic syndrome [22,23]. This study compared the antioxidant capacity of young and ripened *M. calabura* fruit using two different methods: DPPH and ABTS. Generally, both young and ripened fruits have antioxidant activity proportional to the concentration used. The higher concentration of the sample resulted in a higher percentage of inhibition. Table 1 shows that ripened fruits have a higher percentage of inhibition at the same concentration as compared to young fruits. At a concentration of 10 µg/ml, young fruits inhibited the activity of DPPH free radicals by 12.88 ± 1.53%, while ripened fruits reached 23.42 ± 0.70%. The ABTS antioxidant also showed the same activities (Table 2).

Table 1. DPPH antioxidant activity of different maturity stages of the fruit of *M. calabura* (n= 3)

Maturity stages	µg/ml	Inhibition (%)			Mean ± SD
		I	II	III	
Young	10	11.99	14.64	11.99	12.88 ± 1.53
	20	23.83	24.06	23.94	23.95 ± 0.11
	40	35.92	37.41	35.16	36.16 ± 1.14
	80	56.50	55.30	57.53	56.44 ± 1.11
Ripened	10	22.62	23.82	23.82	23.42 ± 0.70
	20	37.41	35.03	33.85	35.43 ± 1.81
	40	69.72	74.16	71.02	71.63 ± 2.29
	80	87.62	83.70	80.58	83.97 ± 3.53
Vitamin C	1	10.78	11.99	11.99	11.59 ± 0.70
	2	35.92	34.70	35.89	35.50 ± 0.70
	4	60.92	59.82	62.21	60.98 ± 1.20
	8	90.14	88.49	90.76	89.80 ± 1.17

The antioxidant capacity of an active compound is reflected in its IC_{50} value. IC_{50} is a concentration that can inhibit 50% of free radical activity. The smaller the IC_{50}, the better the effect. The results showed that in both the DPPH and ABTS tests, ripened fruits had the lowest IC_{50} values compared to young fruit. Ripened and young fruit had IC_{50} values against DPPH of 28.38 ± 0.84 µg/ml and 68.77 ± 1.24 µg/ml and ABTS of 29.92 ± 3.05 µg/ml and 67.46 ± 1.22 µg/ml, respectively (Figure

3). The antioxidant activity of fruit of *M. calabura* was also similar to those found in a previous study. According to Nur et al. (2022), ethanol extract of the fruit of *M. calabura is* capable of reducing hydroxyl radicals with an IC_{50} value was 32.06 µg/ml [24]. Hence, another part, like leaves, is capable of scavenger the DPPH free radical and nitric with IC_{50} value range 17.85 ± 5.40 till 19.77 ± 4.05 µg/ml and 19.90 ± 3.21 till 25.29 ± 5.33 µg/ml, respectively [25].

Table 2. ABTS antioxidant activity of different maturity stages of the fruit of *M. calabura* (n= 3)

Maturity stages	µg/ml	Inhibition (%)			Mean ± SD
		I	II	III	
Young	10	3.32	2.98	1.22	2.51 ± 1.13
	20	10.98	15.11	15.11	13.73 ± 2.38
	40	24.96	25.19	30.92	27.02 ± 3.38
	80	66.00	65.83	61.53	64.46 ± 2.53
Ripened	10	7.41	7.64	15.11	10.05 ± 4.38
	20	35.90	36.37	32.50	34.92 ± 2.11
	40	62.40	56.62	56.62	58.55 ± 3.34
	80	86.62	83.68	83.62	84.64 ± 1.71
Vitamin C	1	7.32	7.32	9.23	7.96 ± 1.10
	2	20.40	20.57	23.24	21.40 ± 1.60
	4	48.14	50.52	49.89	49.52 ± 1.24
	8	86.78	87.73	88.22	87.57 ± 0.73

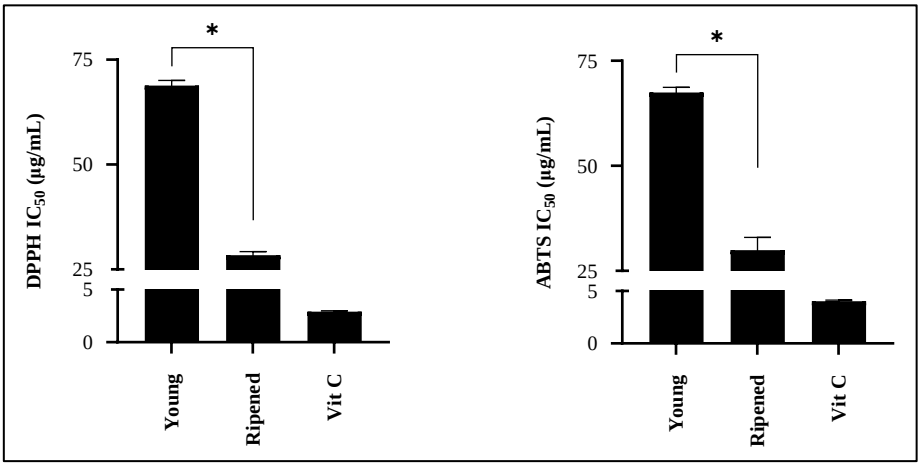

Figure 3. The IC_{50} value of the fruit of *M. calabura* in 2 different maturity stages (n= 3). *Characters represent significant differences between young and ripened group at $p < 0.05$ by unpaired T-test

A negative correlation was observed between the antioxidant capacity of the *M. calabura* fruits' polyphenols and flavonoids at different stages of fruit maturation concerning TP and TF. The polyphenolic concentration decreased during the ripening fruit process, whereas the antioxidant level increased. Phenolic and flavonoid levels were recorded to be significantly higher in the young fruits of *Rubus ellipticus*, *Myrica esculenta*, and *Pyracantha crenulate* [26]. Samaniego et al. (2020) have described that blackberry cultivars' polyphenol, flavonoid, and anthocyanin content decrease with increasing ripening [27]. Dong et al. (2019) demonstrated that the phenolic and antioxidant potency composite values decrease as the degree of fruit maturity increases in *Citrus limon* (L.) Burm. f [28]. As

fruits ripen, their phenols undergo oxidation and contribute to the production of anthocyanins, which accumulate over the course of fruit ripening. Consequently, the phenol concentration in ripened fruits decreases [26].

Thus, specific compounds decrease, and others increase during the fruit ripening process. At this time, TSS levels in the fruit and water content will increase. Based on our results (Table 3), the TTS content of young fruits (49.42 ± 4.92%) was lower than that of ripened fruits (60.75 ± 2.67%). Furthermore, carbohydrate levels in ripened fruit also increase. Increasing carbohydrate levels and simple sugars such as glucose, fructose, and sucrose will increase fruit sweetness. This study showed that SS levels in ripened fruits were much higher than in young fruits.

Table 3. The nutrient composition of the fruit of *M. calabura* in 2 different maturity stages (n= 3)

Parameter	Young	Ripened
Total soluble solid, TSS (%)	49.42 ± 4.92	60.75 ± 2.67*
Soluble sugar, SS (%)	7.13 ± 0.77	12.34 ± 0.76*
Vitamin C (mg/g)	13.38 ± 2.91	21.88 ± 2.73*

*Characters represent significant differences between young and ripened group at $p < 0.05$ by unpaired T-test

Vitamin C levels closely correlate with antioxidant capacity. The level of vitamin C in ripened fruits was much higher than that in young fruits at 21.88 ± 2.73 and 13.38 ± 2.91 mg/g, respectively (Table 3). The antioxidant activity of ripened fruits was higher than that of young fruits, as evidenced by the vitamin C levels. The results obtained are comparable with those reported by Gull et al. (2012), who showed an increasing trend in vitamin C content in guava fruit with maturation [29]. According to Soares et al. (2007), in a study of immature fruit, the amount of vitamin C was 76.8 mg/100 g of the sample, and it became 168.36 mg/100 g at the ripe stages, respectively [30]. Vitamin C, or ascorbic acid, a water-soluble vitamin, plays a role in controlling infections and healing wounds and is a powerful antioxidant that can neutralize harmful free radicals [31,32].

Young to adult people (> 19 years) need 90 mg daily for men and 75 mg for women. Pregnancy and lactation will increase the amount to 85 mg and 120 mg daily, respectively [33]. These studies could confirm our expectation of the benefit value of the fruit of *M. calabura.* Future and in-depth research, including a pharmacological bioassay and randomized clinical trials, is essential to claim the hidden potential medicinal values.

The active compounds, like polyphenols and flavonoids, and the biological activity of the fruit of *M. calabura* depend on the ripeness. In general, ripened fruit has a high vitamin C content and is correlated to antioxidant activity in higher young fruit. The SS content and the TSS also increase in ripened fruit. However, young fruit contains higher polyphenols and flavonoids than ripened fruit. In the future, comprehensive research must be carried out to determine the glycemic index and its potential as a sustainable nutrition and food source.

ACKNOWLEDGEMENTS

The authors wish to thank Almarisah Madani University, South Sulawesi, Indonesia, for providing a research laboratory to carry out this work.

AUTHOR CONTRIBUTIONS

Concept: N.K., A.S., M.I., L.M.; Design: N.K., A.S., M.I., L.M.; Control: N.K., A.S., M.I., L.M.; Sources: H.H., N.K., A.F., A.A.; Materials: H.H., N.K., A.F., W.W., A.N.A., M.I., L.M.; Data Collection and/or Processing: H.H., N.K., A.F., A.N.A.; Analysis and/or Interpretation: A.F., W.W., A.A., M.I., A.S., L.M.; Literature Review: L.L., N.K., M.I., W.W.; Manuscript Writing: H.H., N.K., A.F., W.W., A.N.A., A.S., M.I., L.M.; Critical Review: H.H., N.K., A.F., W.W., A.N.A., A.S., M.I., L.M.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 912-919, 2024
Doi: 10.33483/jfpau.1405409

ORIGINAL ARTICLE / ÖZGÜN MAKALE

STRUCTURAL INSIGHTS AND ANTICANCER POTENTIAL OF MELITTIN IN CD147 INTERACTION

MELİTTİN'İN CD147 İLE ETKİLEŞİMİNE YAPISAL BAKIŞ VE ANTİKANSER POTANSİYELİ

Barış DENK[1]*

[1]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Biochemistry, 03200, Afyonkarahisar, Türkiye

ABSTRACT

Objective: *This study investigates the interaction between melittin (PDB ID: 2MLT), a bioactive peptide from honeybee venom, and CD147 (PDB ID: 5XF0), a glycosylated transmembrane protein implicated in tumor progression.*
Material and Method: *Employing molecular docking and bioinformatics tools, our structural analysis reveals diverse binding features, including hydrogen bonds, salt bridges, and non-bonded contacts, between the CD147 complex and melittin.*
Result and Discussion: *Non-bonded interactions between 2MLT and specific amino acids (Gly181 and Arg201) of CD147 are highlighted, resembling aspects of the CypA/CD147 binding mechanism (Pro180-Gly181 and Arg201). The elevated anticancer potential of 2MLT was substantiated by utilizing the AntiCP 2.0 server and the ENNAACT server, employing machine learning and artificial neural network algorithms. Additionally, hydrophobicity analysis aligns with characteristics associated with anticancer peptides. Notably, thermodynamic stability variations with temperature underscore the robust binding affinity of 2MLT to the 5XF0 receptor. While our study comprehensively explores molecular interactions and predictive analyses, further in vitro and in vivo investigations are crucial to validate these findings for potential therapeutic applications.*
Keywords: *Anticancer peptides, CD147, cyclophilins, melittin, molecular docking*

ÖZ

Amaç: *Bu çalışma, arı zehrinden elde edilen biyoaktif bir peptit olan melittin (PDB ID: 2MLT) ile tümör ilerlemesinde rol oynayan bir glikozile transmembran protein olan CD147 (PDB ID: 5XF0) arasındaki etkileşimi incelemektedir.*
Gereç ve Yöntem: *Moleküler bağlanma ve biyoinformatik araçlar kullanılarak yürütülen yapısal analizimiz, CD147 kompleksi ile melittin arasında hidrojen bağları, tuz köprüleri ve bağlanmamış temaslar dahil olmak üzere çeşitli bağlanma özelliklerini ortaya çıkarmaktadır.*
Sonuç ve Tartışma: *2MLT ile CD147'nin belirli amino asitleri (Gly181 ve Arg201) arasındaki etkileşimlerin, CypA/CD147 bağlanma mekanizmasına (Pro180-Gly181 ve Arg201) benzer şekilde oluştuğu gözlemlenmiştir. 2MLT'nin yüksek antikanser potansiyeli, AntiCP 2.0 server ve ENNAACT server kullanılarak, makine öğrenimi ve yapay sinir ağı algoritmalarını içeren yöntemlerle desteklenmiştir. Ayrıca, hidrofobiklik analizi, antikanser peptitlerle ilişkilendirilen özelliklerle uyumludur. Özellikle, sıcaklıkla olan termodinamik stabilite değişiklikleri, 2MLT'nin 5XF0 reseptörüne güçlü bağlanma eğilimini vurgulamaktadır. Çalışmamız, moleküler etkileşimlerin*

* **Corresponding Author / Sorumlu Yazar:** Barış Denk
e-mail / e-posta: bdenk@aku.edu.tr, **Phone / Tel.:** +905353514002

Submitted / Gönderilme : 15.12.2023
Accepted / Kabul : 03.07.2024
Published / Yayınlanma : 10.09.2024

kapsamlı bir keşfini ve analizlerini sunarken, bu bulguların potansiyel terapötik uygulamalar için geçerliliğini doğrulamak için ileri in vitro ve in vivo çalışmalarının zorunlu olduğunu belirtmektedir.
Anahtar Kelimeler: *Antikanser peptidler, CD147, melittin, moleküler bağlanma, siklofilinler*

INTRODUCTION

Cancer poses a significant threat to the survival of living organisms, with high morbidity and mortality rates. Conventional cancer therapies demonstrate effectiveness primarily against malignant tumors. Yet, their efficacy diminishes in advanced stages due to metastasis, recurrence, heterogeneity, resistance to chemotherapy and radiation therapy, and immune evasion. Additionally, the presence of cancer stem cells has been identified as a contributing factor to treatment failures. In pursuing more successful cancer treatments, numerous studies have identified biomarkers and signaling pathways crucial in promoting malignant features like proliferation, anti-apoptosis, invasion, angiogenesis, therapeutic resistance, and stemness [1,2]. To overcome the limitations of conventional medications and their associated adverse effects, there is a concerted effort to explore alternative treatment strategies. Among these, the utilization of biotoxins, including those derived from animal venom, has gained prominence. These biotoxins, developed by living organisms as a defense mechanism against predators, exhibit toxicological and pharmacological effects [2].

Melittin is a biologically active peptide found in the venom of the honeybee (*Apis mellifera*), constituting approximately half of the venom's composition. This peptide exhibits an amphiphilic structure comprising 26 amino acids [2,3]. The therapeutic potential of melittin has been reported to encompass anti-inflammatory, anti-cancer, and anti-microbial effects [4]. Although the therapeutic potential of melittin is constrained by its hemolytic activity, molecular biology techniques offer avenues for N- and C-terminal adjustments, residue substitutions with both natural and unnatural amino acids, hybridization, cyclization, truncation, arginine enrichment, and the addition of C-terminal cysteine for optimizing the peptides' characteristics [5].

Melittin exhibits a specific binding affinity to phosphatidylcholine membranes [6] and is more attractive to negatively charged membranes within cancer microenvironments. Consequently, its affinity for the membranes of cancer cells, rich in anionic phospholipids, surpasses that of healthy cells [7]. Melittin demonstrates heightened activity in disrupting cancer cells, as evidenced by its targeted and redox-responsive conjugates exhibiting anticancer efficacy against MCF-7, C33A, and HeLa cancer cell lines in a study by Sahsuvar (2023) [8]. The extensive investigation of melittin for the treatment of various cancer types reveals recent advancements, including discussions on its synergistic combination with standard anticancer drugs and the recent progress in formulating a nano-version of melittin to enhance targeted delivery [9].

Further exploration of melittin's direct cytotoxic effects on cancer cells and its diverse immunomodulatory functions is highlighted. Due to its unique dual mechanism of action, involving cell cycle arrest, apoptosis, regulation of cancer cell pathways such as metastasis, angiogenesis, and inflammation through interactions with various signaling molecules, melittin is considered a broad-spectrum antitumor agent [9]. Its impact extends to significant molecular targets associated with growth inhibition and apoptotic induction, including Bax, Bcl-2, caspases, Akt, HIF-α, NF-kB, Wnt, STAT3, matrix metalloproteinases (MMPs), VEGF, and TNF-α [2]. A noteworthy study asserts that melittin-loaded niosomes exhibit more excellent anticancer effects compared to free melittin, underscoring the suitability of niosomes as vesicle carriers for melittin in comparison to its free form [10].

CD147, also known as basigin or EMMPRIN, is a glycosylated transmembrane protein abundant on tumor and stromal cell surfaces, functioning as an inducer of matrix metalloproteinases and a promoter of tumor progression. CD147 is implicated in various mechanisms related to tumor cell invasion, metastasis, and angiogenesis [11]. Recent studies highlight CD147's potential as a biomarker and therapeutic target for various diseases, including cancer, due to its involvement in oncogenic signaling pathways [1].

A study focused on the role of CD147 in the development and diagnosis of hepatocellular carcinoma elucidates its molecular structure and regulatory role in cancer progression [11]. Another

investigation into the expression and functional roles of CD147 in breast cancer cells reveals its involvement in crucial protein modulation associated with functions such as cell migration, invasion, drug resistance, and cancer progression [12].

Initially identified as a regulator of MMP, CD147 emerges as a promising target for cancer therapy due to its engagement in cell-matrix and cell-cell interactions. Beyond MMP regulation, CD147 is overexpressed in cancer cells and plays a regulatory role in cell proliferation, drug resistance, and cell stromal adhesion properties. Additionally, it possesses diverse functions, interacting with various molecular partners to modulate multiple signaling pathways. CD147's role extends to angiogenesis by regulating the production of vascular endothelial growth factor (VEGF) in tumor and stromal cells. It also impacts cancer-associated fibroblasts, promoting tumorigenesis and development. For instance, CD147 expression on melanoma cells induces tumor cell invasion by stimulating fibroblast production of matrix metalloproteinases [11].

Furthermore, the interaction between Cyclophilin A (CypA) and CD147 is pivotal in signal transduction. This interaction, facilitated by binding Pro180 amino acid and subsequent interaction through Pro211, induces signaling. The Glu218 amino acid is crucial for signal response. The CypA/CD147 interaction generates signals outside the cell through proline isomerization, leading to signal transmission inside the cell [1]. Significantly, the CypA/CD147 interaction induces the expression of MMP-2 and MMP-9, essential for the invasion and metastasis of cancer cells [13].

The objective of this study is to elucidate the interaction between melittin and CD147 through the utilization of molecular docking and bioinformatics tools.

MATERIAL AND METHOD

The molecule with Protein Data Bank (PDB) ID 5XF0 represents the three-dimensional structure of the solute CD147's Ig1 domain (residues 99–205 at the C-terminal Ig domain of CD147). This structure was obtained using the nuclear magnetic resonance (NMR) method [14]. The molecule with PDB ID 2MLT corresponds to the three-dimensional structure of melittin, a significant toxin from *Apis mellifera*. This structure, comprising 26 amino acid residues, was determined using X-ray diffraction at a resolution of 2.00 Å [15].

These molecules have been saved in PDB format. It is noted that the structure of 2MLT is a homotetramer composed of chains A and B [15], with chain A selected as a ligand for the docking procedures.

The amino acid sequence of the selected chain (GIGAVLKVLTTGLPALISWIKRKRQQ) was obtained from the PDB. Subsequently, using default settings (Model 1, SVM threshold: 0.45) on the AntiCP 2.0 server [16], sequence-specific parameters (hydrophobicity, hydropathicity, amphipathicity, hydrophilicity, charge, isoelectric point, and molecular weight) were predicted. Hydrophobicity analysis was additionally validated using the ProtScale program (The SIB Swiss Institute of Bioinformatics, Swiss) employing the Kyte-Doolittle method (scoring window size: 3, the relative weight of the window edges compared to the window center: 100%).

The anti-cancer activity of the ligand was investigated using the ENNAACT web server, and the anti-cancer activity scores were predicted to vary within the range of 0 to 1 as normalized sigmoid scores [17].

The amino acid sequence of the ligand was submitted to the PEP-FOLD4 server to predict its secondary structure. The files corresponding to the best model were converted to PDB format and subsequently saved for later use. The sequences and chain IDs of the receptor, 5XF0, and the ligand were verified in PyMOL.

Subsequently, the A chain of 5XF0 was retrieved from the PDB database, and the single-chain form of the ligand was uploaded from the local computer memory to perform docking using the ClusPro 2.0 server [18–21] The docking process was conducted with default settings.

The PDB file of the receptor-ligand complex was uploaded to the Protein Binding Energy Prediction (PRODIGY) server to compare the strength of protein-protein interactions and their thermodynamic stabilities under specific conditions at temperatures of 25°C and 40°C. The free energy change (ΔG, kcal.mol^{-1}) and dissociation constant Kd (M) were predicted from this.

The PDB file of the receptor-ligand complex was analyzed using the Uniprot PDBsum tool. This tool facilitated the visualization of interactions between polypeptide chains, residues, and atoms, including hydrogen bonds, non-bonded contacts, and salt bridges. The interaction region designates the receptor protein chain with ID A and the ligands' protein chain with ID B.

RESULT AND DISCUSSION

The PDB file representing the receptor-ligand interaction was visualized in PyMOL using the molecular surface method. The visualizations included the structural examination of the 5XF0 and 2MLT molecules, revealing their chain structures and interactions (Figure 1).

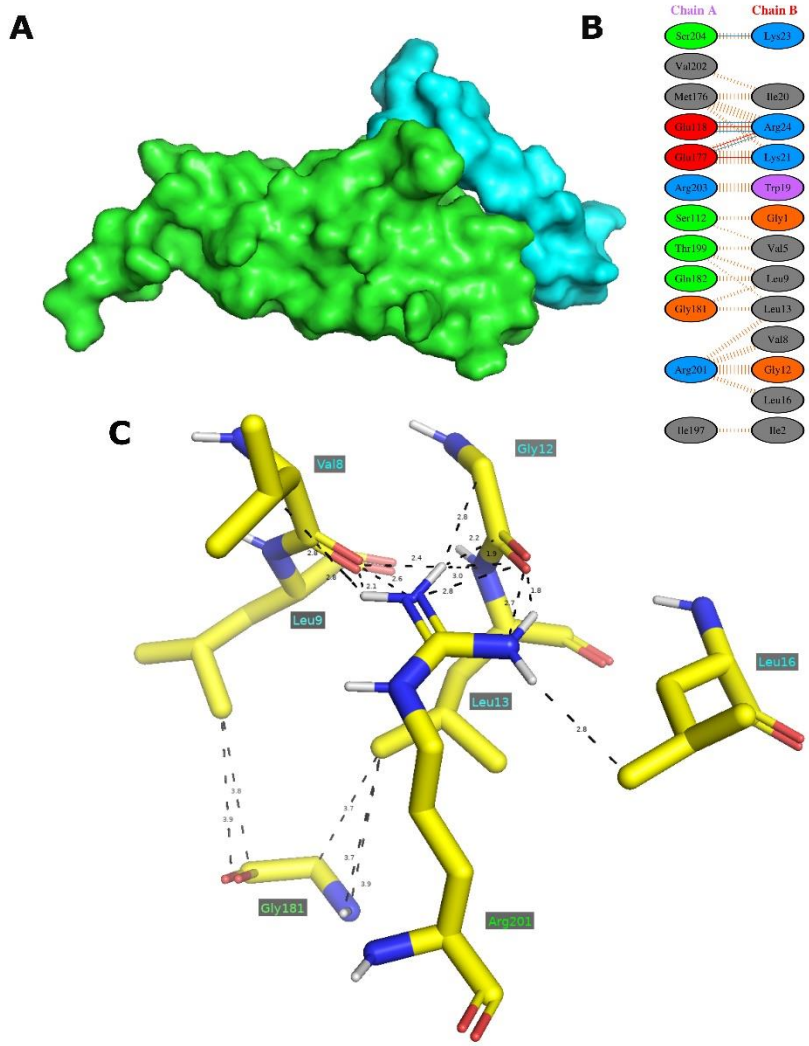

Figure 1. Illustration and molecular interactions of the CD147 Ig1 domain with melittin ligand: docking model and PDBsum analysis

The interaction between the Ig1 domain of CD147 protein and the melittin ligand was investigated. (A) The docking model illustrates the interaction between the IgI domain of the CD147 protein (depicted in green) and the 2MLT ligand (depicted in cyan). (B) The molecular interactions between CD147 (chain A) and the 2MLT ligand (chain B) residues were elucidated using PDBsum. The names of amino acid residues are inscribed within oval shapes of various colors. Key interactions between residues are represented by lines, with color codes indicating salt bridges (in red), hydrogen bonds (in blue), and non-bonded contacts (in orange). (C) The interaction between 5XF0 and 2MLT demonstrates non-bonded contacts involving Gly181 with Leu9 and Leu13, and Arg201 with Leu13, Val8, Gly12, and Leu16. A set of labels represented by green rectangles corresponds to the CD147 protein, while labels depicted in cyan rectangles indicate the amino acids of the 2MLT ligand. The black dashed lines represent non-bonded contacts, and the accompanying numerical values indicate the distances of these interactions in angstroms

The percentage of participating amino acids in the receptor-ligand interaction was delineated. Valine (15%), serine (15%), arginine (15%), glycine (15%), and lysine (10%) collectively constituted 70% of the interaction, with the remaining amino acids (isoleucine, threonine, methionine, lysine, glutamine, tryptophan) contributing to the rest.

The structural analysis revealed four hydrogen bonds between Ser204, Glu118, and Glu17 residues of 5XF0 and Lys23 and Arg24 residues of 2MLT, along with three salt bridges between Glu118 and Glu177 residues of 5XF0 and Arg24 and Lys21 residues of 2MLT. Additionally, 79 non-bonded contacts were identified between various amino acids.

The designed peptide and prediction results obtained from the AntiCP 2.0 server are presented in Table 1, providing the ligand's SVM score, terms describing its interaction with water, and chemical properties. The ligand's high hydrophobicity was observed based on the Kyte–Doolittle method obtained from ProtScale (Figure 2).

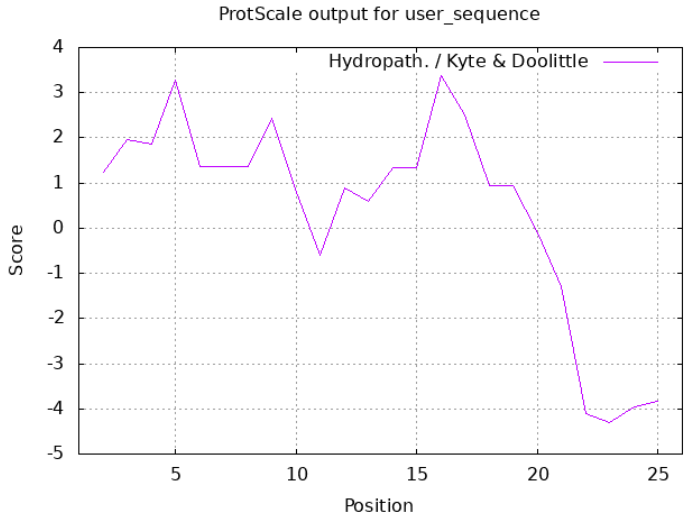

Figure 2. Hydrophobicity analysis of peptide sequence using ProtScale
The peptide sequence (GIGAVLKVLTTGLPALISWIKRKRQQ) underwent hydrophobicity analysis using the ProtScale tool. The horizontal axis represents the amino acid positions, while the vertical axis depicts the hydrophobicity levels. Values above zero correspond to the presence of hydrophobic amino acids within the peptide

The anticancer activity prediction results from the Employing Neural Networks for Anticancer Activity Classification for Therapeutic Peptides (ENNAACT) server are also presented in Table 1, including the ligand's amino acid sequence and a normalized sigmoid score (PROB score) within the range of 0-1.

Table 1. Integrated results of AntiCP 2.0 server for designed peptide and predictions, alongside anticancer activity predictions from the ENNAACT server

ID	Sequence	SVM	Hydro-phobicity	Hydro-pathicity	Amphi-pathicity	Hydro-philicity	Charge	pI	Mol wt	PROB
2MLT	GIGAVLKVLTTGLP ALISWIKRKRQQ	1.0	-0.08	0.27	0.71	-0.20	5.00	12.03	2847.91	0.997

The table presents details regarding the input query sequence. The PROB score from the ENNAACT server is a normalized sigmoid score ranging from 0 to 1. A score of 0 signifies a high probability of being a non-anticancer, whereas a score of 1 denotes a high probability of being an anticancer. Abbreviations: pI, isoelectric point; SVM, support vector machine score

The receptor-ligand complex resulting from docking, with the highest cluster members (model 0), was saved in PDB format. Scores and coefficients for this model are presented in Table 2, indicating that the VdW+Elec mode of the 2MLT model had the highest cluster members.

Table 2. Docking model rankings in various modes and corresponding free energy changes and dissociation constant values at two different temperatures

Receptor-Ligand Complex	Balanced	Electrostatic-favored	Hydrophobic-favored	VdW+Elec	ΔG (kcal.mol^{-1})	Kd (M) at 25.0°C/40.0°C
5XF0-2MLT	112	126	179	297	-9.6	8.4e-08/1.8e-07

The table presents cluster rank values based on the docking model (Model 0) across different modes: Balanced, Electrostatic-favored, Hydrophobic-favored, and VdW+Elec. Moreover, in the VdW+Elec mode, the table includes measurements of free energy changes (ΔG, kcal.mol^{-1}) and dissociation constant Kd (M) values, recorded at two distinct temperatures (25.0°C and 40.0°C)

PRODIGY server outputs for receptor-ligand docking, also presented in Table 2, provided ΔG and Kd values to compare the strength and thermodynamic stability of protein-protein interactions under different temperature conditions. Notably, the 5XF0-2MLT complex exhibited strong ΔG and binding affinity (-9.6 kcal.mol^{-1}; 8.4e-08 M). Additionally, as temperature increased, a consistent decrease in binding affinity between the receptor and ligand was observed, aligning with the patterns of ΔG and binding affinity.

The investigation of the 5XF0 complex and its interaction with the 2MLT molecule, as determined by the PDBsum server, reveals several noteworthy binding characteristics. A thorough analysis of the 5XF0-2MLT interactions unveiled a diverse array of interaction types, including hydrogen bonds (4 occurrences, ranging from a minimum of 2.73 Å to a maximum of 2.85 Å), salt bridges (3 occurrences; ranging from a minimum of 2.59 Å to a maximum of 2.76 Å), and non-bonded contacts (a total of 79 contacts among 22 amino acids; ranging from a minimum of 2.56 Å to a maximum of 3.89 Å). These interactions imply that the specific arrangement of distinct amino acid residues can bolster the stability of the complex and influence binding affinities. Establishing weak interactions originating from hydrogen bonds can guide recognition and binding processes. The potential for hydrogen bonds among the amino acids of the receptor and ligand relies on specific groups in their side chains capable of forming hydrogen bonds, enabling these amino acids to engage in various hydrogen bond formations with each other and other molecules. Salt bridges represent ionic interactions between the receptor and ligand, contributing to increased stability and specific molecular recognition. This aspect is critical in determining the nature of interactions between the receptor and ligand. The distinctive binding characteristics among amino acids may hold mechanistic significance for subsequent investigations.

Increased expression of CypA and CD147 in the signaling cascade of tumor cells triggers cancer pathogenesis [1,22]. CypA facilitates signal transduction by binding to Pro180 and Pro211 of CD147. The crucial role of Glu218 in this mechanism has also been reported [1]. According to a recent study, the CypA/CD147 binding process is regulated by Pro180-Gly181, and Arg201 is identified as an essential residue for binding [23]. Similarly, in our study, the interaction between 5XF0 and 2MLT reveals non-bonded contacts between Gly181 and Leu9, Leu13 and Arg201 with Leu13, Val8, Gly12, and Leu16, indicating specific interactions among amino acids without chemical bonding. The binding of CypA to CD147 induces conformational changes acting as a molecular chaperone, while the non-bonded interactions of 2MLT are a subject for further investigations in subsequent studies.

The SVM score of the ligand is at its maximum level (1.0), indicating a potentially high anticancer capacity for this peptide. Trained explicitly on a dataset of 861 anticancer peptides and 861 non-anticancer peptides using machine learning, the AntiCP 2.0 server [16] performed anticancer scoring for 2MLT. The potential anticancer peptide feature of 2MLT may be explained by its interaction with CD147, yet further *in vitro* and *in vivo* investigations are necessary.

According to the Kyte-Doolittle method, the hydrophobicity values for 2MLT are notably high. This observation suggests the presence of hydrophobic features that tend to bind to the target cell membrane, a characteristic often associated with anticancer peptides. However, it can be argued that the effects of the hydrophobicity feature of 2MLT on its interaction with CD147 cannot be predicted based on the results of this study. Nevertheless, evaluating interaction forms of amino acid residues through hydrogen bonds and salt bridges may provide insights for future studies.

Artificial neural networks that classify anticancer peptides take specific peptide features (e.g., amino acid sequence, hydrophobicity, electric charge) as input. They are trained to classify the anticancer activity based on these features. Due to their ability to learn patterns in large and complex datasets, artificial neural networks can assist in understanding and classifying the properties and activities of anticancer peptides [17]. In our study, the ENNAACT server, trained with neural network algorithms, indicates the high potential anticancer activity of 2MLT due to its high PROB score (0.997), which the AntiCP 2.0 server has corroborated. However, further *in vitro* and *in vivo* investigations are required.

Since 2MLT exhibits the highest cluster members in the VdW+Elec mode, it can be inferred that this mode most strongly supports the interaction of 2MLT. According to the PRODIGY server's results for receptor-ligand docking, lower (negative) ΔG values indicate stronger protein-protein interactions. Lower Kd values signify strong binding (high-affinity interaction). Lower ΔG and Kd values indicate a more robust and more stable interaction. This suggests that 2MLT has a preferred binding affinity to the 5XF0 receptor. The binding energy score of the complex varied with temperature. With the elevation in temperature, a discernible reduction in the binding affinity between the receptor and the ligand was observed. Nevertheless, it can be asserted that the 5XF0-2MLT complex exhibits a diminished sensitivity to the temperature increment. This observation implies a decline in thermodynamic stability concomitant with the temperature rise.

In conclusion, this study delved into the structural aspects and molecular interactions between the 5XF0 complex and the 2MLT molecule, revealing intricate binding features such as hydrogen bonds, salt bridges, and non-bonded contacts. Exploring the interaction between 5XF0 and 2MLT elucidated specific amino acid assemblies influencing complex stability and binding affinities. The study also shed light on the role of the non-bonded interactions of 2MLT, which remains a subject for future investigations. The anticancer peptide (2MLT) demonstrated a high SVM score, suggesting a potential anticancer capacity supported by its interaction with CD147. The study leveraged machine learning and neural networks to classify anticancer peptides, providing insights into 2MLT's possible activity. Analyzing hydrophobicity values and thermodynamic stability variations with temperature also offered valuable perspectives. While the study comprehensively explores molecular interactions and predictive analyses, further *in vitro* and *in vivo* investigations are imperative to validate the findings and translate them into potential therapeutic applications.

AUTHOR CONTRIBUTIONS

Concept: B.D.; Design: B.D.; Control: B.D.; Sources: B.D.; Materials: B.D.; Data Collection and/or Processing: B.D.; Analysis and/or Interpretation: B.D.; Literature Review: B.D.; Manuscript Writing: B.D.; Critical Review: B.D.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 920-928, 2024
Doi: 10.33483/jfpau.1458564

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PHARMACY STUDENTS' KNOWLEDGE, ATTITUDES AND PRACTICES ABOUT PROBIOTICS

ECZACILIK ÖĞRENCİLERİNİN PROBİYOTİKLER HAKKINDA BİLGİ, TUTUM VE UYGULAMALARI

Aslınur ALBAYRAK[1]*, Şimal MÜLAZIM[2]

[1]Suleyman Demirel University, Faculty of Pharmacy, Department of Clinical Pharmacy, 32200, Isparta, Türkiye
[2]Suleyman Demirel University, Faculty of Pharmacy, 32200, Isparta, Türkiye

ABSTRACT

Objective: *Probiotics are an important and fast-growing functional food group. Pharmacy students, as pharmacists of the future, need to have sufficient knowledge on this subject. Therefore, this study aims to evaluate pharmacy students' knowledge, attitudes, and practices about probioti cs.*
Material and Method: *A cross-sectional survey study was conducted among students of Suleyman Demirel University Faculty of Pharmacy in Türkiye between 9 October and 30 October 2023. The paper-based questionnaires consisted of 23 questions and were administered for approximately 10-15 minutes.*
Result and Discussion: *The questionnaires were answered by 347 (74.7%) students. The mean±SD of the knowledge score was 5.14±1.53. The majority of respondents (79%) correctly identified probiotics. Approximately half of the participants knew the type of bacteria used in probiotic production The mean±SD of the attitude score was 11.13±1.44. Most respondents (91.9%) believed that probiotic consumption was beneficial for health. The mean±SD of the practice score was 3.72±2.77. 45.2% of respondents stated that they had used probiotic supplements before and 31.1% stated that they had sought additional information about probiotics from various sources. 40.6% of the respondents stated that they recommend probiotics to their family/close relatives. This study showed that although the attitudes of pharmacy students were acceptable, they had some knowledge deficiencies and their practices were poor. To increase the knowledge and practices of pharmacy students about probiotics, this subject should be given more space in undergraduate education and relevant scientific events should be organized.*
Keywords: *Attitude, knowledge, pharmacy students, practices, probiotics*

ÖZ

Amaç: *Probiyotikler önemli ve hızla büyüyen fonksiyonel bir gıda grubudur. Geleceğin eczacıları olarak eczacılık öğrencilerinin bu konuda yeterli bilgiye sahip olmaları gerekmektedir. Bu nedenle bu çalışmada eczacılık öğrencilerinin probiyotiklere ilişkin bilgi, tutum ve uygulamalarının değerlendirilmesi amaçlanmaktadır.*
Gereç ve Yöntem: *Kesitsel bir anket çalışması Türkiye'de Süleyman Demirel Üniversitesi Eczacılık Fakültesi öğrencileri arasında 9 Ekim - 30 Ekim 2023 tarihleri arasında gerçekleştirildi. Kağıt bazlı anketler 23 sorudan oluşmaktaydı ve yaklaşık 10-15 dakika süreyle uygulandı.*
Sonuç ve Tartışma: *Anketler 347 (%74.7) öğrenci tarafından yanıtlanmıştır. Bilgi puanının ortalama±SS'si 5.14±1.53 idi. Ankete katılanların çoğunluğu (%79) probiyotikleri doğru bir şekilde*

* **Corresponding Author / Sorumlu Yazar:** Aslınur Albayrak
e-mail / e-posta: aslinuralbayrak@sdu.edu.tr, **Phone / Tel.:** +902462110346

Submitted / Gönderilme : 25.03.2024
Accepted / Kabul : 03.07.2024
Published / Yayınlanma : 10.09.2024

tanımlamıştı. Katılımcıların yaklaşık yarısı probiyotik üretiminde kullanılan bakteri türünü biliyordu. Tutum puanının ortalama±SS'si 11.13±1.44 idi. Katılımcıların çoğu (%91,9) probiyotik tüketiminin sağlığa faydalı olduğuna inanıyordu. Uygulama puanının ortalama±SS'si 3.72±2.77 idi. Ankete katılanların %45.2'si daha önce probiyotik takviyesi kullandığını ve %31.1'i probiyotikler hakkında çeşitli kaynaklardan ek bilgi aradığını belirtti. Katılımcıların %40.6'sı ailesine/yakın akrabalarına probiyotik önerdiğini belirtti. Bu çalışma eczacılık öğrencilerinin tutumlarının kabul edilebilir olmasına rağmen bilgi eksikliklerinin olduğunu ve uygulamalarının zayıf olduğunu gösterdi. Eczacılık öğrencilerinin probiyotikler konusundaki bilgi ve uygulamalarını arttırmak için lisans eğitiminde bu konuya daha fazla yer verilmeli ve konuyla ilgili bilimsel etkinlikler düzenlenmelidir.

Anahtar Kelimeler: *Bilgi, eczacılık öğrencileri, probiyotik, tutum, uygulama*

INTRODUCTION

The desire for a healthy diet has led to an increase in people's interest in nutritional supplements and functional foods [1,2]. The term functional food refers to foods that contain these ingredients naturally or are enriched with healthy ingredients, and are foods that can be effective in protecting against diseases and improving the quality of life. Probiotics are an important and fast-growing functional food group [2,3]. Probiotic is defined as ‘‘Probiotic is live microorganisms which when administered in adequate amounts confer a health benefit on the host’’according to The Food and Agriculture Organization/World Health Organization [4]. Studies have proven that lactic acid bacteria, bifidobacteria, *Saccharomyces cerevisiae, Saccharomyces boulardii*, *Streptococcus lactis*, *Escherichia coli*, etc. can be used as probiotics [5].

Probiotics are beneficial in many diseases such as prophylaxis of diarrhea, treatment of inflammatory bowel diseases, gastric ulcer treatment, urogenital infections, obesity treatment, liver diseases, hypercholesterolemia, and diabetes prevention [5,6]. The effects of different types of probiotics also vary [7].

Since probiotics are generally sold through pharmacies and pharmacists are health consultants that are easily accessible to the public [8,9], pharmacists need to keep their knowledge on this subject up to date and provide accurate information to patients [10,11]. Therefore, pharmacy students, as pharmacists of the future, need to have sufficient knowledge on this subject [12,13]. It is also important to identify students' deficiencies in probiotics and include them in the pharmacy curriculum. As far as is known, there is no study evaluating the use of probiotics only in pharmacy faculty students. This study aims to evaluate pharmacy students' knowledge, attitudes, and practices about probiotics.

MATERIAL AND METHOD

Study Design

This cross-sectional survey was conducted among the students of Suleyman Demirel University Faculty of Pharmacy in Türkiye between 9 October and 30 October 2023. Ethical approval for the research was received from Suleyman Demirel University Clinical Research Ethics Committee (No:156 / Date:21.07.2023). Undergraduate students over the age of 18 studying at Suleyman Demirel University Faculty of Pharmacy were included in the research, but postgraduate students were not included.

Sample Size

According to the Raosoft sample size calculator, the sample size was calculated as minimum 211 students with a 5% margin of error, 95% confidence interval, and 50% response rate [14].

Data Collection

The survey was prepared in Turkish by revising two previously validated current studies and taking the opinions of two pharmacists and a pharmacologist academician [15,16]. The questions were mostly created from the study conducted by Ong et al. [16], and questions were selected to measure students' general probiotic knowledge, attitudes and practices. A pilot study was conducted with 30 students to examine the questions in terms of content and understandability. The questions were found

to be clear and understandable. Cronbach's alpha score was calculated for internal consistency and was found to be 0.76.

Before the questionnaires were distributed, students were informed about the study and informed consent was obtained. The paper-based questionnaire consisted of 23 questions and was administered for approximately 10-15 minutes. The first 3 questions were about demographic information.

8 questions were about knowledge, 3 questions were about attitude and 9 questions were about practice.

Statistical Analysis

Data were analyzed with IBM SPSS Statistics for Versions 20.0. Quantitative variables were defined as mean-standard deviation (SD), median-interquartile range (IQR) and quantitative variables as percentages, respectively. The normality of the data was determined by the Kolmogorov Smirnov test. Variables were compared using Mann-Whitney U test for comparison of two groups with non-parametric data." The Chi-square test was used to compare categorical variables. p value <0.05 was considered statistically significant.

In the knowledge section, correct answers were scored as 1 and incorrect answers as 0. The attitudes section was recorded on a 5-point Likert scale (5 = strongly agree, 4 = agree, 3 = neutral, 2 = disagree, 1 = strongly disagree). In the practices section, the positive answer was 1 and the negative answer was 0. Scores only for the "frequency of probiotic supplement consumption" question were as follows (4 = every day, 3 = every 2-6 days, 2 = every 1-3 weeks, 1 = every 3-4 weeks, 0 = did not consume). The total score was a maximum of 8 for knowledge, 15 for attitudes and 12 for practice. ≥80% of the total score was attributed to a good level of knowledge, attitude, and practice.

RESULT AND DISCUSSION

The questionnaires were answered by 347 (74.7%) students. The majority of respondents (68.6%) were female students and the median age was 21 (IQR,19-22) years. Participation rates of students in the study according to academic years: 88 (25.4%) students in the 1st year, 80 (23.1%) students in the 2nd year, 68 (19.6%) students in the 3rd year, 58 (16.7%) students in the 4th year, 53 (15.3%) students in the 5th year.

Knowledge

The mean±SD of the knowledge score was 5.14±1.53. The majority of respondents (79%) correctly identified probiotics. Approximately half of the participants knew that the type of bacteria used in probiotic production was *Lactobacillus acidophilus*. Most respondents (77.5%) knew that probiotics can reinforce the defenses of the mucosal barrier in the digestive tract. 44.7% of the respondents disagreed and answered correctly that probiotics disrupt the balance of normal microorganisms in the digestive system. 38.9% of respondents agreed on the immune benefits of probiotics. 42.1% of the respondents believed that probiotics should be consumed regularly. Table 1 shows students' knowledge about probiotics.

Attitudes

The mean±SD of the attitude score was 11.13±1.44. Most respondents (91.9%) believed that probiotic consumption was beneficial for health. 37.7% of respondents agreed that probiotics may prevent the side effects of antibiotics. Table 2 shows students' attitudes about probiotics.

Practices

The mean±SD of the practice score was 3.72±2.77. 45.2% of the respondents stated that they had used probiotic supplements before. 31.1% of respondents declared that they sought additional information about probiotics from various sources. 40.6% of the respondents stated that they recommend probiotics to their family/close relatives. Most respondents (73.2%) stated that they had not used probiotic supplements in the last month. 73% of the students declared that they consumed yogurt as a probiotic product. 17.6% of the survey participants stated that they used probiotic products to prevent

digestive system complaints due to infections in the last month, 32.3% of the respondents to strengthen the immune system, and 9.8% of the respondents to prevent the side effects of antibiotics. Table 3 shows students' practices about probiotics.

Table 1. Students' knowledge about probiotics

Questions	Items	n (%)
Which of the following best defines probiotics?	Antibiotics prescribed to treat infections caused by bacteria.	36 (10.4)
	Live microorganisms that can be beneficial to humans when digested.	274 (79)
	A vitamin supplement that benefits human health.	37 (10.7)
Which of the following is a type of bacteria that can be used in probiotic production?	*Lactobacillus acidophilus*	173 (49.9)
	Campylobacter jejuni	25 (7.2)
	Listeria monocytogenes	36 (10.4)
	Staphylococcus aureus	48 (13.8)
	Proteus mirabilis	65 (18.7)
Which of the following foods can be a natural source of probiotics?	Yogurt	330 (95.1)
	Fish and poultry	10 (2.9)
	Fruits and vegetables	7 (2)
Probiotics can increase the defense of the mucous lining of the digestive system.	Yes	269 (77.5)
	No	13 (3.7)
	Don’t know	65 (18.7)
Probiotics are known to disrupt the balance of normal microorganisms found in the digestive system.	Yes	89 (25.6)
	No	155 (44.7)
	Don’t know	103 (29.7)
Inhibition of pro-inflammatory cytokines and promotion of anti-inflammatory cytokines are immune benefits of probiotics.	Yes	135 (38.9)
	No	19 (5.5)
	Don’t know	193 (55.6)
Probiotic consumption should be done regularly.	Yes	146 (42.1)
	No	124 (35.7)
	Don’t know	77 (22.2)
The benefits of probiotics vary depending on their species.	Yes	302 (87)
	No	10 (2.9)
	Don’t know	35 (10.1)

Table 2. Students' attitudes about probiotics

Questions	Items	n (%)
Consuming probiotics is beneficial for health.	Strongly agree	100 (28.8)
	Agree	219 (63.1)
	Uncertain	24 (6.9)
	Disagree	3 (0.9)
	Strongly disagree	1 (0.3)

Table 2 (*continue*). Students' attitudes about probiotics

Questions	Items	n (%)
Probiotics may be an alternative option in preventing digestive system complaints due to pathogenic infections.	Strongly agree	42 (12.1)
	Agree	180 (51.9)
	Uncertain	107 (30.8)
	Disagree	16 (4.6)
	Strongly disagree	2 (0.6)
Probiotics may prevent the side effects of antibiotics.	Strongly agree	31 (8.9)
	Agree	100 (28.8)
	Uncertain	142 (40.9)
	Disagree	70 (20.2)
	Strongly disagree	4 (1.2)

Table 3. Students' practices about probiotics

Questions	Items	n (%)
Have you used probiotic supplements before?	Yes	157 (45.2)
	No	190 (54.8)
Have you sought additional information about probiotics from various sources?	Yes	108 (31.1)
	No	239 (68.9)
Have you ever recommended probiotics to your family/close relatives?	Yes	141 (40.6)
	No	206 (59.4)
Which pharmaceutical form of probiotic supplement did you use last month?	Capsule	18 (5.2)
	Tablet	20 (5.8)
	Dust	24 (6.9)
	Liquid	31 (8.9)
	I didn't use	254 (73.2)
How often have you used probiotic supplements in the last month?	Daily	11 (3.2)
	Every 2-6 days	35 (10.1)
	Every 1-3 weeks	21 (6.1)
	Every 3-4 weeks	26 (7.49)
	I didn't use	254 (73.2)
What type of probiotic product have you consumed most frequently in the last month?	Yogurt	256 (73.8)
	Kefir	20 (5.8)
	Tarhana	6 (1.7)
	Pickle	37 (10.7)
	Boza	1 (0.3)
	I didn’t use	27 (7.8)
For the last month, I have been consuming probiotic products to prevent digestive system complaints due to pathogenic infections.	Yes	61 (17.6)
	No	286 (82.4)
For the last month, I have been consuming probiotic products to strengthen the immune system.	Yes	112 (32.3)
	No	235 (67.7)
For the last month, I have been consuming probiotic products to prevent the side effects of antibiotics.	Yes	34 (9.8)
	No	313 (90.2)

Table 4. Comparison of demographic characteristics with knowledge, attitudes and practices about probiotics

Variables	Knowledge Level			Attitudes Level			Practices Level		
	Poor (%) n	Good (%) n	p	Poor (%) n	Good (%) n	p	Poor (%) n	Good (%) n	p
Gender									
Male	91 (83.5)	18 (16.5)	0.276[a]	73 (67)	36 (33)	0.06[c]	106 (97.2)	3 (2.8)	0.762[d]
Female	185 (77.7)	53 (22.3)		134 (56,3)	104 (43,7)		228 (95.8)	10 (4.2)	
Age years (median± IQR)	20 (19-22)	22 (20-22)	<0.001[b]	20 (19-22)	21 (20-22)	0.007[b]	21 (19-22)	21 (21-22)	0.104[b]
Academic year									
First year	77 (87.5)	11 (12.5)	0.002[c]	61 (69.3)	27 (30.7)	0.012[c]	87 (98.9)	1 (1.1)	0.112[c]
Second year	70 (87.5)	10 (12.5)		52 (65)	28 (35)		79 (98.8)	1 (1.2)	
Third year	54 (79.4)	14 (20.6)		38 (55.9)	30 (44.1)		65 (95.6)	3 (4.4)	
Fourth year	37 (63.8)	21 (36.2)		24 (41.4)	34 (58.6)		53 (91.4)	5 (8.6)	
Fifth year	38 (71.7)	15 (28.3)		32 (60.4)	21 (39.6)		50 (94.3)	3 (5.7)	

IQR: Interquartile range [a] Continuity Correction test, [b] Mann Whitney U test, [c] Pearson Chi Square test, [d] Fisher's Exact test

Older age (median 20, vs. 22, p<0.001) and higher academic years (4th, and 5th grade, vs. 1st, 2nd, 3rd grade p =0.002) were found to be associated with better knowledge levels. Older age (median 20 vs. 22, p=0.007) was found to be associated with better attitude level. Additionally, respondents in the fourth year were associated with better attitude levels than other classes (p=0.012). The variables were not found to be significant in terms of practice level (Table 4).

This study showed that although the attitudes of pharmacy students were acceptable, they had some knowledge deficiencies and their practices were poor. Studies similar to this study showed that students' knowledge about probiotics was poor or limited [17-19]. In the study conducted by AbuKhader et al. [17], it was revealed that 70% of medical students and 59.4% of dentistry students correctly identified probiotics, and in the study conducted by Babina et al. [18], 77.4% of dentistry students identified probiotics correctly.

In the study conducted by Wilson et al. [20] among health professionals, 55.7% of dietitians, 76.3% of pediatricians, and 70% of general practitioners knew the definition of probiotics. In our study, probiotic definition was made correctly by pharmacy students at a higher rate (79%) compared to most studies. In the study conducted by Rahmah et al. [15] in health sciences students, 97.7% of the students knew that probiotics can strengthen the defenses of the mucous membrane of the digestive system, and 66.7% of the students knew that probiotics increase the secretion of anti-inflammatory cytokines and antibodies. Additionally, 66.7% of the students knew that probiotics should be consumed regularly. In our study, these questions were answered at a lower rate (77.5%, 38.9%, and 42.1% respectively). This shows that the students in this study have some lack of knowledge about the mechanism of action of probiotics.

In most studies, respondents had neutral or positive attitudes about probiotics, as in this study [15,16,18,21]. However, in this study they agreed at a lower rate (37.7%) with the idea that probiotics can prevent the side effects of antibiotics. Antibiotic-associated diarrhea may occur while being treated with antibiotics. This can affect patients at any time during or after treatment. Antibiotic-associated diarrhea occurs when antibiotics change the diversity and number of bacteria in the stomach and disrupt the ecosystem of the intestinal microbiota [5,22]. In a meta-analysis of randomized placebo-controlled

trials, the probiotic proved effective for antibiotic-associated diarrhea in adults [23]. Additionally, probiotics have been shown to significantly prevent the risk of developing *C. difficile*-associated diarrhea in patients taking antibiotics [24,25].

In this study, 45.2% of the students had used probiotic supplements before. This rate was lower than in studies conducted in other countries [15,16], it was found to be higher (20%) than in a study conducted on university students in Türkiye [26]. The low consumption of probiotic supplements by students in this study could be due to the fact that they were not known, were not needed, or were considered expensive. In this study, 40.6% of the students recommended probiotics to their family/relatives. In a study conducted on nutrition and dietetics students, this rate was 69% [27], while in a study conducted on doctor and dentist students [17], this rate was 63.5% and 50.8%, respectively. The rate in this study was lower than in other studies. This may be because students do not know the health benefits of probiotics. Additionally, since probiotics may have some risks [28], students may have refrained from recommending them. Most of the students consumed yogurt as the most probiotic product (79%) in the last month. This situation is not surprising because yogurt is a frequently consumed product in Türkiye [29,30]. Research conducted in 15 countries revealed that those who consume the most yogurt live in the Netherlands, France, Türkiye, Spain, and Germany [29].

In this study, parallel to other studies, no statistical relationship was found between gender and attitude level [16,21]. Older age was also associated with better knowledge and attitudes, contrary to other studies [15,21,31]. Similar to the study by Ong et al. [16], respondents in the fourth grade had better knowledge and attitude levels than other grades.

One of the limitations of this study was that it was single-center. This situation may prevent the generalizability of the results. There is a need to conduct multicenter national surveys. Additionally, some items were removed from the original scale. Therefore, it is necessary to verify the factor structures with exploratory and confirmatory factor analyses and to conduct appropriate validity and reliability analyses.

This study showed that although the attitudes of pharmacy students were acceptable, they had some knowledge deficiencies and their practices were poor. Pharmacy faculty students have a critical role in the healthcare system as future pharmacists. To increase the knowledge and practices of pharmacy students about probiotics, this subject should be given more space in undergraduate education and relevant scientific events should be organized.

ACKNOWLEDGEMENTS

Authors would like to thank all students who participated in this study.

AUTHOR CONTRIBUTIONS

Concept: A.A.; Design: A.A., Ş.M; Control: A.A., Ş.M.; Sources: A.A., Ş.M.; Materials: A.A., Ş.M.; Data Collection and/or Processing: Ş.M.; Analysis and/or Interpretation: A.A.; Literature Review: A.A., Ş.M.; Manuscript Writing: A.A., Ş.M.; Critical Review: A.A., Ş.M.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Ethical approval for the research was received from Suleyman Demirel University Clinical Research Ethics Committee (No:156 / Date:21.07.2023).

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 929-939, 2024
Doi: 10.33483/jfpau.1478733

ORIGINAL ARTICLE / ÖZGÜN MAKALE

NETWORK TOXICOLOGY FOR THE CARDIOVASCULAR TOXICITY ANALYSIS OF TYROSINE KINASE INHIBITORS

TİROZİN KİNAZ İNHİBİTÖRLERİNİN KARDİYOVASKÜLER TOKSİSİTE ANALİZİ İÇİN AĞ TOKSİKOLOJİSİ

Fuat KARAKUŞ[1]*

[1]Van Yuzuncu Yil University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 65080, Van, Türkiye

ABSTRACT

Objective: *This study aims to explore potential molecular mechanisms and targets of cardiovascular toxicities caused by tyrosine kinase inhibitors. Therefore, toxicogenomic data mining was conducted focusing on sunitinib, sorafenib, pazopanib, axitinib, and their associations with cardiovascular diseases.*
Material and Method: *Common genes between tyrosine kinase inhibitors and cardiovascular diseases were uncovered via comparative toxicogenomic databases. Additionally, protein-protein and gene-gene interactions were identified using STRING and GeneMANIA, respectively. Subsequently, hub proteins associated with tyrosine kinase inhibitor-induced cardiovascular diseases were determined through Metascape. Transcription factors and microRNAs related to this toxicity were identified using ChEA3 and MIENTURNET, respectively. Finally, gene ontology enrichment analysis and the most associated molecular pathways were identified using the DAVID database and Metascape, respectively.*
Result and Discussion: *Toxicogenomic data mining revealed six genes common between tyrosine kinase inhibitors and cardiovascular diseases, with five of these genes (FLT1, FLT4, KDR, MAPK1, and MAPK3) identified as hub genes. Physical interaction was dominant among these hub genes (77.64%). Sunitinib, sorafenib, pazopanib, and axitinib generally downregulated the activities of these proteins. SOX17 and SOX18 were prominent among transcription factors, while hsa-miR-199a-3p was the most important microRNA associated with this toxicity. Moreover, the Ras signaling pathway was mostly associated with tyrosine kinase inhibitor-induced cardiovascular toxicities. These findings make a substantial contribution to understanding the processes underlying cardiovascular diseases induced by sunitinib, sorafenib, pazopanib, and axitinib. They also reveal novel potential therapeutic targets, including genes, proteins, transcription factors, microRNAs, and pathways.*
Keywords: *Cardiovascular disease, in silico data mining, tyrosine kinase inhibitors*

ÖZ

Amaç: *Bu çalışma, tirozin kinaz inhibitörlerinin neden olduğu kardiyovasküler toksisitelerin potansiyel moleküler mekanizmalarını ve hedeflerini araştırmayı amaçlamaktadır. Bu nedenle, sunitinib, sorafenib, pazopanib, axitinib ve bunların kardiyovasküler hastalıklarla ilişkilerine odaklanarak toksikogenomik veri madenciliği yapılmıştır.*
Gereç ve Yöntem: *Tirozin kinaz inhibitörleri ile kardiyovasküler hastalıklar arasındaki ortak*

* **Corresponding Author / Sorumlu Yazar:** Fuat Karakuş
e-mail / e-posta: fuatkarakus@yyu.edu.tr, **Phone / Tel.:** +904324445065

Submitted / Gönderilme : 05.05.2024
Accepted / Kabul : 03.07.2024
Published / Yayınlanma : 10.09.2024

genler, karşılaştırmalı toksikogenomik veritabanları aracılığıyla belirlenmiştir. Ayrıca, protein-protein etkileşimleri ve gen-gen etkileşimleri sırasıyla STRING ve GeneMANIA kullanılarak belirlenmiştir. Daha sonra, tirozin kinaz inhibitörü ile ilişkilendirilmiş kardiyovasküler hastalıklara ait merkezi proteinler Metascape kullanılarak belirlenmiştir. Bu toksisite ile ilişkili transkripsiyon faktörleri ve mikroRNA'lar sırasıyla ChEA3 ve MIENTURNET kullanılarak belirlenmiştir. Son olarak, gen ontolojisi zenginleştirme analizi ve en çok ilişkilendirilen moleküler yollar sırasıyla DAVID veritabanı ve Metascape kullanılarak belirlenmiştir.

Sonuç ve Tartışma: *Toksikogenomik veri madenciliği, tirozin kinaz inhibitörleri ile kardiyovasküler hastalıklar arasında altı ortak geni ortaya çıkardı; bunlardan beşi (FLT1, FLT4, KDR, MAPK1 ve MAPK3) merkezi genler olarak belirlendi. Bu merkezi genler arasında fiziksel etkileşim baskın olarak gözlemlendi (%77.64). Sunitinib, sorafenib, pazopanib ve axitinib genel olarak bu protein aktivitelerini azaltmaktadır. Transkripsiyon faktörleri arasında SOX17 ve SOX18 öne çıkmaktadır, hsa-miR-199a-3p ise bu toksisite ile en önemli mikroRNA'dır. Ayrıca, Ras sinyal yolunun tirozin kinaz inhibitörleri ile ilişkilendirilen kardiyovasküler toksisitelerle çoğunlukla ilişkilendirildiği görülmüştür. Bu bulgular, sunitinib, sorafenib, pazopanib ve axitinib tarafından indüklenen kardiyovasküler hastalıkların altında yatan süreçleri anlamada önemli bir katkı yapmaktadır. Ayrıca, genler, proteinler, transkripsiyon faktörleri, mikroRNA'lar ve yollar da dahil olmak üzere yeni potansiyel terapötik hedefleri ortaya koymaktadır.*

Anahtar Kelimeler: *İn siliko veri madenciliği, kardiyovasküler hastalıklar, tirozin kinaz inhibitörleri*

INTRODUCTION

Tyrosine kinase inhibitors (TKIs) are widely used in clinical practice to treat various cancers, ranging from blood malignancies to advanced solid tumors. Compared to conventional chemotherapy agents such as doxorubicin, TKIs are generally considered safer. However, despite their perceived safety, TKIs are often administered for prolonged periods without a prescribed upper limit on dosage. Consequently, some TKIs still pose significant risks of cardiac adverse events. For example, sunitinib (SUN) has been linked to congestive heart failure in approximately 4.1% of cases in a meta-analysis involving 6935 patients, while sorafenib (SRF) has been shown to cause myocardial ischemia in around 2.7–3% of participants in clinical trials. Additionally, both SUN and SRF are associated with hypertension in up to 47% of patients, likely due to their inhibitory effects on vascular endothelial growth factor (VEGF) signaling [1-5]. A systematic review reported sufficient evidence of high- and all-grade hypertension for pazopanib (PAZ), axitinib (AXI), and SRF. It also indicated probable evidence of all-grade congestive heart failure or left ventricular ejection fraction decline for PAZ and SUN [6]. Another study reported that hypertension was the most commonly reported cardiovascular event and was most frequently associated with SRF and PAZ in 1624 pediatric adverse events linked to TKIs in pediatrics [7]. A case study documented two cases of acute heart failure following PAZ treatment [8]. Another case study reported a patient with metastatic renal cell carcinoma who suddenly developed life-threatening hyperkalemia following the initiation of AXI treatment [9].

TKI-induced cardiotoxicity encompasses two distinct categories: "on-target" and "off-target" effects. Within these categories, cardiac cells exhibit both adaptive and maladaptive reactions to the pharmacological impact of TKIs. Physiological reactions inherent to the heart, such as hypertrophic responses, activation of fetal gene programs, initiation of unfolded protein responses, and stimulation of antioxidant defenses, are frequently triggered by external chemical stressors and may serve to modulate cardiotoxicity adaptively over time. The manifestation of TKI-induced cardiotoxicity is influenced by both the pharmacological inhibition of intended targets or unintended off-targets and the stress responses elicited within cardiac cells [1,10].

To improve the quality of life and clinical treatment of patients with TKI-induced cardiotoxicity, it is necessary to understand the molecular mechanisms of TKI-induced cardiotoxicity. The present study utilized network toxicology strategies to investigate the mechanism of TKI-induced cardiovascular toxicity. Network toxicology is a scientific discipline that employs computer modeling and bioinformatics methods to study the toxic impacts of chemical substances on living organisms. By constructing interaction networks between chemical substances and molecules within organisms,

network toxicology predicts and assesses chemical substance toxicity, aiding in understanding toxicity mechanisms, screening for potentially harmful substances, and more [11]. In addition to network construction, data mining techniques are integral in extracting and analyzing vast datasets to identify patterns and relationships in biological systems relevant to toxicity. Furthermore, *in silico* analysis plays a crucial role by using computational methods to simulate and predict the effects of chemical substances on biological pathways, providing insights into toxic mechanisms and aiding in the identification of biomarkers and therapeutic targets.

The purpose of this research is to gain insight into the toxic mechanisms of TKI-induced cardiotoxicity, elucidate the toxicological profile of four TKIs (SUN, SRF, PAZ, and AXI), and predict their molecular mechanisms. Thus, it provides genomic biomarkers for further *in vitro* and *in vivo* studies.

MATERIAL AND METHOD

Identification of Overlapping Genes Between TKIs and Cardiovascular Diseases

The identification of genes associated with the TKIs (SUN, SRF, PAZ, and AXI) and their connection to cardiovascular diseases (CVDs) was facilitated through the use of the Comparative Toxicogenomics Database (CTD; https://ctdbase.org) and its tools. CTD collects and integrates diverse data on chemical exposures and their biological impacts across different species. This involves manually curating and interconnecting data on chemicals, genes, phenotypes, anatomies, diseases, taxa, and exposures found in published literature [12].

For the analysis of the genes related to the TKIs, each names of TKIs were entered one by one in the "Chemicals" section of the CTD, and all resulting genes were downloaded. The genes associated with CVDs were obtained from the “Direct Evidence” section of CTD, where “M” stands for “marker/mechanism” and “T” stands for “therapeutic.” To obtain common genes related to the four TKIs and CVDs, the Jvenn tool (https://jvenn.toulouse.inrae.fr/app/index.html) was used. It can process up to six lists of input and show the results using either the Edwards-Venn or classical layouts [13]. All findings presented in this study are based on data obtained in April 2024.

Drug-Gene Binary Interaction Analysis

To establish correlations between genes linked to CVDs and genes associated with the TKIs, a manual analysis was conducted using CTD (https://ctdbase.org). This involved scrutinizing the "gene interaction" card in the CTD chemical profile, specifically identifying interactions between the genes and TKIs in terms of protein activity, mRNA expression, and protein expression. The resulting table enumerates the interactions between TKIs and common genes, excluding interactions involving a combination of two or more chemicals and their collective impact on the genes [12].

Protein-Protein Interaction and Centrality Analysis

For protein-protein interactions (PPI) of the common genes between TKIs and CVDs, String v.12.0 (https://string-db.org/cgi) was used [14]. The STRING database systematically collects and integrates protein-protein interactions-both physical interactions and functional associations.

For the analysis, the protein set was entered into the “Multiple Proteins by Names/Identifiers” section, and *Homo sapiens* was selected as the target species. The minimum required interaction score was set to 0.4. The final PPI network was constructed using Cytoscape version 3.10.1 (http://www.cytoscape.org/). Cytoscape is an open-source software for interactive analysis, integration, and visualization of network data [15]. Furthermore, the Molecular Complex Detection (MCODE) algorithm was utilized through Metascape to pinpoint densely interconnected network components. Metascape, accessible via the web at https://metascape.org/gp/index.html#/main/step1, is a web-based platform engineered to deliver thorough annotation and analysis of gene lists. In its design, Metascape integrates functional enrichment, interactome analysis, gene annotation, and membership search functionalities, harnessing the resources of over 40 distinct knowledgebases within a unified portal. Additionally, it streamlines comparative analyses of datasets derived from various independent and orthogonal experiments [16].

Gene Network Analysis

The common genes between TKIs and CVDs, which were put into GeneMANIA (http://genemania.org), were used to study the network of gene-gene interactions. GeneMANIA identifies additional genes associated with a given set of input genes by leveraging an extensive array of functional association data. This dataset encompasses diverse sources such as protein-protein and genetic interactions, pathways, co-expression patterns, co-localization tendencies, and similarities in protein domains. Currently, it maps 166,691 genes from nine organisms [17]. In this study, *Homo sapiens* was chosen as the target organism for analysis, and an automatically selected weighting method was employed.

Analysis of Transcription Factors and MicroRNAs

The common genes between TKIs and CVDs were input into ChIP-X Enrichment Analysis Version 3 (ChEA3) (https://maayanlab.cloud/chea3) to identify the transcription factors (TFs) responsible for their regulation. ChEA3 serves as a web-based tool for analyzing transcription factor (TF) enrichment, organizing TFs linked to gene sets submitted by users. Its background database comprises diverse gene set libraries derived from various origins, encompassing TF-gene coexpression data sourced from RNA-seq investigations, TF-target associations established through ChIP-seq studies, and TF-gene cooccurrence patterns derived from gene lists contributed by the community [18].

Next, the common genes were also subjected to the MIcroRNA Enrichment TURned NETwork (MIENTURNET) tool (http://userver.bio.uniroma1.it/apps/mienturnet/), and *Homo sapiens* was selected as the target to determine potential miRNA networks from miRTarBase that were experimentally confirmed. MIENTURNET uses computationally predicted or experimentally validated miRNA-target interactions from several organisms, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Danio rerio* [19]. In the analysis, the threshold for the minimum number of miRNA-target interactions was set at 2, and the adjusted p-value (FDR) was 0.5.

Functional Enrichment Analysis of Common Genes

Gene Ontology (GO) term enrichment analysis was conducted on annotated genes related to TKIs and CVDs using DAVID (https://david.ncifcrf.gov/tools.jsp). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) offers a comprehensive suite of functional annotation resources, enabling researchers to elucidate the biological significance inherent in extensive gene lists [20]. In this analysis, the common gene list was input into DAVID, and *Homo sapiens* was selected as the target species. For gene ontology analysis, the top 5 biological processes, cellular components, and molecular functions were determined, with a p-value < 0.05 and false discovery rate (FDR) < 0.05 set as the cutoff criteria.

For molecular pathways analysis, Metascape was employed [16]. In this analysis, the common gene list was input into Metascape, and *Homo sapiens* was selected as the target species. The significance of the results was determined by p and q values. P-values were computed utilizing the cumulative hypergeometric distribution, and q-values were determined employing the Benjamini-Hochberg method to control the false discovery rate (FDR) via sequential modified Bonferroni correction for multiple hypothesis testing, thereby addressing the issue of multiple comparisons [21].

RESULT AND DISCUSSION

Common Genes Associated with TKIs and CVDs

Searching the CTD database showed that SUN, SRF, PAZ, and AXI target 6220, 196, 24, and 11 genes, respectively. Additionally, the number of CVD-associated genes was 3.79 million; 1674 of them were marked as “markers/mechanisms” and/or “therapeutics” in the "Direct Evidence" section. Six genes were common between the four TKIs and CVDs, alphabetically: Cytochrome P450 3A4 (CYP3A4), Mitogen-activated protein kinase 1 (MAPK1, ERK2), Mitogen-activated protein kinase (MAPK3), Vascular endothelial growth factor receptor 1 (FLT1, VEGFR1), Vascular endothelial

growth factor receptor 2 (KDR, VEGFR2), and Vascular endothelial growth factor receptor 3 (FLT4, VEGFR3) (Figure 1B).

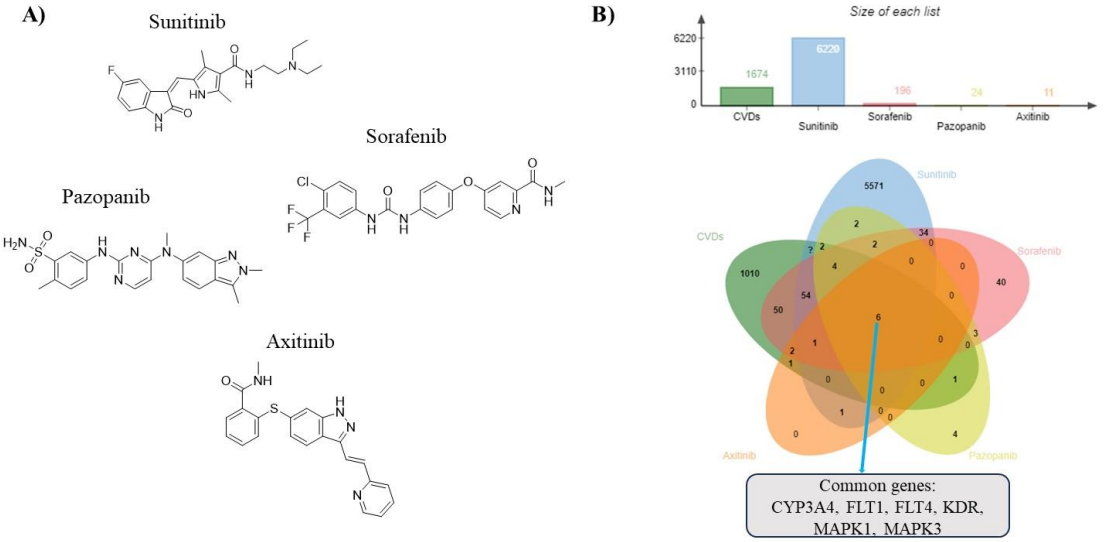

Figure 1. A) Tyrosine kinase inhibitors (TKIs) often associated with cardiovascular toxicity, **B)** Common genes between TKIs and CVDs

TKIs-Gene Binary Interaction Analysis Results

Individual chemical-gene interactions were analyzed to uncover potential overlaps in protein activity, protein expression, and mRNA expression (Table 1). The findings suggest that the examined TKIs can downregulate the activity, mRNA expression, and protein expression of the five hub genes. Given that TKIs also target these hub genes in cancer cells and reduce their activity and/or expression, their ability to downregulate these genes in the cardiovascular system may indicate on-target toxicity.

Table 1. TKIs-gene binary analysis results

	FLT1 (VEGFR1)			**FLT4** (VEGFR3)			**KDR** (VEGFR2)			**MAPK1** (ERK2)			**MAPK3**		
TKIs	P.A.	mR. E.	P. E.	P. A.	mR. E.	P. E.	P. A.	mR. E.	P. E.	P. A.	mR. E.	P. E	P. A.	mR. E.	P. E.
SUN	↓	↓		↓		↓	↓	↓		↓			↓		
SRF	↓			↓			↓			↓		↓	↓		
PAZ	↓						↓			↓			↓		
AXI	↓			↓			↓			↓			↓		

P. A. = Protein activitiy. mR. E. = mRNA expression. P. E. = Protein expression

PPI, Centrality Analysis, and Gene-Gene Network

The PPI network analysis depicted 6 nodes and 7 edges, as shown in Figure 2A (upper panel), with a significant PPI enrichment p-value of 0.00263. Additionally, centrality analysis was conducted to identify hub proteins, revealing five hub proteins associated with TKIs-induced CVDs (Figure 2A, lower panel).

To construct a connected network from shared genes, the GeneMANIA online plug-in was utilized. The findings indicated that a majority of genes linked to the examined TKIs were involved in physical interactions (77.64%), whereas other interaction types were less prominent (co-expression (8.01%); predicted interactions (5.37%); colocalization (3.63%); genetic interactions (2.87%); pathway (1.88%); shared protein domains (0.60%) (Figure 2B). These outcomes emphasize the prevalence of physical interactions among hub genes associated with CVDs, highlighting the pivotal role of direct molecular associations in the pathogenesis induced by TKIs.

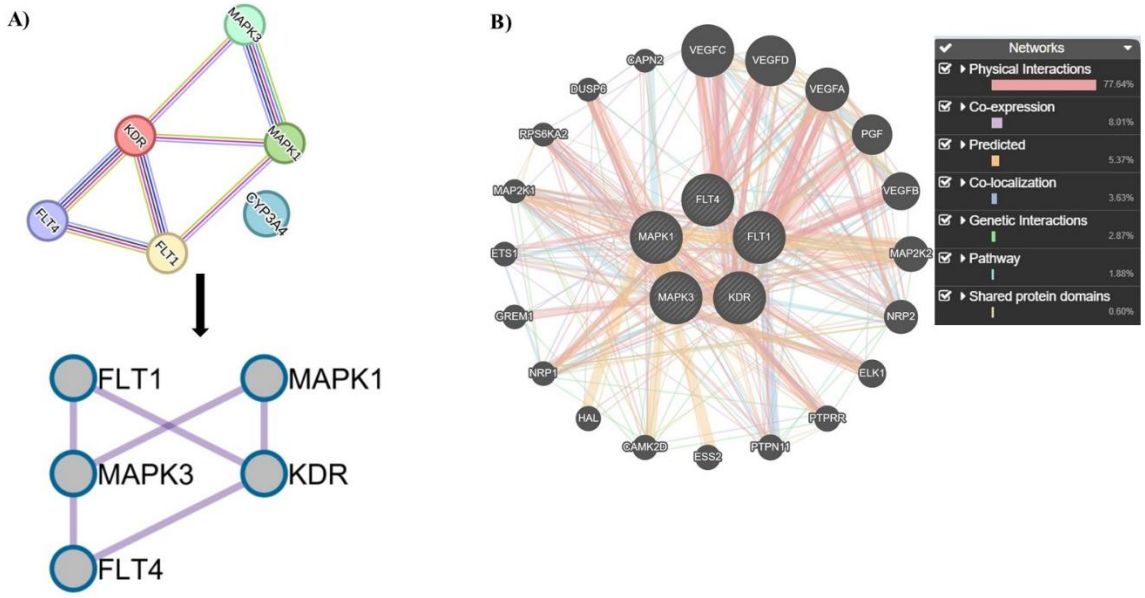

Figure 2. A) PPI analysis of common genes and hub genes, **B)** Gen-gen interaction analysis of the hub genes

Core Transcription Factors and miRNAs Involved in CVDs Induced by TKIs

After analyzing transcription factors (TFs) for five hub genes in ChEA3, the top 10 TFs were identified. Subsequently, nodes and edges representing the relationships between TFs and hub genes were manually prepared in Excel and schematized in Cytoscape 3.10.1, as shown in Figure 3A. The results are sorted by mean rank (Table 2). 'MeanRank' refers to the mean rank of each TF across all libraries containing that TF, serving as the score by which a composite list of TFs is reranked. In Figure 3A, green nodes represent TFs, whereas light brown nodes represent hub genes. These TFs may serve as potential targets for treating patients with TKIs-induced CVDs. Although there have not been any studies on some Table 2 TFs, there are studies highlighting the relationship between these TFs and CVDs. For instance, it is suggested that genetic variations in SOX18 and SOX17 in humans contribute to congenital heart disease [22,23]. Another study reported that SOX7 deficiency causes ventricular septal defects [24]. Other TFs, such as HAND1, play an essential role in cardiac morphogenesis [25].

Table 2. The top 10 TFs associated with to five hub genes

Rank	TF	Mean Rank	Overlapping Genes
1	BCL6B	2.667	FLT1, FLT4, KDR
2	SOX18	3.667	FLT1, FLT4, KDR
3	SOX17	28.25	FLT1, FLT4, KDR
4	HAND1	35.0	FLT1, KDR
5	SOX7	45.0	FLT1, FLT4, KDR
6	FOXE1	51.67	FLT1, FLT4, KDR
7	GSC	54.0	FLT1, KDR
8	SALL4	62.5	FLT4, KDR, MAPK1
9	ANHX	100.0	KDR
10	LHX1	101.0	KDR

As a result of miRNA-target analysis, the top 10 miRNAs were identified and are shown in Figure 3B. Among these 10 miRNAs, hsa-miR-199a-3p emerged as the most important miRNA in TKIs-induced CVDs. Some studies suggest that hsa-miR-199a-3p might be beneficial. For instance, Joris et al. [26] reported that it could help with repair after a heart attack by promoting heart muscle cell growth.

This is according to a study highlighting the role of hsa-miR-199a-3p in regulating endothelial nitric oxide synthase pathway, which is crucial for blood vessel health. Eulalio et al., [27] reported that hsa-miR-199a stimulated marked cardiac regeneration and almost complete recovery of cardiac functional parameters after myocardial infarction in mice.

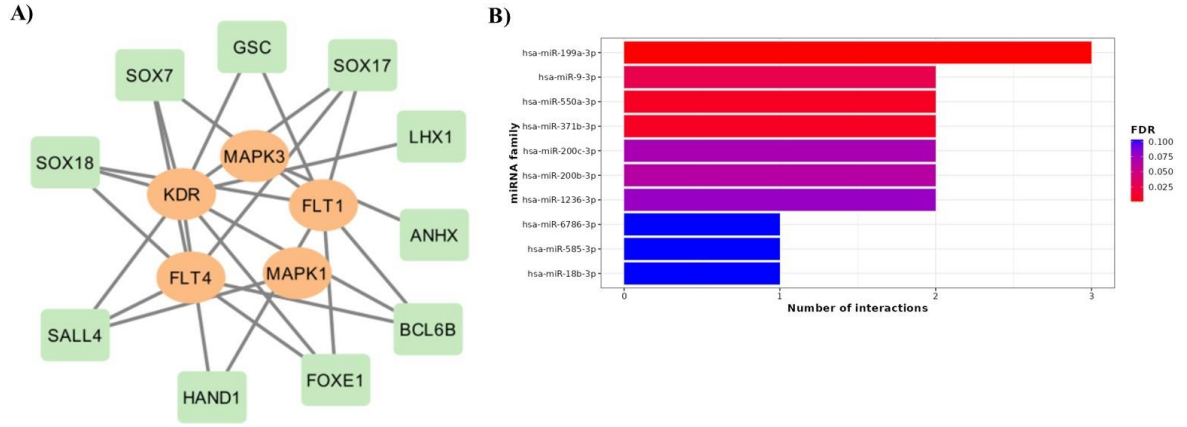

Figure 3. A) Core transcription factors associated with five hub genes obtained from ChEA3 and prepared in Cytoscape, **B)** Core miRNAs associated with five hub genes obtained from MIENTURNET

GO Enrichment Analyses and Molecular Pathways of Hub Genes

The top five enriched terms associated with TKIs-induced CVDs, identified in each GO category (biological processes, cellular components, and molecular functions) using the DAVID web tool, are outlined in Table 3. The findings underscore phosphorylation, a fundamental cellular process involving the addition of phosphate groups to proteins, as a primary biological process associated with hub genes. This suggests that dysregulation of phosphorylation pathways may play a pivotal role in the pathogenesis of CVDs resulting from exposure to SUN, SRF, PAZ, and AXI.

Furthermore, the analysis identified the receptor complex, a dynamic assembly of proteins that mediate cellular signaling and responses to extracellular stimuli, as the most significant cellular component involved in these four TKI-induced CVDs (Table 3). This highlights the importance of receptor-mediated signaling pathways in the development and progression of CVDs associated with TKI exposure.

Moreover, vascular endothelial growth factor-activated receptor activity emerged as the predominant molecular function implicated in these four TKI-induced CVDs, underscoring the critical role of VEGF signaling in vascular homeostasis and endothelial function. Dysregulation of VEGF receptor activity may disrupt angiogenesis and vascular integrity, contributing to the pathophysiology of TKI-induced CVDs. These insights shed light on the molecular mechanisms underlying the adverse cardiovascular effects of TKIs and may inform strategies for mitigating their cardiotoxicity (Table 3).

The molecular pathway associated with the five hub genes determined via Metascape highlighted 'Ras signaling' as the top pathway, with Log10(p) value of -11.11 and Log10(q) value of -7.22 (Figure 4 and 5).

Ras signaling is a crucial pathway involved in regulating various cellular processes, including cell growth, differentiation, and survival. The Ras protein acts as a molecular switch, cycling between an active (GTP-bound) and inactive (GDP-bound) state, and plays a central role in transmitting extracellular signals to the nucleus, thereby influencing gene expression and cellular behavior. The central role of Ras in pathologic and physiologic cardiac hypertrophy has been demonstrated in the literature. The dysregulation of Ras signaling has been implicated in several pathological conditions, including hypertrophy, fibrosis, and cardiomyopathy [28-31]. Activation of Ras signaling can lead to cardiac hypertrophy, a condition characterized by an increase in the size of individual cardiac muscle

cells. This is often a compensatory response to increased workload or pathological stimuli, such as hypertension or myocardial infarction. Prolonged hypertrophy can ultimately lead to heart failure. Ras signaling has been linked to the activation of fibroblasts and the production of extracellular matrix proteins, contributing to cardiac fibrosis. Fibrosis is the excessive deposition of collagen and other matrix components in the myocardium, leading to stiffening of the heart muscle and impaired function [28-31]. Given the role of Ras signaling in TKIs-induced cardiovascular pathology, it has emerged as a potential therapeutic target for the treatment of various CVDs. Strategies aimed at modulating Ras activity or downstream effectors may offer new avenues for intervention in conditions such as heart failure, myocardial infarction, and cardiac hypertrophy induced by TKIs.

Table 3. The top 5 gene ontology enrichments, include biological processes, cellular components, and molecular functions associated with the five hub genes (https://david.ncifcrf.gov/tools.jsp)

No.	ID	Biological Processes	*P* value	Bonferroni	Benjamini	FDR	Fisher Exact
1	GO:0016310	phosphorylation	$5.3x10^{-6}$	$9.6x10^{-4}$	$7.5x10^{-4}$	$5.4x10^{-4}$	$2.0x10^{-7}$
2	GO:0038084	vascular endothelial growth factor signaling pathway	$8.2x10^{-6}$	$1.5x10^{-3}$	$7.5x10^{-4}$	$5.4x10^{-4}$	$1.4x10^{-8}$
3	GO:0048010	vascular endothelial growth factor receptor signaling pathway	$2.3x10^{-5}$	$4.2x10^{-3}$	$1.3x10^{-3}$	$9.4x10^{-4}$	$6.8x10^{-8}$
4	GO:0035924	cellular response to vascular endothelial growth factor stimulus	$2.8x10^{-5}$	$5.2x10^{-3}$	$1.3x10^{-3}$	$9.4x10^{-4}$	$9.1x10^{-8}$
5	GO:0080090	regulation of primary metabolic process	$4.6x10^{-5}$	$8.4x10^{-3}$	$1.7x10^{-3}$	$1.2x10^{-3}$	$1.9x10^{-7}$
		Cellular Component					
1	GO:0043235	receptor complex	$1.0x10^{-3}$	$3.5x10^{-2}$	$3.6x10^{-2}$	$3.1x10^{-2}$	$2.1x10^{-5}$
2	GO:0005769	early endosome	$2.2x10^{-3}$	$7.5x10^{-2}$	$3.8x10^{-2}$	$3.3x10^{-2}$	$6.7x10^{-5}$
3	GO:0005925	focal adhesion	$4.2x10^{-3}$	$1.4x10^{-1}$	$3.8x10^{-2}$	$3.3x10^{-2}$	$1.7x10^{-4}$
4	GO:0031143	pseudopodium	$4.4x10^{-3}$	$1.4x10^{-1}$	$3.8x10^{-2}$	$3.3x10^{-2}$	$1.1x10^{-5}$
5	GO:0005901	caveola	$1.8x10^{-2}$	$4.7x10^{-1}$	$1.1x10^{-1}$	$9.4x10^{-2}$	$2.0x10^{-4}$
		Molecular Function					
1	GO:0005021	vascular endothelial growth factor-activated receptor activity	$1.6x10^{-6}$	$6.7x10^{-5}$	$6.7x10^{-5}$	$6.0x10^{-5}$	$1.0x10^{-9}$
2	GO:0019838	growth factor binding	$4.1x10^{-5}$	$1.8x10^{-3}$	$6.9x10^{-4}$	$6.1x10^{-4}$	$1.6x10^{-7}$
3	GO:0004714	transmembrane receptor protein tyrosine kinase activity	$4.8x10^{-5}$	$2.1x10^{-3}$	$6.9x10^{-4}$	$6.1x10^{-4}$	$2.0x10^{-7}$
4	GO:0005524	ATP binding	$2.1x10^{-4}$	$8.8x10^{-3}$	$2.2x10^{-3}$	$2.0x10^{-3}$	$2.0x10^{-5}$
5	GO:0004713	protein tyrosine kinase activity	$3.5x10^{-4}$	$1.5x10^{-2}$	$3.0x10^{-3}$	$2.7x10^{-3}$	$4.1x10^{-6}$

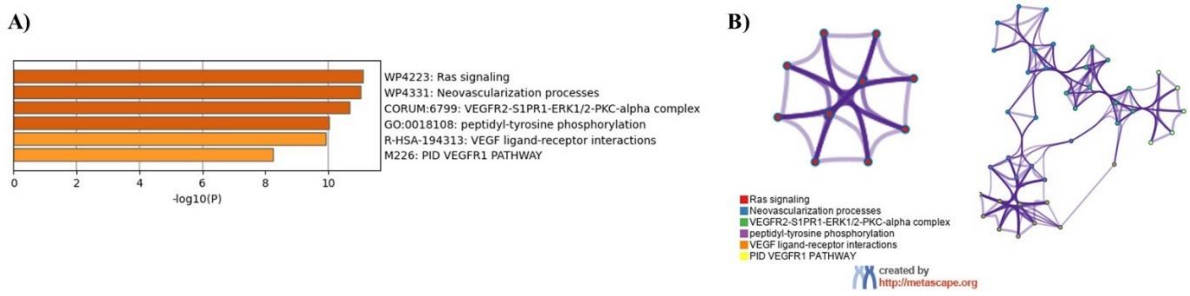

Figure 4. A) The top molecular pathways associated with five hub genes obtained from Metascape, **B)** The pathways associated with five hub genes are colored by its *p*-value obtained from Metascape

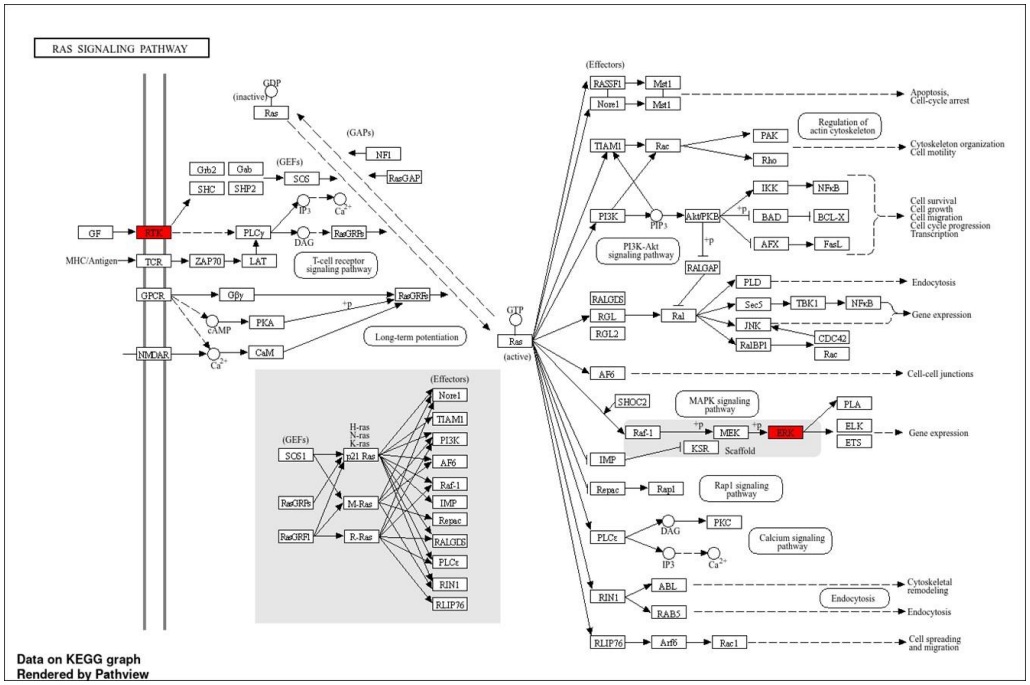

Figure 5. Ras signaling pathway downloaded from KEGG pathway (https://www.genome.jp/kegg/) [32]

In conclusion, this study employed network toxicology and bioinformatics approaches to elucidate the molecular mechanisms underlying cardiovascular toxicity induced by TKIs such as SUN, SRF, PAZ, and AXI. Through comprehensive data mining and analysis, we identified FLT1, FLT4, KDR, MAPK1, and MAPK3 as hub genes/proteins, SOX17 and SOX18 as transcription factors, and hsa-miR-199a-3p as a key microRNA, along with pathways implicated in TKI-induced cardiotoxicity. Notably, Ras signaling emerged as a pivotal pathway in mediating these adverse effects. These findings not only enhance our understanding of TKI-associated cardiovascular diseases but also highlight potential targets for future therapeutic interventions aimed at mitigating TKI-induced cardiotoxicity and improving patient outcomes.

AUTHOR CONTRIBUTIONS

Concept: F.K.; Design: F.K.; Control: F.K.; Sources: F.K.; Materials: F.K.; Data Collection and/or Processing: F.K.; Analysis and/or Interpretation: F.K.; Literature Review: F.K.; Manuscript Writing: F.K.; Critical Review: F.K.; Other: -

CONFLICT OF INTEREST

The author declares that there are no actual, potential or perceived conflicts of interest.

ETHICS COMMITTEE APPROVAL

The author declares that ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 940-948, 2024
Doi: 10.33483/jfpau.1443799

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SILDENAFIL DECREASED TNF-α AND IL-6 LEVELS IN CD-INDUCED ACUTE TOXICITY

SİLDENAFİL CD İLE İNDÜKLENEN AKUT TOKSİSİTEDE TNF-α VE IL-6 DÜZEYLERİNİ DÜŞÜRÜR

Ahmet Hüsamettin BARAN[1]*, Ahmet BERK[2], Ayşe Burçin UYUMLU[3], Özgen Arslan SOLMAZ[4], Göknur YALIM[5]

[1]Republic of Türkiye Ministry of Health, Türkiye Medicines and Medical Devices Agency, 06520, Ankara, Türkiye
[2]Department of Pharmacy, BattalGazi State Hospital, 44210, Malatya, Türkiye
[3]Inönü University, Faculty of Pharmacy, Department of Biochemistry, 44280, Malatya, Türkiye
[4]The Health Sciences University, Elazig Health Application and Research Center, Elazig Fethi Sekin City Hospital, Department of Pathology, 23200, Elazig, Türkiye
[5]Yuksek Ihtisas University, Faculty of Medicine, Internal Medical Sciences, Department of Pharmacology, 06530, Ankara, Türkiye

ABSTRACT

Objective: *This study aimed to evaluate the effects of sildenafil (SIL) on inflammation and histopathological changes in cadmium (Cd)-induced toxicity in female rats.*
Material and Method: *Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) levels were measured to assess the degree of inflammation. Histopathological changes in the liver, lungs and kidneys were also assessed.*
Result and Discussion: *SIL significantly reduced the cellular release of TNF-α and IL-6, which have been implicated in the pathogenesis of Cd-induced tissue damage. When SIL was administered alone, it showed histopathological effects similar to the control group. However, it was found that co-administration of SIL with Cd prevented portal vein dilation and central vein enlargement in the liver, prevented necrosis in kidney tissue, but did not affect the lung. Although SIL has variable protective effects on tissues, our results are in support of the idea that the use of SIL in tissue damage management can be investigated for its efficacy in modulating oxidative stress-induced proinflammatory cytokine activation in vivo and ultimately help prevent Cd-induced tissue damage. Our study has shown that SIL can reduce Cd-induced acute toxicity in rats. SIL may be use as a protective agent against toxicity of heavy metals.*
Keywords: *Cadmium, liver enzymes, oxidative stress, sildenafil, tissue damage*

ÖZ

Amaç: *Bu çalışmada, dişi sıçanlarda kadmiyum (Cd) kaynaklı toksisitede sildenafilin (SIL) inflamasyon ve histopatolojik değişiklikler üzerindeki etkilerinin değerlendirilmesi amaçlanmıştır.*
Gereç ve Yöntem: *İnflamasyon derecesini değerlendirmek için interlökin-6 (IL-6) ve tümör nekroz faktörü-alfa (TNF- α) seviyeleri ve karaciğer, akciğer ve böbreklerdeki histopatolojik değişiklikler*

* **Corresponding Author / Sorumlu Yazar:** Ahmet Hüsamettin Baran
e-mail / e-posta: eczabaran@gmail.com, **Phone / Tel.:** +905534514950

Submitted / Gönderilme : 27.02.2024
Accepted / Kabul : 08.07.2024
Published / Yayınlanma : 10.09.2024

This article has been derived from PhD thesis of Ahmet Hüsamettin Baran.

değerlendirilmiştir.
Sonuç ve Tartışma: *SIL, Cd ile indüklenen doku hasarının patogenezinde rol oynayan TNF-α ve IL-6'nın hücresel salınımını önemli ölçüde azaltmıştır. SIL tek başına uygulandığında, kontrol grubuna benzer histopatolojik etkiler göstermiştir. Bununla birlikte, SIL'in Cd ile birlikte uygulanmasının karaciğerde portal ven genişlemesini ve merkezi ven büyümesini önlediği, böbrek dokusunda nekrozu önlediği, ancak akciğeri etkilemediği bulunmuştur. SIL'in dokular üzerinde değişken koruyucu etkileri olmasına rağmen, sonuçlarımız doku hasarı yönetiminde SIL kullanımının in vivo oksidatif stres kaynaklı proinflamatuar sitokin aktivasyonunu modüle etmedeki etkinliğinin araştırılabileceği ve nihayetinde Cd kaynaklı doku hasarını önlemeye yardımcı olabileceği fikrini desteklemektedir. Çalışmamız, SIL'in sıçanlarda Cd kaynaklı akut toksisiteyi azaltabildiğini göstermiştir. SIL, ağır metallerin toksisitesine karşı koruyucu bir ajan olarak kullanılabilir.*
Anahtar Kelimeler: *Doku hasarı, kadmiyum, karaciğer enzimleri, oksidatif stres, sildenafil*

INTRODUCTION

Cadmium (Cd) is a carcinogenic heavy metal that is commonly found in the earth's crust and has significant harm to human, animal and plant health [1]. As a result of industrial activities Cd, which spreads as a contaminant to air, water and soil, causes serious health problems on living organisms due to its toxicity and bioaccumulation in the ecosystem [2]. The mechanisms that trigger oxidative stress and initiate the inflammatory process underlie the nephrotoxicity, hepatotoxicity and lung toxicity observed as a result of environmental and occupational exposure to Cd [3-6].

Cadmium exposure causes an oxidant/antioxidant imbalance in the organism and increases peroxide formation. This leads to the production of high levels of hydroperoxides and disruption of lipid, carbohydrate and protein metabolism. On the other hand, it has been shown to increase the levels of biological indicators such as tumor necrosis factor alpha (TNF-α) and interleukins which are responsible for inflammation, apoptosis and tumor development [1-6].

The phosphodiesterase 5 (PDE5) enzyme is a cyclic nucleotide phosphodiesterase found in many tissues, including neurons, smooth muscle cells, and the brain. It neutralizes the second messenger 3′-5′ cyclic guanosine monophosphate (cGMP), by hydrolyzing it to guanosine monophosphate (GMP) [7]. Sildenafil (licensed as Viagra®) is a selective and potent PDE5 inhibitor, extending the half-life of endogenous cGMP and expanding corpus cavernosum vascular smooth muscles. As a PDE5 inhibitor, SIL is now frequently used to treat pulmonary hypertension and erectile dysfunction [8,9]. In some recent studies, there have been suggestions that the use of SIL may have anti-inflammatory and antioxidant activities [10-12]. However, the protective effect of SIL against multi-organ damage in rats has not been adequately studied.

The aim of this study was to evaluation of the effect of the use of SIL on acute lung, liver and kidney damage induced by a single dose of Cd administered to female rats, and its anti-inflammatory potential, using interleukin-6 (IL-6) and TNF-α as inflammation markers and histopathological analysis of the tissues.

MATERIAL AND METHOD

Although different exposure routes of Cd are mentioned in literature reviews, human intoxication generally occurs orally. In experimental models, the intraperitoneal route is a preferred one to induce acute Cd toxicity [13-16]. The route of administration and dosage of SIL in our study were established on the basis of previous studies [17-19].

All animals received humane care according to the guidelines established by the Committee for the Institutional Use and Care of Laboratory Animals of the University of Fırat, and the same committee approved the experimental protocol (Ethical Approval Number; 2016/151). Female Sprague-Dawley rats (180-220g) were obtained from Fırat University Laboratory of Experimental Animals. The animals were fed a standard pellet diet. Water was provided ad libitum. They were kept in plastic cages at a temp of 21 ± 2°C with a 12-hour light/dark period and an air humidity of 30% to 70%. The rats were divided into four experimental groups. All groups contained seven animals. Group I: 0.5 ml/rat of saline solution

was injected i.p. on the 7th day to the Control group. Group II: 3.7 mg/kg single dose of Cd was injected i.p. on the 7th day to the Cd group. Group III: SIL group was administered with 5 mg/kg/day of SIL in drinking water for 10 days. Group IV: Cd+SIL group was given 5 mg/kg/day of SIL drinking water during 10 days and a single i.p. injection of 3.7 mg/kg of Cd on day 7 (Figure 1). To finish experimenting, the animals were under anesthesia by i.p. administration of 50 mg/kg ketamine and 5 mg/kg xylazine and liver, lungs and kidneys removed for further analysis. Tissue samples had a storage temperature of -80°C. To prevent deformation of the fragile molecules, an experimental environment was prepared with ice water before removing the lung, kidney and liver tissues from the -80°C deep freezer, and all procedures were performed in this environment. Lung, kidney and liver tissues were removed, weighed on a precision balance and placed in plastic tubes at a weight of approximately 100 mg. The tubes were homogenized with a homogenizer after the addition of 1 ml of phosphate buffer and sonication. After centrifugation, supernatants were collected in Eppendorf tubes and used to measure biochemical parameters.

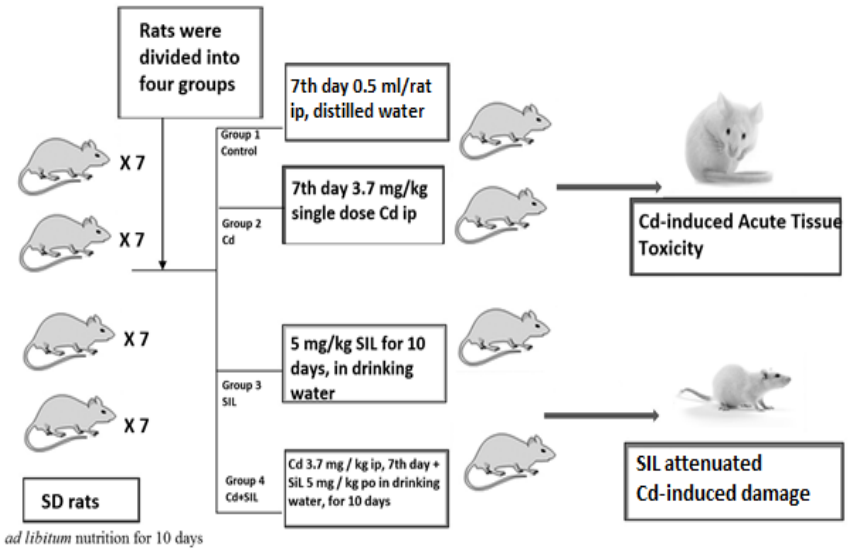

Figure 1. Scheme of the experimental design

This experimental protocol was designed taking into account similar studies [20-23]. The fact that Cd accumulates mainly in the kidney and liver, where metallothionein levels are high, dictated the choice of tissues analyzed. Furthermore, Cd hepatotoxicity is the main cause of acute Cd lethality and the liver is the main target organ of toxicity after acute Cd poisoning [24].

Cytokine Analysis

Enzyme-linked immunosorbent assay (ELISA) kits obtained from Shanghai Yehua Biological Technolgy were used for IL-6 and TNF-α determinations. For this, Samples previously kept at -20°C were allowed to come to room temperature. The kits were removed from 4°C and kept at room temperature for 30 min. Chromogen B in the kits was kept in a closed box as it is light sensitive. Sera were centrifuged at 2500 g for 20 min. Supernatants were separated. Dilution of standard solutions was started. For this, 120 µl of the original standard in the kit was taken and mixed with 120µl of standard dilute in an ependorf tube. Then 120 µl of the mixture was taken and mixed with the same amount of standard dilution each time. Samples and solutions were then loaded onto the ELISA plate. 50 µl of standards were loaded and 50 µl of streptomycin HRP was added. The blind portion was left blank and 40 µl serum and 10 µl TNF-α antibody for TNF-α measurement and IL-6 antibody solution for interleukin 6 were added. Finally, 50 µl streptavidin-HRP was added and the layer was covered. Shaken gently and incubated at 37°C for 1 hour. The washing solution was prepared by mixing 1/30 (v/v) with distilled water and applied 5 times for 30 s to all wells in the incubated layers. For color formation, 50 µl of chromogen A was first added to all wells. Then 50 µl of chromogen B was added and shaken

carefully. 37°C incubation was allowed for 10 min. In the meantime, the assay was performed in the dark to avoid light exposure. After incubation, 50 µl of the stop solutions were added and read immediately at 450 nm. The data were calculated in the coordinate plane according to the standards. Results were expressed in pg/ml for TNF-α and IL-6.

Histopathological Analysis

Tissue samples were fixed in 10% buffered neutral formalin and embedded in paraffin. 5-micron thick sections were cut from each paraffin block using a fully automated rotary microtome (Leica Biosystems RM2245 Semi-Automated Rotary Microtome) and stained with hematoxylin-eosin. Preparations were then examined by light microscopy (Leica Dm 200 Led Light Microscope) and photographed using an Olympus 3E03784 camera.

Statistical Analysis

Statistical analysis was performed using the INSTAT automated software package (GraphPad Prism Inc., San Diego, CA, USA). All values are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) has been used when comparing differences between groups, and Tukey post hoc test was used when comparing paired groups. Statistically significant was defined as a P value of <0.05.

RESULT AND DISCUSSION

The biochemical findings related to all groups studied are summarized in Table 1. There are no significant differences in inflammation markers in SIL group compared to the control group. Management of Cd increased TNF-α levels in the liver (1.6- fold), lung (2.0-fold), and kidney (2.1-fold) tissues. SIL treatment significantly inhibited Cd-induced TNF-α increase in the liver (20.2%), lung (36.8%), and kidney (34.7%) tissues. The IL-6 levels were higher in the liver (1.9-fold), lung (1.8-fold), and kidney (1.4-fold) tissues in Cd group when compared to the control. SIL treatment significantly decreased Cd-induced IL-6 increase in the liver (33.1%), lung (24.7%), and kidney (14.7%) tissues. The results of the analysis of TNF-α and IL-6 biomarkers are shown in Table 1. TNF-α, liver (ANOVA F= 22.8, p= 0.0036), lung (ANOVA F= 33.228, p= 0.001), and kidney (ANOVA F= 66.28, p= 0.001). IL-6, liver (ANOVA F= 85.95, p=0.0009), lung (ANOVA F= 24.62, p=0.0079) and kidney (ANOVA F= 36.14, p= 0.0027).

Table 1. Effect of SIL on inflammation markers in Cd induced toxicity

Groups		Control	SIL	Cd	SIL + Cd
TNF-α (pg/ml)	Liver	59.39±7.62	56.2±5.48	94.61±14.52***	75.49±8.97**
	Lung	36.78±7.43	34.81±6.72	75.13±10.24***	47.53±9.26***
	Kidney	43.37±6.25	41.89±5.62	89.14±9.74***	58.24±7.21***
IL-6 (pg/ml)	Liver	230.1±24.13	218.32±28.93	430.16±32.15***	287.92±25.16***
	Lung	118.25±20.16	111.44±21.25	209.15±27.87***	157.42±25.63**
	Kidney	167.3±12.35	153.27±13.27	228.01±17.29***	194.52±14.62**

Abbreviations: TNF-α, tumor necrose factor; IL-6, interleukin-6. Values are represented as mean±SEM. **Significant change in comparison with control at P <0.01; ***Significant change in comparison with control at P <0.001

Lung, liver and kidney tissues were examined for inflammation and necrosis. As a result of the histopathological examination of the kidney, lung and liver tissues of the control group, a normal histopathological appearance was found. Histopathological examinations of the kidney, lung and liver tissues of the SIL group revealed normal histopathological features similar to the control group. Histopathological examination revealed of kidney, lung and liver tissues of the Cd group; enlargement of the portal (Figure 2C1) and central vein (Figure 2C2) and necrosis of single cells were observed (Figure 2C3). Inflamed areas and hemorrhage were detected in the lung tissue (Figure 3C). Focal

necrosis areas were detected in the kidney tissue (Figure 4C). It was observed that Cd toxicity had the potential to cause multiple organ damage in all three groups. The results of the histopathological examination of the SIL+Cd group; necrosis in the liver tissue (Figure 2D), chronic inflammation and hemorrhage in the lung tissue (Figure 3D), and normal histopathological appearance in the kidney tissue (Figure 4D) were observed. Cd administration caused significant pathological changes in female rat liver (Figure 2C1, 2C2, 2C3). Co-administration of SIL with Cd had no effect on necrosis formation, but reversed portal vein dilation and central vein enlargement caused by Cd (Figure 2D). Cd administration caused significant pathological changes in female rat lung compared to the control group (ANOVA F = 12.25, p = 0.0077) (Figure 3C). The administration of cadmium resulted in significant pathological changes in the kidney tissue in comparison with the control group (Figure 4C). Co-administration of SIL with Cd prevented necrosis caused by Cd when given alone (Figure 4D). When the three tissues were compared, it was determined that SIL caused a partial improvement in liver histopathology, had no positive effect on lung tissue, and reversed the effect of Cd in kidney tissue. The protective effect of SIL was most pronounced in kidney tissue.

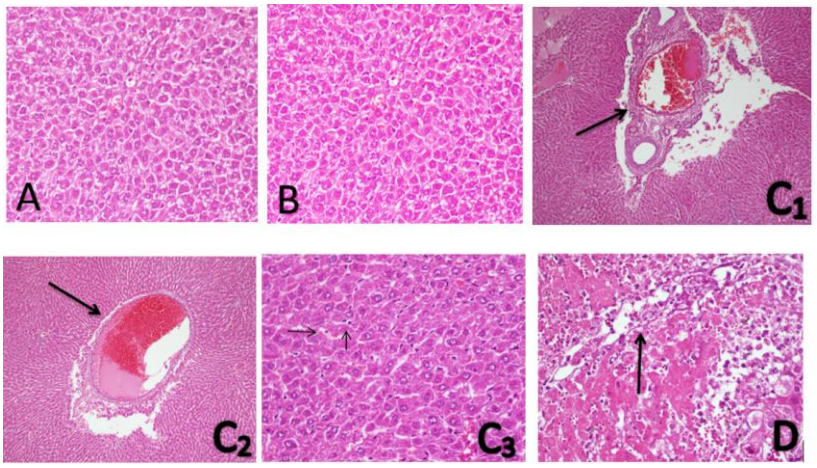

Figure 2. Liver tissue histopathology examination

A) Liver-control; Normal histopathological appearance in liver tissue B) Liver-SIL; Normal histopathological appearance in liver tissue C1) Liver-Cd; Portal vein dilation in liver tissue C2) Liver-Cd; Central vein enlargement in liver tissue C3) Liver-Cd; single cell necrosis in liver tissue D) Liver-SIL+Cd; Necrosis in liver tissue

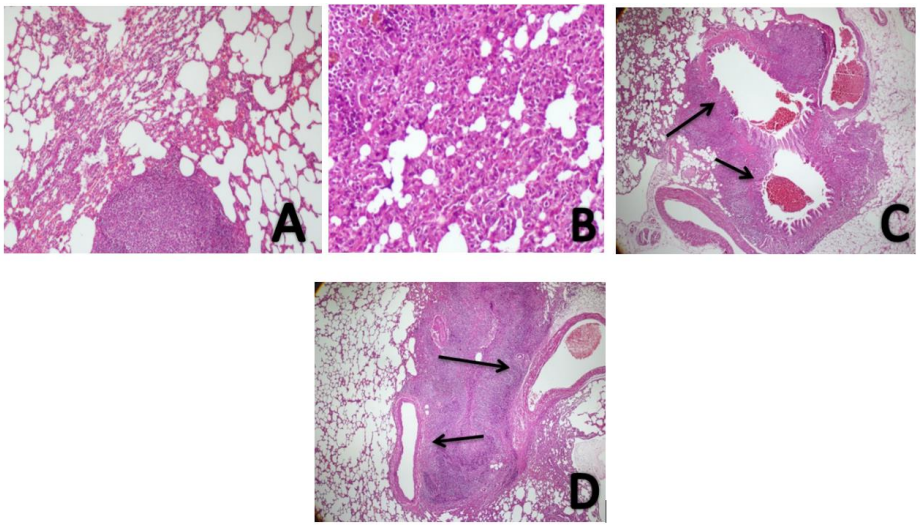

Figure 3. Lung tissue histopathology examination

A) Lung-control; Normal histopathological appearance in lung tissue B) Lung-SIL; Normal histopathological appearance in lung tissue C) Lung-Cd; Chronic inflammation, hemorrhage in lung tissue D) Lung-SIL+Cd; Chronic inflammation, hemorrhage in lung tissue

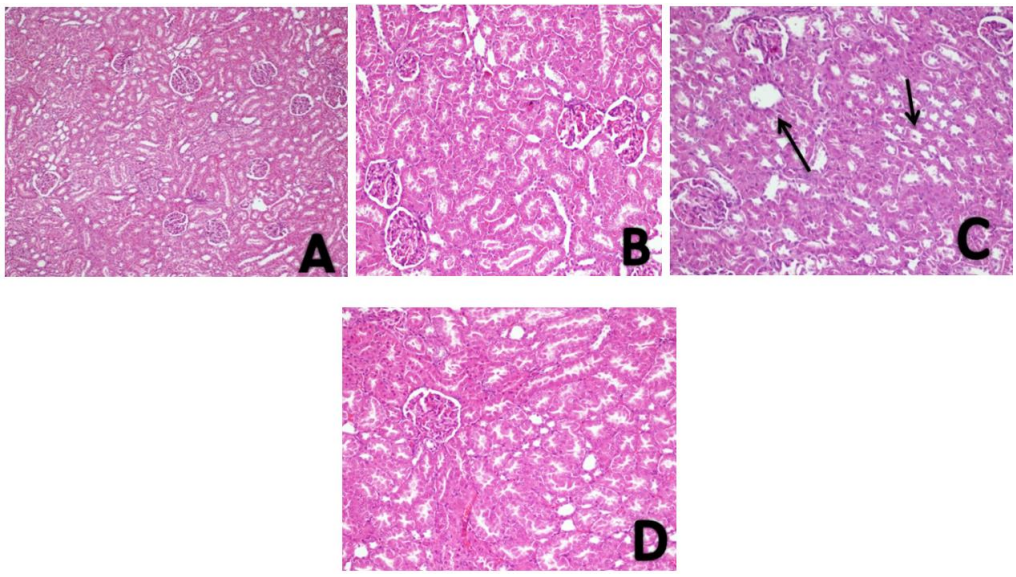

Figure 4. Kidney tissue histopathology examination
A) Kidney-control; Normal histopathological appearance in kidney tissue B) Kidney-SIL; Normal histopathological appearance in kidney tissue C) Kidney-Cd; Focal necrosis in kidney tissue
D) Kidney-SIL+Cd; Normal histopathological appearance in kidney tissue

Some researchers have suggested that Cd toxicity varies according to gender differences. In a study examining the impact gender differences on liver and kidney accumulation of cadmium [25–27], they found that significantly higher Cd is accumulated in the liver tissues of female rats 1 hour after subcutaneous Cd injection compared to males. They observed that Cd levels in female rats remained high for at least 10 days [25]. Therefore, we preferred to use female rats in our study.

On the basis of the results of our study, we have the following suggestions treatment with SIL may reduce the inflammatory response and prevent multiple organ damage to liver, lung and kidneys due to Cd in the rat model. As a result of histopathological evaluations, we detected focal necrosis in rat kidney tissue, chronic inflammation in lung tissue, hemorrhage, portal vein dilatation, dilation of the central vein and single cell necrosis in liver tissue. We found that SIL significantly reduced the severity of Cd damage partly in the liver tissue but mostly in the kidney tissue. Inflammation development is accompanied by inflammatory cell infiltration and inflammatory cytokine release. Cd resulted in a significant increase in the levels of IL-6 and TNF-α in the all tissues. SIL significantly reduced inflammation in the group where Cd and SIL were administered together.

Similar to our study, Fang et al. also investigated the protective role of a single i.p. dose of SIL administration in the inflammatory acute lung injury model induced by sodium taurocholate and performed histopathological analysis together with evaluation of the inflammatory parameters such as IL-6 and TNF-α. They observed low levels of lung damage and inflammation in rats treated with SIL [8]. In another study, SIL administration in the model of lung ischemia-reperfusion injury has been shown to reduce inflammation and formation of reactive oxygen species [28]. In addition to these findings investigating the effect of SIL and febuxostat on doxorubicin-induced nephrotoxicity in rats, the effect of SIL (5 mg/kg; p.o.) administration for 21 days on nephrotoxicity, oxidative stress markers, TNF-α and inflammatory mediators were examined.

SIL and/or febuxostat application with doxorubicin has led to a significant reduction in the nephrotoxicity markers and inflammatory mediators, and the return of oxidative stress biomarkers to their normal values. In addition, SIL and febuxostat improved the histological changes caused by doxorubicin [17]. Furthermore, investigating the protective role of SIL on the kidneys, SIL has been shown to reduce renal tubular damage and apoptosis in an experimental model of cisplatin-induced nephrotoxicity [29]. Similar to our study, the protective role of SIL against hepatic fibrosis induced by bile duct ligation in rats was investigated and it was found that SIL administration significantly reduced

high TNF-α values [18]. In many studies investigating the effects of SIL on inflammation, histopathological evaluations have found that SIL has a protective effect by reducing inflammation [30-32].

In our study, unlike previous studies [33,34], SIL did not have a hepatotoxic effect on the liver when administered alone or together with Cd, but it did not prevent the formation of necrosis in the liver. Neutrophil infiltration and proinflammatory cytokines like TNF-α and IL-6 are often associated with Cd-induced toxicity. Although the role of TNF-α and IL-6 in both pathological and physiological situations has not been fully elucidated, the decrease in levels of these cytokines after tissue damage is suggestive of the involvement of cellular repair mechanisms. Whereas, when inflammatory cytokines are produced in abundance, pathological conditions such as dermal toxicity, liver toxicity, rheumatoid arthritis, atherosclerosis, kidney and lung toxicity can occur [35-37]. The increase in the levels of TNF-α and of IL-6 observed in Cd-treated rats in our study indicates the initiation of inflammation leading to cellular damage, and the decrease in levels with SIL administration suggests that repair mechanisms are induced.

SIL has been shown to have protective ability in exposure to various inflammatory stimuli. However, the curative effect of SIL on Cd-induced tissue damage and the mechanism of this effect have not been studied. In this trial, we have demonstrated for the first time that protective effect of PDE5I SIL against tissue damage by an inhibitory effect on IL-6 and TNF-α activity in Cd-induced acute toxicity.

Based on the biochemical and histopathological findings of our study, we can suggest that SIL has a protective effect on the liver, lung and kidney against acute toxicity induced by Cd. SIL reverses Cd-induced increases in IL-6 and TNF-α levels, thereby exerting its anti-inflammatory effect. It also prevents cell death by inhibiting inflammation and has a protective effect as confirmed by liver and kidney histopathological analysis. Therefore, we concluded SIL may be use as a protective agent against toxicity of heavy metals.

AUTHOR CONTRIBUTIONS

Concept: A.H.B., G.Y.; Design: A.H.B., G.Y.; Control: A.H.B., G.Y.; Sources: A.H.B.; Materials: A.H.B., G.Y.; Data Collection and/or Processing: A.H.B., A.B., A.B.U., Ö.A.S., G.Y.; Analysis and/or Interpretation: A.H.B., A.B., A.B.U., Ö.A.S., G.Y.; Literature Review: A.H.B., G.Y.; Manuscript Writing: A.H.B.; Critical Review: A.H.B., G.Y.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Ethical approval of this study was obtained from the Committee for the Institutional Use and Care of Laboratory Animals of the University of Fırat (Ethical Approval Number; 2016/151).

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 949-961, 2024
Doi: 10.33483/jfpau.1487169

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF THE CIRCADIAN OSCILLATION PATTERN OF UNFOLDED PROTEIN RESPONSE SIGNALING COMPONENTS IN HUMAN EMBRYONIC KIDNEY HEK293 CELLS

HEK293 İNSAN EMBRİYONİK BÖBREK HÜCRELERİNDE KATLANMAMIŞ PROTEİN YANITI SİNYALİ BİLEŞENLERİNİN SİRKADİYEN SALINIM MODELİNİN BELİRLENMESİ

Yalcin ERZURUMLU[1]*, Hatice Kubra DOGAN[2], Deniz CATAKLI[3]

[1]Suleyman Demirel University, Faculty of Pharmacy, Department of Biochemistry, 32260, Isparta, Turkey
[2]Suleyman Demirel University, Institute of Science, Department of Bioengineering, 32260, Isparta, Turkey
[3]Suleyman Demirel University, Faculty of Medicine, Department of Pharmacology, 32260, Isparta, Turkey

ABSTRACT

Objective: *The circadian rhythm is one of the primary regulatory systems with near 24-hour oscillations. It has a crucial role in regulating physiological conditions in the human body, including body temperature and the secretion of hormones. Numerous disorders, such as cancer and diabetes, have been linked to disruptions of the cellular circadian rhythm. Herein, we aimed to investigate the relationship between the circadian rhythm and unfolded protein response (UPR) signaling, which is one of the important physiological mechanisms in mammalian cells and has recently been associated with drug resistance, invasion and metastasis in cancer.*
Material and Method: *Human embryonic kidney cell line HEK293 was provided from the American Type Culture Collection and propagated in DMEM containing 10% FBS and growth ingredients. For in vitro circadian synchronization, cells were exposed to 50% and then the oscillation pattern of gene and protein expression of UPR-related target genes was analyzed by agarose gel electrophoresis and immunoblotting, respectively. The oscillation pattern was commented on through curve-fitting analysis.*
Result and Discussion: *Our findings demonstrated that UPR components, including IRE1α, XBP-1s, eIF2α, phospho(Ser51)-eIF2α, PERK, ATF4, GADD34 and ATF6, tightly exhibit oscillation patterns under a circadian rhythm on a 48-hour time scale like the PER1 gene that is a core component of the circadian rhythm. Moreover, endoplasmic reticulum (ER) stress genes, BiP/GRP78 and CHOP, were similar to UPR components under the circadian rhythm. Additionally, we found the activation of UPR signaling harmoniously modulated with the circadian rhythm. Present data indicated that the expression level of UPR components exhibited strict oscillation under the circadian rhythm. Our findings may guide experimental studies of new-generation UPR-targeted drugs to be developed to treat various pathologies in accordance with the circadian rhythm.*
Keywords: *Circadian clock, ER stress, unfolded protein response*

ÖZ

Amaç: *Sirkadiyen ritim, yaklaşık 24 saatlik salınımlara sahip temel düzenleyici sistemlerden biridir. Vücut ısısı ve hormon salgılanması da dahil olmak üzere insan vücudundaki fizyolojik koşulların düzenlenmesinde çok önemli bir role sahiptir. Kanser ve diyabet de dahil olmak üzere çok sayıda rahatsızlık hücresel sirkadiyen ritmin bozulmasıyla ilişkilendirilmiştir. Bu çalışmada memeli*

* **Corresponding Author / Sorumlu Yazar:** Yalcin Erzurumlu
e-mail / e-posta: yalcin.erzurumlu@gmail.com, **Phone / Tel.:** +905448878439 / +902462110345

Submitted / Gönderilme : 20.05.2024
Accepted / Kabul : 08.07.2024
Published / Yayınlanma : 10.09.2024

hücrelerinde önemli fizyolojik mekanizmalardan biri olan ve son zamanlarda kanserde ilaç direnci, invazyon ve metastaz ile ilişkilendirilen katlanmamış protein yanıtı (UPR) sinyali ile sirkadiyen ritim arasındaki ilişkiyi araştırmayı amaçladık.

Gereç ve Yöntem: *İnsan embriyonik böbrek hücre hattı HEK293 Amerikan Tipi Kültür Koleksiyonundan sağlandı ve hücreler %10 FBS ve büyüme bileşenleri içeren DMEM besi ortamı içinde çoğaltıldı. İn vitro sirkadiyen senkronizasyon için hücreler %50 at serumuna maruz bırakıldı ve ardından UPR ile ilişkili hedef genlerin gen ifadesi ve protein düzeyindeki salınım modeli sırasıyla agaroz jel elektroforezi ve immünoblotlama ile analiz edildi. Salınım modeli eğri uydurma analizi yoluyla değerlendirildi.*

Sonuç ve Tartışma: *Bulgularımız, IRE1α, XBP-1s, eIF2α, fosfo(Ser51)-eIF2α, PERK, ATF4, GADD34 ve ATF6 dahil olmak üzere UPR bileşenlerinin, sirkadiyen ritmin çekirdek komponentlerinden PER1 geni gibi 48 saatlik bir zaman ölçeğinde sirkadiyen ritim altında sıkı bir şekilde salınım modelleri sergilediğini gösterdi. Ayrıca endoplazmik retikulum (ER) stres genleri, BiP/GRP78 ve CHOP da sirkadiyen ritim altında UPR bileşenlerine benzer şekilde davrandı. Ek olarak UPR sinyalinin aktivasyonunun sirkadiyen ritimle uyumlu bir şekilde modüle edildiğini bulduk. Mevcut veriler, UPR bileşenlerinin ekspresyon seviyesinin sirkadiyen ritim altında katı salınım sergilediğini gösterdi. Bulgularımız, çeşitli patolojileri sirkadiyen ritme göre tedavi etmek için geliştirilecek yeni nesil UPR hedefli ilaçların deneysel çalışmalarına yol gösterebilir.*

Anahtar Kelimeler: *ER stres, katlanmamış protein yanıtı, sirkadiyen saat*

INTRODUCTION

Circadian rhythms are identified as near-24-hour oscillations present in almost all physiological processes in the human body and brain [1]. The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the master circadian pacemaker. It is hierarchically organized and synchronized by light-dark cycles by neural and neuroendocrine pathways [2,3]. The circadian clock system also regulates many physiological conditions like body temperature, blood pressure, sleep/wake cycle, immune responses, and synthesis and secretion of hormones, such as cortisol and melatonin [4].

Cellular-level oscillations are originated by circadian clock genes such as clock circadian regulator (CLOCK), period circadian regulator 1-3 (Per 1-3), cryptochrome genes (CRY1 and CRY2) and brain and muscle ARNT-like1 (Bmal1) rhythmically regulating their transcription as well as the transcription of various clock-controlled genes [2,5]. The circadian timing system in mammals has long been a research subject due to its potential involvement in the pathogenesis of different diseases. While proper coordination of cellular- and tissue-level clocks is crucial for maintaining homeostasis, disruption of cellular circadian rhythm has been implicated in the pathogenesis of many diseases, including cardiovascular diseases, epilepsy, Alzheimer's disease, diabetes and cancer [6-8]. Therefore, it is extremely important to understand the relationship between circadian rhythm and physiologically important mechanisms in mammalian cells, such as endoplasmic reticulum-associated degradation (ERAD) and unfolded protein response (UPR) signaling and to characterize their oscillation patterns under a circadian rhythm [9]. In this context, it will be essential to determine oscillation pattern maps for the use of drugs that pharmacologically target these mechanisms to obtain more therapeutically effective results.

The endoplasmic reticulum (ER) has multiple roles in eukaryotic cells and is responsible for coordinating several functions, including protein synthesis, transport and folding, lipid and steroid biosynthesis, carbohydrate metabolism and Ca^{+2} storage [10-13]. UPR signaling organized in the ER plays a vital role in the re-adaptation of the ER against altered cellular physiological conditions and also mitigates stress responses through setting the ER capacity [14]. UPR signaling orchestrates the ER membrane resident three transmembrane proteins, inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [15]. Accumulating misfolded or unfolded proteins in the ER lumen or specific stressful conditions activates the UPR signaling through autophosphorylation and dimerization or trimming of ER stress sensor proteins. Subsequently, downstream effectors coordinate the continuation of signal transmission and eventually, specialized transcription and translational programs are activated in the cells [15]. Excessive activation of the UPR signaling has been associated with emerging acquired drug

resistance, cancer cell invasion and metastasis [14,16]. Unusual alterations in the UPR signaling have been associated with numerous diseases, including neurodegenerative diseases, diabetes, inflammatory disease and cancer [17-20]. Therefore, determining the circadian oscillation pattern of UPR components is crucial in terms of organizing the use of UPR-targeted drugs according to the oscillatory pattern. However, the detailed behavior of the UPR signaling under circadian oscillation currently remains unclear.

In the present study, we investigated the daily physiological oscillation pattern of UPR signaling under circadian rhythmicity in human embryonic kidney HEK293 cells. For this aim, we evaluated the oscillation pattern of UPR signaling-related genes, IRE1α, XBP-1s, PERK, ATF4, GADD34, ATF6 and also ER stress genes, BiP/GRP78 and CHOP. Additionally, protein expression levels of some downstream effects of UPR signaling components, XBP-1s, eIF2α, phospho(Ser51)-eIF2α and BiP/GRP78 were tested by immunoblotting. Our results revealed a detailed physiological release pattern of UPR signaling. Present data indicated that the expression level of UPR components exhibited strict oscillation under circadian rhythm. Our findings may guide experimental studies of new-era UPR-targeted drugs in treating several pathologies.

MATERIAL AND METHOD

Material

Rabbit polyclonal antibodies anti-BiP/GRP78 (11587-1-AP)(1:10000), anti-Bmal1 (14268-1-AP)(1:2000) and anti-XBP-1s (24868-1-AP)(1:2500) were provided from Proteintech, anti-eIF2α (#9722)(1:2000) and anti-phospho-eIF2α (Ser51) (#9721)(1:2000) from Cell Signaling Technology. Mouse monoclonal β-actin antibody (A5316)(1:10000) was obtained from Sigma Aldrich. Horseradish peroxidase (HRP)-conjugated anti-mouse (#31430)(1:5000) or anti-rabbit (#31460)(1:5000) IgG (H+L) was purchased from Thermo Scientific.

Cell culture materials and reagents, including plastic ingredients, growth media, fetal bovine serum (FBS) and additional growth requirements were obtained from Sartorius. Horse serum was obtained from Biowest.

Cell Culture

Human embryonic kidney cell line HEK293 (CRL-1573TM) was obtained from the American Type Culture Collection (ATCC, USA). Cells were routinely propagated in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 5 mg ml^{-1} penicillin/streptomycin and 2 mM L-glutamine (Lonza). Cells were kept in a humidified atmosphere of 5% CO_2 and 95% air at a temperature of 37°C. The absence of mycoplasma contamination was periodically verified using MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza).

In vitro Circadian Synchronization of HEK293 Cells

Circadian synchronization was performed as previously described [9,21]. Cells were seeded on 10mm cell culture dishes. At confluence, the cells were propagated for 6 days and then cells were subjected to 50% horse serum (serum shock) or 10% FBS containing regular media for 2 hours for circadian synchronization. After serum shock synchronization, the shock medium was replaced with a serum-free medium. Cells were collected at 4-hour intervals for 48 hours and lysed for RNA extraction and protein isolation.

Total RNA Extraction and Complementary DNA Synthesis

Total RNA was extracted from cells by Monarch® Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. The concentration and purity of isolated RNAs were determined by a micro-spectrophotometer (Allsheng). Complementary DNA was synthesized using 1 μg RNA by iScript™ cDNA Synthesis kit (Bio-Rad). The reaction mix was incubated for 5 min at 25°C, 20 min at 46°C and 1 min at 95°C, respectively.

Reverse Transcription PCR (RT-PCR) and Agarose Electrophoresis

Specific primers were used for the PCR amplification of interested genes with Taq DNA polymerase (Thermo Scientific). The following PCR conditions were used: pre-denaturation at 95°C for 5 min, followed by 36 cycles of denaturation 30 s at 95°C, annealing 30 s at 60°C and extension 30 s 72°C and final extension 2 min at 72°C. After that, PCR products were electrophoretically separated in electrophoresis on 2% agarose gel containing ethidium bromide (Et-Br) and visualized by UV-transilluminator (Vilber Lourmat). The density of PCR amplicons was quantified with ImageJ software (National Institutes of Health, USA) (http://imagej.nih.gov/ij/). Primers were designed specifically for genes of interest, including PER1, IRE1α, XBP-1s, PERK, ATF4, GADD34, ATF6, BiP/GRP78, CHOP and RPLP0 and verified the optimized working. The primer sequences are available upon request. RPLP0 was used for the housekeeping gene. Each cDNA sample was analyzed in triplicates for each PCR.

Immunoblotting Assay

Cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer (1xPBS, 1% nonidet P-40, 0.5% 7-DOC and 0.1% SDS, pH 8.0). After removing the insoluble phase by centrifugation at 14.000 rpm for 20 min at 4°C, total protein concentrations were determined by the bicinchoninic acid (BCA) (Thermo Scientific) assay. Typically, 25-40 μg of total cellular protein was used in immunoblotting studies. Protein samples were denatured in 4x Laemmli buffer at 70°C for 15 min and were separated on hand-cast polyacrylamide gels. Separated proteins were transferred to an Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-Tween) for 1 h at room temperature and then incubated with the primary antibody prepared in PBS–Tween containing 5% skim milk for 1-2 h at room temperature or 4°C overnight. Secondary antibodies were applied for 1 h at room temperature. Protein bands were monitored using an enhanced chemiluminescence (ECL) solution (Thermo Scientific) in the Fusion Pulse system (Vilber Lourmat). The protein bands were quantitated by Image StudioTM Lite (LI-COR®) (https://www.licor.com/bio/image-studio-lite/).

Curve Fitting Analysis

Curve fitting analysis was carried out as previously described [9]. The circadian oscillation was conducted using a non-linear curve fitting algorithm using GraphPad Prism and MATLAB software, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the three replicates. Genes showing an R2 correlation greater than 0.8 in non-linear curve fitting analyses were kept.

Statistical Analysis

The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test with a minimum of 95% confidence interval by GraphPad Prism 7 (GraphPad Software, La Jolla CA, USA, www.graphpad.com). The significant level was set at 5% and probability values of $p<0.05$ were considered statistically significant for all tests.

RESULT AND DISCUSSION

Verifying the Circadian Synchronization of HEK293 Cells

To determine the success of the circadian synchronization of HEK293 cells, we applied the serum shock protocol as previously described and then investigated the expression level of the PER1 gene depending on the change over time [9,21]. PER1 gene is a characteristic circadian oscillator and is also rhythmically transcribed under circadian rhythms [22]. Therefore, PER1 is generally used to determine circadian synchrony in mammalian cells. The success of serum shock-mediated circadian synchronization of HEK293 cells was verified by examining the oscillation of PER1 expression. Our findings demonstrated that PER1 exhibited rhythmic oscillation, which peaked at 8-16 h and 32-40 h (Figures 1A, 1B). These results confirmed the achievement of circadian synchronization of HEK293 cells.

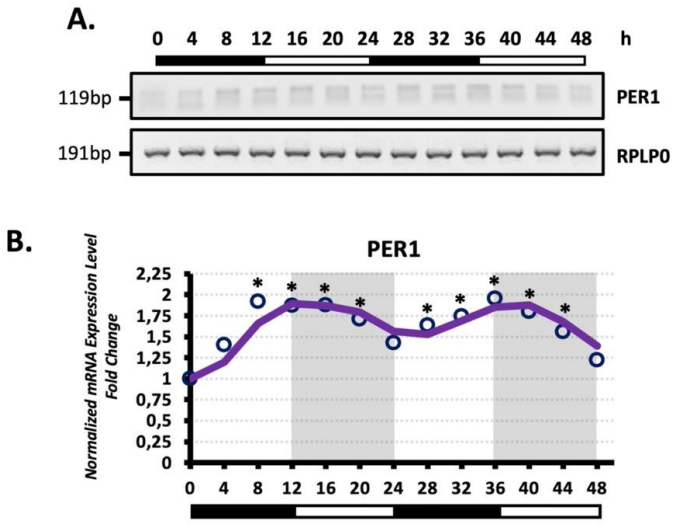

Figure 1. Verifying the circadian programming of HEK293 cells. **A,** Rhythmic mRNA expression levels of PER1 in the HEK293 cells across a 48 h circadian cycle. The total RNA was extracted from the HEK293 cells collected every 4 h during a 48 h circadian period and complementary DNA was synthesized and then target genes were amplified using specific primers. PCR amplicons were visualized on ethidium bromide-containing agarose gel. **B,** Relative gene expression quantities corresponding to three biological replicates were collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and the housekeeping gene RPLP0 was used as a loading control. Fold changes in mRNA expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled, via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the replicates. Genes showing an R^2 correlation greater than 0.8 in non-linear curve fitting analyses were kept. White-grey/black scale represents 4-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group. (*p<0.05) (n = 3)

Investigation of the Circadian Oscillation Pattern of UPR and ER Stress Genes

Expression levels of UPR signaling-related genes, including IRE1α, XBP-1s, PERK, ATF4, GADD34 and ATF6, were analyzed in serum shock-mediated synchronized HEK293 cells. Our results showed that all tested UPR genes tightly exhibited oscillation under the circadian rhythm similar to the PER1 expression pattern, which peaked at 8-16 h and 32-40 h and troughed at 24 h and 48 h (Figures 2A, 2B).

Next, we tested the ER stress-associated critical components, BiP/GRP78 and CHOP expression under the circadian rhythm in HEK293 cells. Consistent with the pattern of UPR genes, BiP/GRP78 and CHOP genes peaked at 8-16 h and 32-40 h and troughed at 24 h and 48 h (Figures. 3A, 3B).

Determination of the Protein Expression Levels of UPR Signaling-related Proteins under Circadian Oscillation

We investigated the protein expression level of some critical signal mediators of the UPR pathway, XBP-1s, a downstream effector of IREα signaling, and total-eIF2α, phospho(Ser51)-eIF2α for PERK signaling and also ER stress-related molecular chaperone BiP/GRP78 in serum shock mediated synchronized HEK293 cells by immunoblotting (Figures. 4A, 4B). Our results revealed that consistent with the mRNA data, XBP-1s, total-eIF2α, phospho (Ser51)-eIF2α and BiP/GRP78 proteins strongly exhibited robust circadian oscillation in synchronized HEK293 cells. The protein expression level of all tested downstream effectors of UPR and BiP/GRP78 peaked at 8-12 h and 34-32 h and troughed at 16-20 h and 36 h (Figures 4A, 4B).

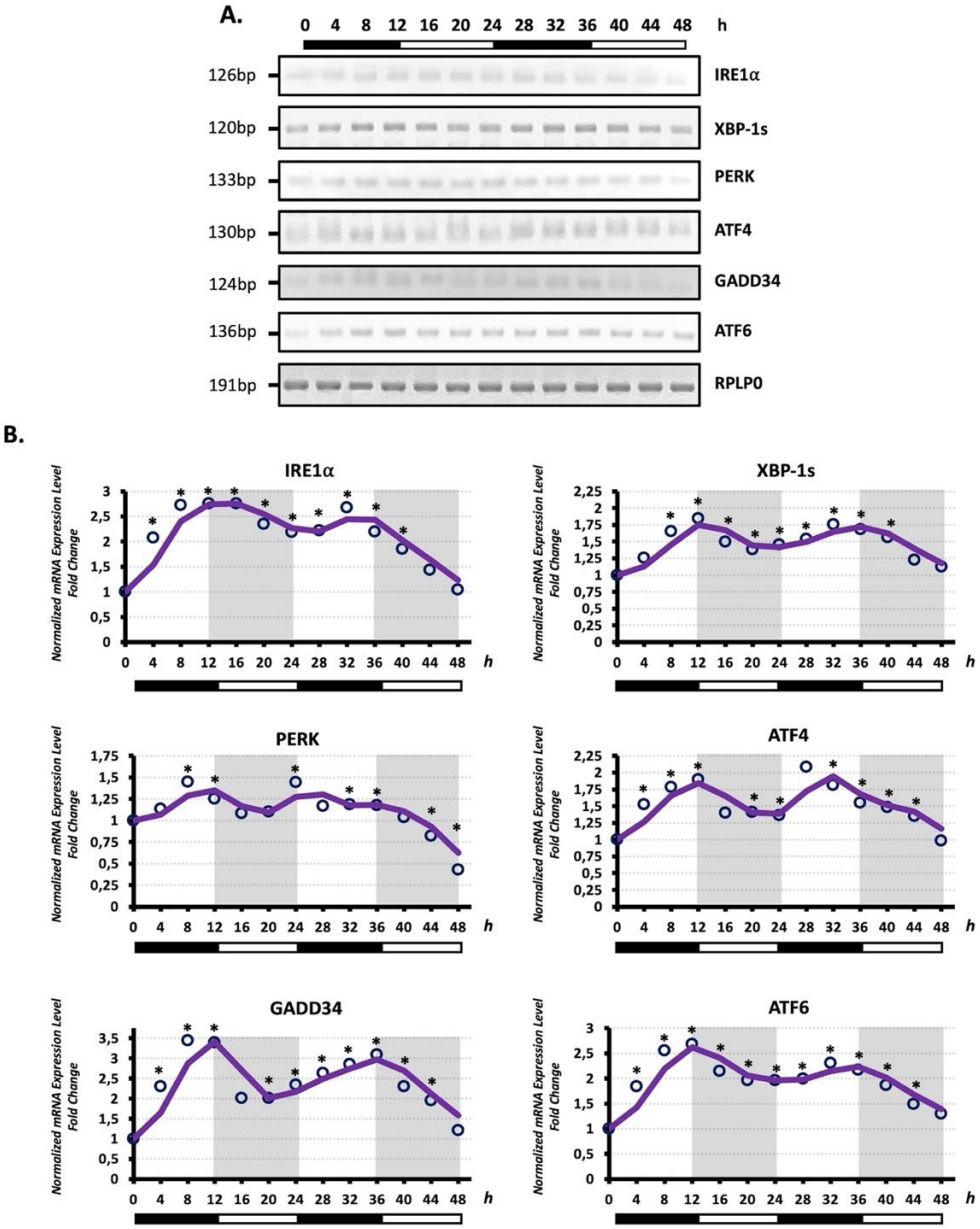

Figure 2. Evaluation of the circadian rhythmicity of the UPR components. **A,** Rhythmic mRNA expression levels of IRE1α, XBP-1s, PERK, ATF4, GADD34 and ATF6 in the HEK293 cells across a 48 h circadian cycle. The total RNA was extracted from the HEK293 cells collected every 4 h during a 48 h circadian period and complementary DNA was synthesized and then target genes were amplified using specific primers. PCR amplicons were visualized on ethidium bromide-containing agarose gel. **B,** Relative gene expression quantities corresponding to three biological replicates were collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and the housekeeping gene RPLP0 was used as a loading control. Fold changes in mRNA expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled, via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the replicates. Genes showing an R^2 correlation greater than 0.8 in non-linear curve fitting analyses were kept. White-grey/black scale represents 4-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group (*$p<0.05$) (n = 3)

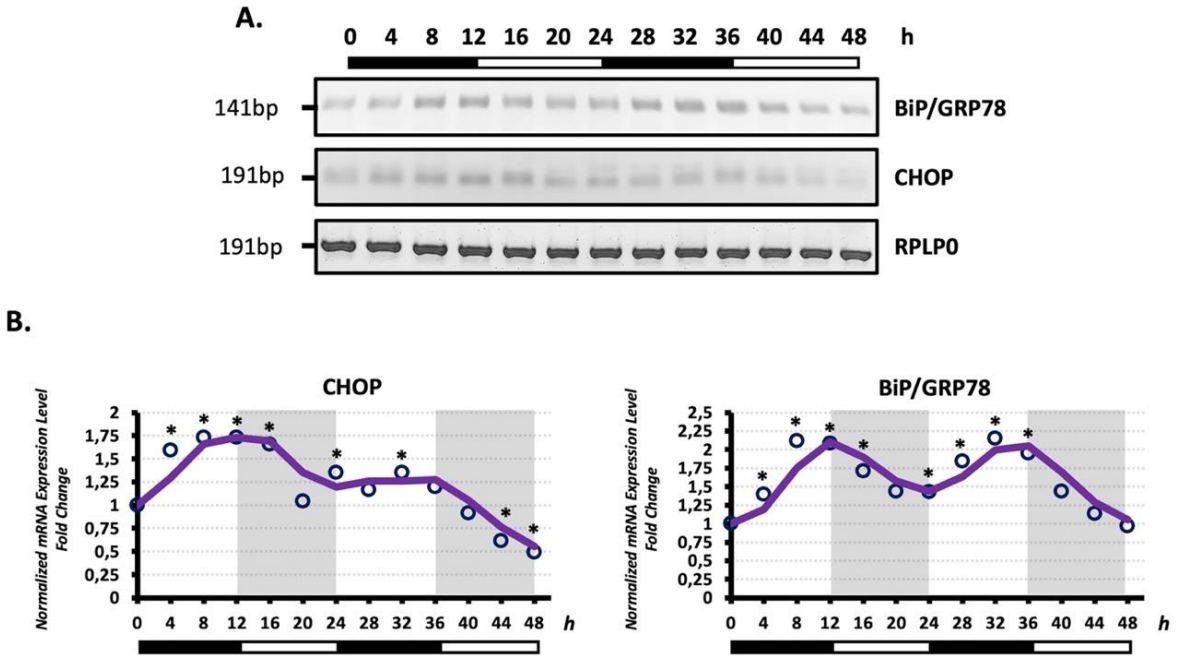

Figure 3. Evaluation of the circadian rhythmicity of BiP/GRP78 and CHOP**. A,** Rhythmic mRNA expression levels of BiP/GRP78 and CHOP in the HEK293 cells across a 48 h circadian cycle. The total RNA was extracted from the HEK293 cells collected every 4 h during a 48 h circadian period and complementary DNA was synthesized and then target genes were amplified using specific primers. PCR amplicons were visualized on ethidium bromide-containing agarose gel. **B,** Relative gene expression quantities corresponding to three biological replicates were collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and housekeeping gene RPLP0 was used as a loading control. Fold changes in mRNA expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled, via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the replicates. Genes showing an R^2 correlation greater than 0.8 in non-linear curve fitting analyses were kept. White-grey/black scale represents 4-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group. (*$p<0.05$) (n = 3)

The circadian system is a master regulatory system for nearly all physiological conditions and also consists of 24-hour. At the cellular level, molecular clocks originate circadian rhythms to promote coordination between internal time and the external world. This system mediates the regulation of the target genes by the circadian transcription factors, including CLOCK, BMAL1, PER1/2 and CRY1/2 [1]. The irregular circadian rhythmicity has been linked to increased susceptibility to digestive, immune, cardio-metabolic and neurological disorders, chronic conditions, such as diabetes, obesity, depression and bipolar disorder, as well as cancer [6,23-29]. Research studies have demonstrated that targeting the molecular and cellular mechanisms underlying circadian pathophysiology can be an alternative treatment way against diseases [6]. Therefore, understanding the circadian rhythmicity of physiologically important cellular mechanisms is very valuable as it has the potential to offer a new and promising treatment approach.

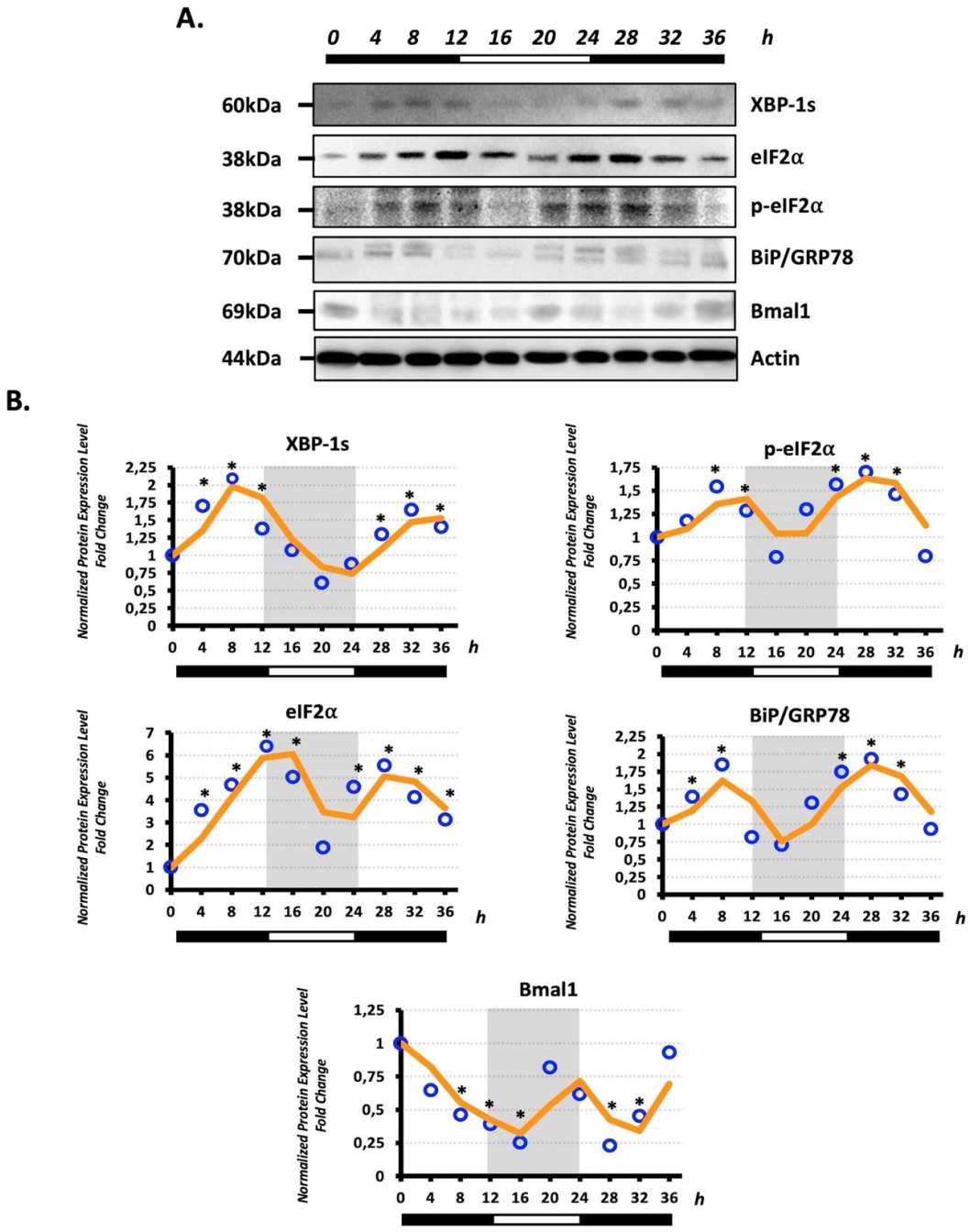

Figure 4. Expression level of UPR signaling-related proteins under circadian rhythmicity in HEK293 cells. Samples were collected every 4 h in a 36 h circadian period and then protein levels of XBP-1s, eIF2α, p-eIF2α, BiP/GRP78 and Bmal1 were analyzed by immunoblotting assay. Beta-actin was used as a loading control. **B.** Protein expression levels of XBP-1s, eIF2α, p-eIF2α, BiP/GRP78 and Bmal1 were quantified corresponding to three biological replicates collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and normalized using the beta-actin expression. Fold changes in protein expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data, including the replicates. The white/black scale represents 12-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group. (*$p<0.05$) (n = 3)

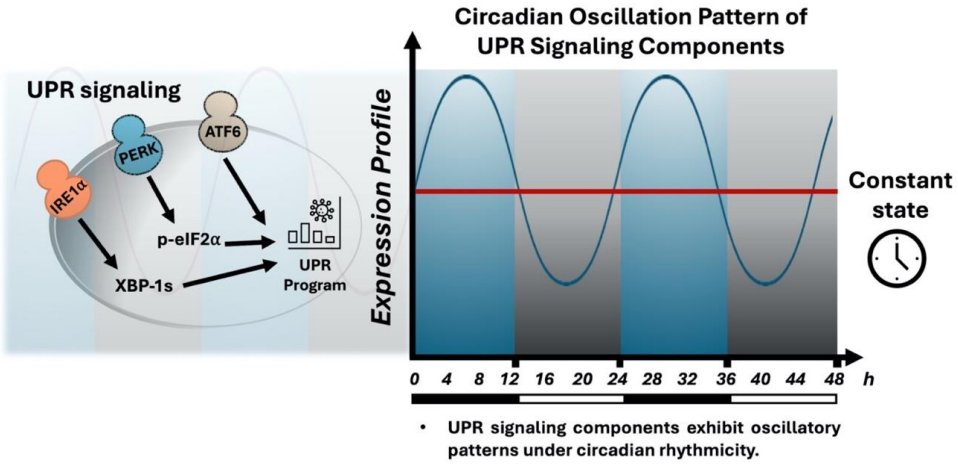

Figure 5. Representation of the expressional behavior of UPR signaling members under a circadian rhythm

Interest in the molecular biology of UPR signaling is exponentially growing based on the characterized roles of ER stress in numerous diverse pathologies, such as neurodegeneration, inflammation, diabetes, acquired drug resistance, invasiveness and metastasis capability-related roles in human cancer cells as well [30]. UPR activation can occur in various ways, such as elevated metabolic and oxidative stress, protein misfolding in the ER, impaired ER-associated degradation (ERAD) and altered ER Ca^{2+} stocks [31,32].

UPR signaling is coordinated by ER sensor transmembrane proteins, IRE1α, PERK and ATF6 and these proteins normally keep inactive form through interacting with BiP/GRP78. Upon ER stress, BiP/GRP78 is released from the sensor proteins. In this process, IRE1α and PERK proteins are activated by homodimerization and autophosphorylation and their downstream activators are warned. The activated PERK protein phosphorylates eIF2α at the serine 51 position, attenuating global translation in the cells. Phosphorylated eIF2α causes the selective increment in the translation of some of the group mRNAs, such as ATF4. GADD34, the phosphatase of phospho-eIF2α, negatively regulates the PERK signaling [19]. Additionally, PERK signaling controls the transcription of CHOP, a basic leucine zipper-containing transcription factor that promotes apoptosis in cells. Moreover, it increases the GADD34 expression [33,34]. IRE1α proteins function as a kinase and also as endoribonuclease enzyme. Activated IRE1α catalyzes the processing of the unspliced XBP1 (XBP-1u) to the spliced XBP1 (XBP1s). XBP-1s function as a transcription factor responsible for the transcription of UPR target genes [31,35]. Also, IRE1α can regulate of the subset of mRNAs and miRNAs through its IRE1α-dependent decay (RIDD) [35]. ATF6 activation allows the transition to the cis-Golgi compartment, which is cleaved by site-1 protease (S1P) and site-2 protease (S2P). The cleaved cytosolic N-terminal fragment translocates to the nucleus and coordinates the specialized transcriptional program of several genes, including ER chaperones and protein-folding enzymes [15,36]. Therefore, UPR signaling can lead to the adaptation or elimination of eukaryotic cells through reprogramming the cellular mechanism in response to cellular stresses and altered physiological conditions.

Herein, we clarified the circadian oscillation pattern of UPR signaling components in HEK293 cells at that transcriptional and translational levels. Our findings indicated that UPR components, including IRE1α, XBP-1s, PERK, ATF4, GADD34, and ATF6, robustly exhibit oscillation patterns under a circadian rhythm on a 48-hour time scale; likewise, the PER1 gene is the master component of the circadian rhythm (Figure 1a, b, 2a, b). Besides that, ER stress genes, BiP/GRP78 and CHOP were also manners similar to UPR components under the circadian rhythm (Figure 3a, b). To determine the relationship between UPR activation and circadian rhythmicity, we tested the downstream effectors of IRE1α and PERK branches of UPR signaling on a 36-hour time scale by immunoblotting. Similar to the oscillation pattern of mRNA expression of UPR components, protein levels of downstream effectors

of UPR signaling, XBP-1s, eIF2α, phospho(Ser51)-eIF2α and BiP/GRP78 peaked at 8-12 h and 28-32 h and troughed at 16-20 h and 36 h (Figure 4a, b). On the other hand, transcriptional activator circadian clock protein Bmal1 peaked at 20-24 h and 36 h and troughed at 12-16 and 28-32 h (Figure 4a, b). These results showed that UPR components and ER stress protein BiP/GRP78 exhibit oscillatory patterns not only at the transcriptional level but also consistently at the translational level.

Studies have demonstrated that ER stress is regulated in a circadian manner as other physiological processes like the daily oscillation of metabolism and hormonal rhythm [37]. Today, the enhancing effect of the UPR signal on the levels of ERAD target genes is well understood, which is one of the significant protein quality control mechanisms in eukaryotic cells. It rigorously degrades the misfolded proteins and also selectively regulates the steady-state level of physiologically essential protein levels [38,39]. Recent research has suggested the regulatory links between certain components of the ER protein quality control mechanism and the circadian rhythm. Guo et al., (2020) showed that circadian control of Bmal1 through ubiquitination is facilitated by E3 ubiquitin ligase Hrd1, a key member of the ERAD pathway [40]. Kim et al. (2021) revealed the regulation of hepatic lipid and glucose homeostasis by the E3 ubiquitin ligase HRD1/Sel1L-controlled CREBH/PPARα transcriptional program in vitro and in vivo models [41]. Furthermore, Erzurumlu et al., (2023) reported that the mRNA and protein expression levels of ERQC components are extensively regulated under circadian rhythmicity. Moreover, the main E3 ligase enzymes associated with ERAD, Hrd1, and gp78, were suggested to regulate circadian oscillation through regulation of the stability of Bmal1, which is a master transcription factor known to regulate circadian rhythm [9]. 12 h period rhythmic activation of the IRE1α branch of UPR has been associated with the regulation of lipid metabolism in mouse liver [42]. Additionally, activation of UPR in cancer cells has been shown to lead to a 10-hour shift in circadian oscillation [43]. All these studies suggest that ER-mediated coordinated signaling mechanisms, which have physiological importance, are periodically regulated by circadian rhythmicity. Thus, a detailed characterization of the oscillation pattern of these mechanisms is essential for understanding the human pathologies associated with the circadian rhythm.

Our study revealed the detailed oscillation pattern of UPR components and also indicated the expression of UPR members is regulated at the transcriptional and translational levels in human embryonic kidney HEK293 cells with robust rhythmicity (Figure 5). Furthermore, IRE1α and PERK signaling activation also exhibited regular rhythmicity with circadian oscillation. In light of all this literature information, we hope that the present study will provide essential oscillatory map data for understanding the relationship between UPR and circadian rhythm in the molecular pathophysiology of many diseases, including cancer.

UPR is one of the most essential evolutionary conserved signaling mechanisms in mammalian cells. Today, we know that UPR, in addition to being a mechanism for maintaining cellular homeostasis and an adaptation mechanism against cellular stresses in healthy cells, is also a vital mechanism for cancer cells and is one of the essential mechanisms that limit the success of cancer treatments, such as the development of drug resistance. Moreover, the impairment in the UPR signaling has been associated with numerous pathophysiological diseases. The present *in vitro* study revealed the robust oscillation patterns of UPR signaling components under a circadian rhythm in healthy embryonic kidney cells. We hope that our research will guide experimental treatment protocols that will be designed according to the circadian rhythm map of the UPR in UPR-focused pharmacological interventions.

ACKNOWLEDGEMENTS

We thank Dr. Signem Oney Birol (Department of Molecular Biology and Genetics, Faculty of Science, Mehmet Akif Ersoy University) for allowing us to access the use of the chemiluminescence monitoring system. This study was partially supported by Suleyman Demirel University internal funds (TSG-2021-8302, TAB-2020-8253).

AUTHOR CONTRIBUTIONS

Concept: Y.E.; Design: Y.E.; Control: Y.E.; Sources: Y.E.; Materials: Y.E.; Data Collection and/or Processing: Y.E., D.C., H.K.D.; Analysis and/or Interpretation: Y.E.; Literature Review: Y.E., D.C., H.K.D.; Manuscript Writing: Y.E.; Critical Review: Y.E., D.C., H.K.D.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 962-974, 2024
Doi: 10.33483/jfpau.1464923

ORIGINAL ARTICLE / ÖZGÜN MAKALE

QUALITY BY DESIGN ASSISTED RP-HPLC METHOD FOR ESTIMATION OF TERIFLUNOMIDE AND ITS PROCESS IMPURITIES IN DRUG SUBSTANCE

TERİFLUNOMİD VE İLAÇ MADDESİNİN ÜRETİMİNDEKİ SAFSIZLIKLARININ TAHMİNİ İÇİN TASARIMLA KALİTE DESTEKLİ RP-HPLC YÖNTEMİ

Vasudha DADI[1], Saritha MEDAPATI[2], Jagadeesh Kumar BARATAM[2], Hemant Kumar TATAPUDI[4], Gangu Naidu CHALLA[3], Srinivasa Rao YARRAGUNTLA[1], Bhagavan Rajesh Babu KOPPISETTY[1]*

[1]Department of Pharmaceutical Analysis, Vignan Institute of Pharmaceutical Technology, Visakhapatnam, Andhra Pradesh, 530046, India
[2]Department of Pharmacy Practice, Vignan Institute of Pharmaceutical Technology, Visakhapatnam, Andhra Pradesh, 530046, India
[3]Department of Basic Sciences and Humanities (BS&H), Division of Chemistry, Vignan's Institute of Information Technology VIIT(A), Visakhapatnam, Andhra Pradesh, 530046, India
[4]Department of Pharmaceutical Technology, NSHM Knowledge campus, Kolkata, West Bengal, 700053, India

ABSTRACT

Objective: *In this research, a validated RP-HPLC method for analyzing teriflunomide drug substance and its associated process-related impurities was developed with the assistance of the Quality by Design (QbD) approach.*
Material and Method: *The QbD methodology employs statistical design of experiments to establish a robust method within a defined "design space." This design space outlines the experimental parameters' range within which alterations will not significantly impact the results. Chromatographic separation was done on HPLC system connected to a PDA detector, and the column used was the C18 YmC-Triart with specifications of 125 cm x 4.0 mm x 5.0 µm. The optimized mobile phase consisted of 0.5% triethylamine buffer with pH 4.0 (± 0.05) and acetonitrile in a 65:35 v/v ratio, flow rate of 1.0 ml/min. Detection wavelength of 210 nm.*
Result and Discussion: *The developed RP-HPLC method successfully achieved high resolution, specificity, linearity, precision, accuracy, and robustness in quantifying both teriflunomide and its impurities simultaneously. Using a design of experiments (DoE) approach, critical method parameters were systematically identified and optimized, ensuring accurate and precise determination of impurity levels across the drug substance lifecycle. This validated method provides a thorough approach to ensuring the quality and safety of teriflunomide drug substances by delivering reliable data on impurity profiles. By applying Quality by Design (QbD) principles, not only does the method enhance understanding of the analytical process, but it also supports ongoing improvement and lifecycle management of the procedure.*
Keywords: *Method development, QbD, related impurities, RP-HPLC, teriflunomide*

* **Corresponding Author / Sorumlu Yazar:** Bhagavan Rajesh Babu Koppisetty
e-mail / e-posta: koppisettybrbabu@gmail.com, **Phone / Tel.:** +919490597959

Submitted / Gönderilme : 06.04.2024
Accepted / Kabul : 09.07.2024
Published / Yayınlanma : 10.09.2024

ÖZ

Amaç: *Bu araştırmada, teriflunomid ilaç maddesini ve onun süreçi ile ilişkili safsızlıkları Tasarımla Kalite (QbD) yaklaşımı yardımıyla analiz etmek için doğrulanmış bir RP-HPLC yöntemi geliştirildi.*
Gereç ve Yöntem: *QbD metodolojisi, tanımlanmış bir "tasarım alanı" dahilinde sağlam bir yöntem oluşturmak için deneylerin istatistiksel tasarımını kullanır. Bu tasarım alanı, değişikliklerin sonuçları önemli ölçüde etkilemeyeceği deneysel parametre aralığının ana hatlarını çizer. Kromatografik ayırma, bir PDA detektörüne bağlı bir HPLC sistemi üzerinde gerçekleştirildi ve kullanılan kolon, 125 cm x 4.0 mm x 5.0 µm spesifikasyonlarına sahip C18 YmC-Triart'tı. Optimize edilmiş mobil faz, pH 4.0 (± 0.05) olan %0.5 trietilamin tamponundan ve 65:35 v/v oranında, 1.0 ml/dak akış hızında asetonitrilden oluşuyordu. 210 nm algılama dalga boyu.*
Sonuç ve Tartışma: *Geliştirilen RP-HPLC yöntemi, hem teriflunomidin hem de onun safsızlıklarının aynı anda ölçülmesinde yüksek çözünürlük, özgüllük, doğrusallık, kesinlik, doğruluk ve sağlamlığı başarıyla elde etti. Bir deney tasarımı (DoE) yaklaşımı kullanılarak, kritik yöntem parametreleri sistematik olarak tanımlandı ve optimize edildi; böylece ilaç maddesi yaşam döngüsü boyunca safsızlık seviyelerinin doğru ve kesin olarak belirlenmesi sağlandı. Doğrulanmış bu yöntem, safsızlık profilleri hakkında güvenilir veriler sunarak teriflunomid ilaç maddelerinin kalitesini ve güvenliğini sağlamaya yönelik kapsamlı bir yaklaşım sağlar. Tasarımla Kalite (QbD) ilkelerini uygulayarak, yöntem yalnızca analitik sürecin anlaşılmasını geliştirmekle kalmaz, aynı zamanda prosedürün sürekli iyileştirilmesini ve yaşam döngüsü yönetimini de destekler.*

Anahtar Kelimeler: *İlgili safsızlıklar, QbD, RP-HPLC, teriflunomid, yöntem geliştirme*

INTRODUCTION

Teriflunomide (TFN), the primary active metabolite of leflunomide, is a drug commonly employed for the management of rheumatoid arthritis. Its main mechanism involves the inhibition of mitochondria's dihydroorotate dehydrogenase, an important enzyme in the *de novo* synthesis of pyrimidines. This inhibition results in the suppression of activated T type cells and B type cells and reduces the movement of lymphocytes to the central nervous system. TFN is also believed to have other immunological effects, including the suppression of cyclooxygenase-2 and protein tyrosine kinases. TFN belongs to a immunomodulatory drugs that function by impeding pyrimidine formation. It has proven efficient in the treatment of multiple sclerosis and rheumatoid arthritis. Physically, TFN is a white, tasteless, and odorless substance that does not readily absorb moisture. Its chemical name is "(*Z*)-2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl)phenyl]but-2-enamide", and it has a molecular weight of 270.2 g/mol with a chemical formula of $C_{12}H_9N_2O_2F_3$.

By literature review we found that a validated LC-MS method for quantification of Teriflunomide and its metabolite in in-vivo conditions [1]. A validated HPLC technique was established for the quantification of methotrexate and TFN in formulations [2]. A validated LC-MS method was developed for estimation of TFN and other compounds [3]. A validated LC-MS/MS method was developed for estimation of TFN in human biological fluids [4-5]. Koppisetty et al., 2023 has developed a HPLC method for quantitative separation of TFN and its process impurities in drug substance [6]. Nukendra et al., 2017 had developed QbD based validated UPLC method, which reported forced degradation products and those degradation products were characterized using standards [7]. Suneetha et al., [8] has worked on separation of TFN with a single unknown process related impurity, the work of suneetha et. al is not suitable for determination of all process related impurities of TFN. Literature review shows that there is no work on a validated RP-HPLC method for impurity profiling in TFN.

The primary purpose of this study is to systematically investigate and develop a reliable RP-HPLC method for quantifying TFN in the presence of its distinct production related impurities. In addition, the research outlines the synthetic pathway of these impurities, as depicted in Figure 1. This study adopts an analytical approach based on Quality by Design (QbD) principles [9-11] to accurately determine TFN in the presence of its production related impurities. Furthermore, the analytical method was subjected to validation in accordance with the ICH guidelines [12,13] to ensure its accuracy and reliability.

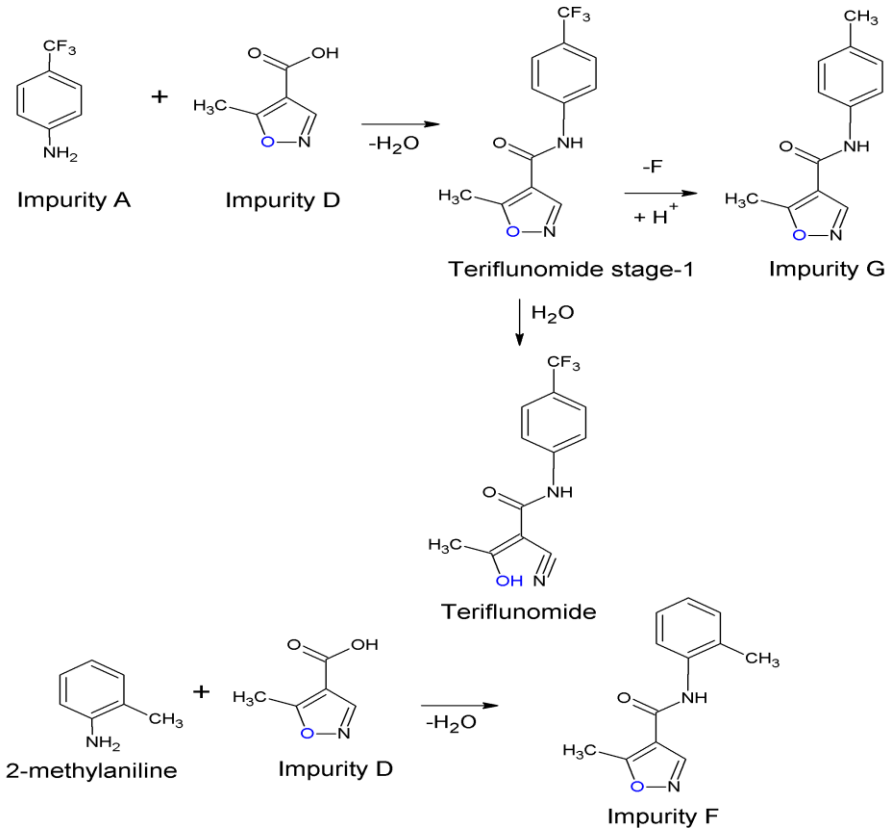

Figure 1. The novel synthetic pathway of TFN and its related impurities

MATERIAL AND METHOD

Chemicals and Reagents

The working standard Terflunomide and its related impurity standards, Impurity-D "(5-methylisoxazole-4-carboxylic acid)",Impurity-F"(5-methyl-*N*-[2-(trifluoromethyl) phenyl]isoxazole-4-carboxamide)", Impurity-G "(5-methy-*N*-(4-methylphenyl) isoxazole-4carboxamide)", Impurity-A "(4-(trifluoromethyl)aniline)" were received as a gift from synpure labs India pvt. Ltd. High-quality analytical grade reagents, including Triethylamine, Acetonitrile (ACN), Orthophosphoric acid (OPA), HCL, NAOH, and H_2O_2 were procured from Merck. Milli-Q grade water was utilized for the analysis.

Analytical Quality by Design

Analytical Target Profile (ATP) and Critical Analytical Attribute (CAA)

Establishing the definition of ATP represents the initial stage in adopting a Quality by Design (QbD) approach. This entails the careful selection of elements, including Critical Method Parameters (CMP) and Critical Analytical Attributes (CAA), to attain the desired ATP. Variations in CMPs can influence CAAs, and as such, they should be closely examined and managed to ensure the intended quality.

Every CMP was categorically assigned as either high or low risk levels, taking into consideration the likelihood of risk occurrence and the seriousness of potential impacts on the CAAs. Potential CAAs were identified among numerous quality attributes using relevant information and from available literature. These attributes, directly impact the analytical method's quality and safety. In this particular study, specific factors such as Relative Standard Deviation (RSD), assay on a dried basis, and tailing factor were designated as CAAs.

Risk Assessment (RA)

The ICH Q8 and Q9 guideline outline a risk-based strategy for defining the Quality Target Product Profile (QTPP), which is equivalent to the ATP. The guidelines place strong emphasis on ensuring that analytical methods are rugged and robust, with a specific focus on RA studies. In the context of method development, RA was conducted to pinpoint the factors with higher risk levels that could impact the process. For this RA study, a Pareto analysis was employed. A Pareto diagram was created to identify the CMPs that have a significant impact on the CAAs) when quantifying TFN.

Design of Experiment (DoE)

A critical phase in the process involved the identification of the CMPs that have an impact on the CAAs, such as RSD, assay on a dried basis, and tailing factor. To achieve improved values for RSD, assay on a dried basis, and tailing factor, a Design of Experiments (DoE) approach was employed. This approach involved varying the flow rate, adjusting the pH of the buffer, and modifying the buffer concentration [9]. Following the initial preliminary studies, a 2^3 total factorial design was employed to optimize the method. A total of eight experiments were conducted to investigate the impact of three experimental variables: pH (X1), flow rate (X2), and buffer concentration (X3) on the CAAs, specifically RSD (Y1), assay on a dried basis (Y2), and tailing factor (Y3), at three different levels. The data generated from these experimental runs were analyzed using Design-ExpertR software (version 11, Stat-Ease, Minneapolis, USA).

Statistical Analysis and Optimization

Statistical analysis and the method optimization were conducted using Design-ExpertR software, specifically version 11 from M/s Stat-Ease in Minneapolis, USA. The results from the eight experimental trials conducted as part of the 2^3 total factorial design underwent Multiple Linear Regression Analysis. ANOVA was carried out, and any observed variations were considered statistically significant if the p-value was below 0.05.

Experimental

Instrumentation

The HPLC Waters Alliance 2695 system was utilized for the analysis, coupled with a PDA model 2998, and the empower chromatographic software. Several other pieces of equipment were utilized for the analysis, including an analytical balance from Shimadzu in Japan, a pH meter (Elico, model LI-120), an oven from Thermolab, and a hot air oven from Heraeus.

Chromatographic Conditions

The RP-HPLC method was created and subjected to validation using a Waters HPLC system in conjunction with a YMC-triart column measuring 125cm x 4.0 mm x 5.0 µm. The method was configured with the following parameters: 1.0 ml/min flow rate, a detection wavelength of 210 nm, analysis at ambient temperature, a 10µl injection volume, and a total run time of 20 minutes. The mobile phase consisted of 0.5% triethylamine buffer with pH adjusted to 4.0 (± 0.05) using diluted OPA. Isocratic elution was employed with the mobile phase composed of a 65:35 v/v ratio of buffer and ACN. Notably, the dilution of both standard and sample solutions was carried out using the mobile phase.

Standard Solution

Approximately 50.0 mg of TFN standard was carefully weighed and transferred into a 100 ml flask. It was then dissolved in approximately 15 ml of ACN and subsequently brought to the mark with diluent to achieve the desired volume. To obtain a standard solution of 50 µg/ml, 10.0 ml of the previously prepared solution was further diluted to 100 ml using the diluent.

Sample Solution

Approximately 50.0 mg of the TFN drug substance was precisely weighed and then transferred into a 100 ml flask. It was dissolved using around 15 ml of ACN and then brought up to the mark by

adding the diluent to reach the desired volume. Subsequently, a 10.0 ml aliquot of the API sample solution was further diluted to 100 ml with the diluent, resulting in a 50 µg/ml API sample solution. This process of preparing the solution of drug substance was carried out in duplicate for subsequent analysis.

System Suitability Solution

To assess the HPLC system's performance, six consecutive injections were made, starting with a blank injection, followed by standard solution injections. The system is considered acceptable for analysis under the following conditions: a) theoretical plate count of at least 2000. b) The tailing factor should be between 0.8 and 1.5. c) The RSD% for the TFN peak area for six replicate injections should be below 2%.

Validation of Method

Specificity

To validate the analytical method's selectivity for determining the assay content in TFN, the following steps were taken: a) We created distinct sample solutions for TFN and its associated impurities (Impurity-D, Impurity-F, Impurity-G, and Impurity-A) in a suitable diluent. These solutions were then prepared and analyzed. b) Additionally, we analyzed duplicate spiked sample solutions, where each specified impurity was added to TFN sample solutions to demonstrate the method's selectivity.

Precision

System precision was assessed by injecting a standard TFN solution at a concentration of 50 µg/ml, conducting six determinations to ensure the system's suitability. Method precision was determined by analyzing a standard TFN solution at a concentration of 50 µg/ml through six replicate determinations to measure assay accuracy. Intermediate precision was evaluated by examining the variability in results obtained for TFN. This was accomplished by analyzing six replicate sample preparations, all prepared at the working concentration, with different analysts using different instruments on separate days.

Linearity

To establish the method's linearity, TFN solutions spanning the range of 35 µg/ml to 60 µg/ml, corresponding to 70.0% to 120.0% of the TFN working concentration, were employed. Sample solutions of TFN within this specified range were prepared and subjected to duplicate analysis in accordance with the method.

Accuracy

To assess accuracy, samples were prepared at three different concentration levels, and each level's solutions were replicated three times, resulting in a total of nine determinations. These concentrations included 80.0%, 100.0%, and 120.0% of the working concentration.

Robustness

To evaluate the method's robustness in determining the assay, we conducted an analysis of the TFN standard solution while intentionally modifying parameters such as pH, flow rate, and buffer strength. The changes made to the method were used to derive system suitability results, demonstrating the method's robustness.

Solution Stability

Samples of the TFN solution and standard solution, both at 100.0% of the working concentration, were maintained at room temperature (between 22-27°C). The solutions' stability was assessed at three different time points: initially, after 24 hours, and after 48 hours.

RESULT AND DISCUSSION

Quality by Design Paradigm

The QbD paradigm is a comprehensive and technical approach that employs various statistical, economic, and planning tools, as well as risk assessment techniques, to establish a design space, control strategy, and continuous improvement in various industries. QbD is widely used to enhance method robustness, bridge quality gaps, and reduce instances of failure. In this study, an AQbD approach was applied to plan and execute the development of an analytical method for estimating teriflunomide in the presence of process-related impurities. In the QbD framework, the establishment and execution of an ATP were outlined as the primary phase in the development of the analytical method to attain the specified objectives. The ATP provides a systematic framework for the development of a method, guiding the process. It aided in selecting CAAs necessary for quantifying TFN. In order to reduce inaccuracies and pinpoint potential high-risk factors impacting the proposed quantification of TFN in CAAs, a comprehensive risk assessment study was undertaken. A Pareto chart was formulated specifically for this analysis. The Pareto chart is a valuable tool for assessing the significant factors, where effects surpassing the Bonferroni Limit are highly likely to be significant, those exceeding the t-value limit are potentially significant, and effects below the t-value limit are unlikely to be significant. The Pareto chart indicates that each of the chosen factors has significant effects on the selected responses, with their importance ranked in a descending order.: For response 1, X2 > X3 >X2X3>X1X3 (Figure 2). For response 2, X3 > X1>X1X2 (Figure 3). For response 3 only X3 has significant effect as shown in Figure 4. The model's validity was confirmed through the ANOVA using Design Expert software. The equation, enables predictions to be made regarding the response at particular levels of each factor. The equation functions as a useful tool to evaluate the relative influence of the factors by analyzing and comparing their coefficients. If the model's p-value is greater than 0.05, it indicates that the factors have an insignificant effect on the response, signifying the method's robustness. The low standard deviation (expressed as the percentage coefficient of variance, CV) and the satisfactory precision demonstrate a strong correlation between the experimental data and the fitted models. Synergism and antagonism effects are indicated by the positive and negative signs in the equation, respectively. The desirability value, which is closest to 1 as shown in Figure 5, conveys the dependability of the optimization approach. These results revealed that the actual values of the variables align well with the predicted values of the method.

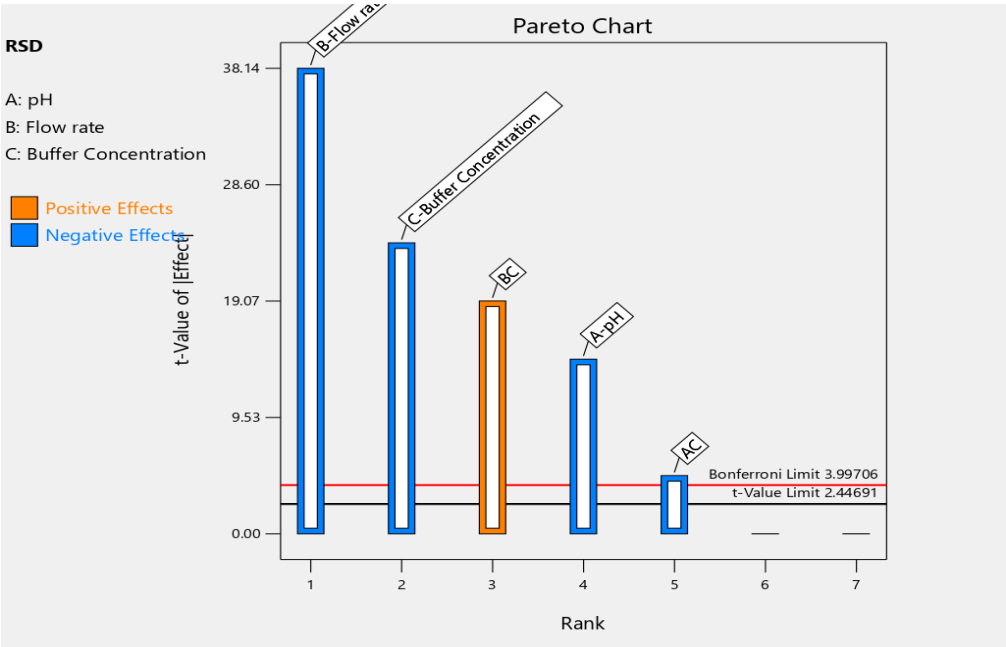

Figure 2. Effect of factors and interaction on responses 1:RSD

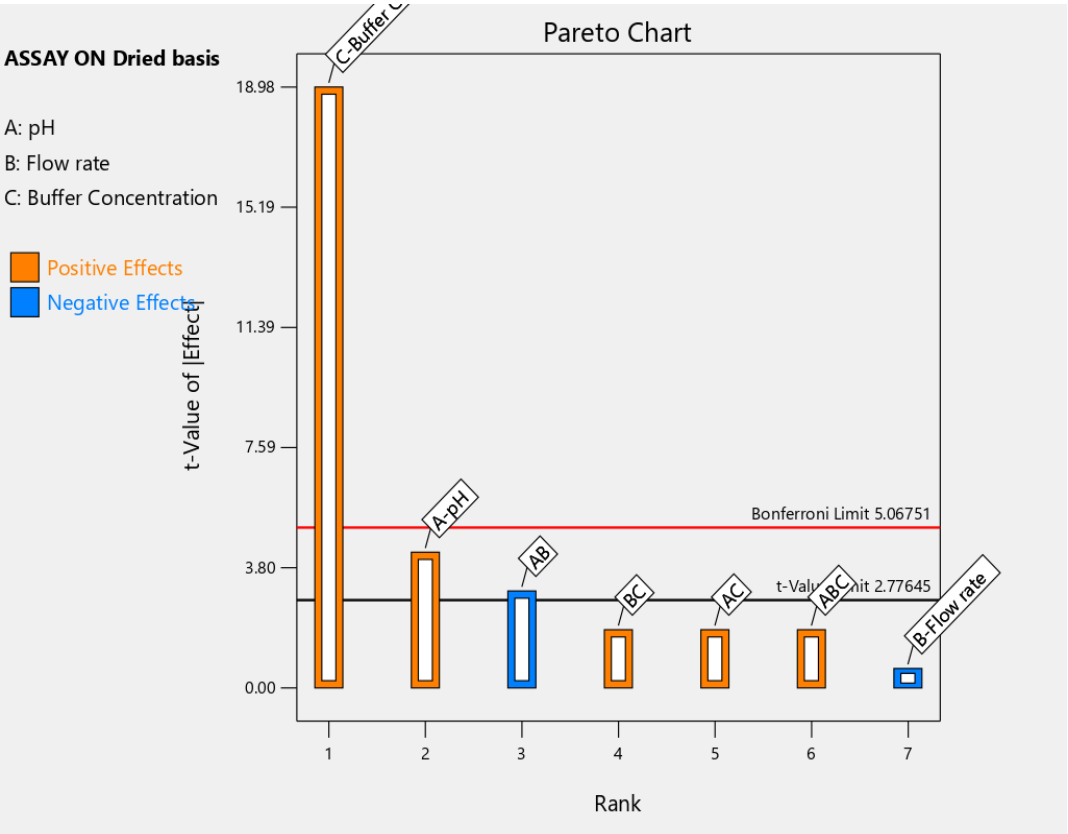

Figure 3. Effect of factors and interaction on response 2: Assay

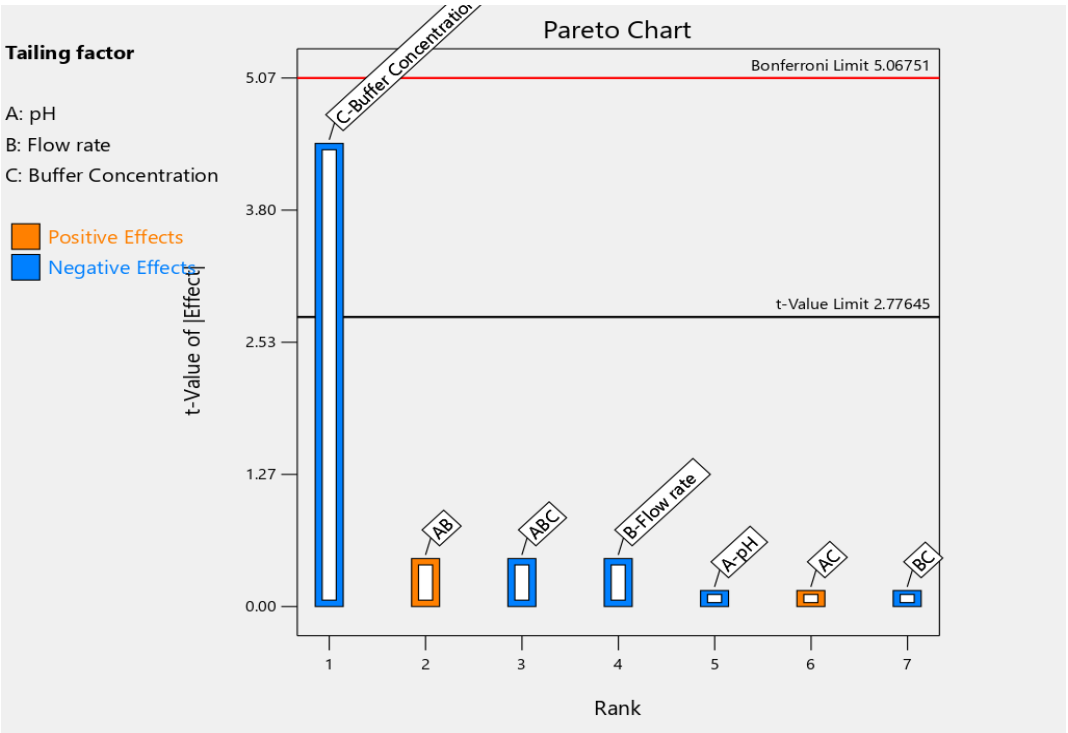

Figure 4. Effect of factors and interaction on responses 3: Tailing factor

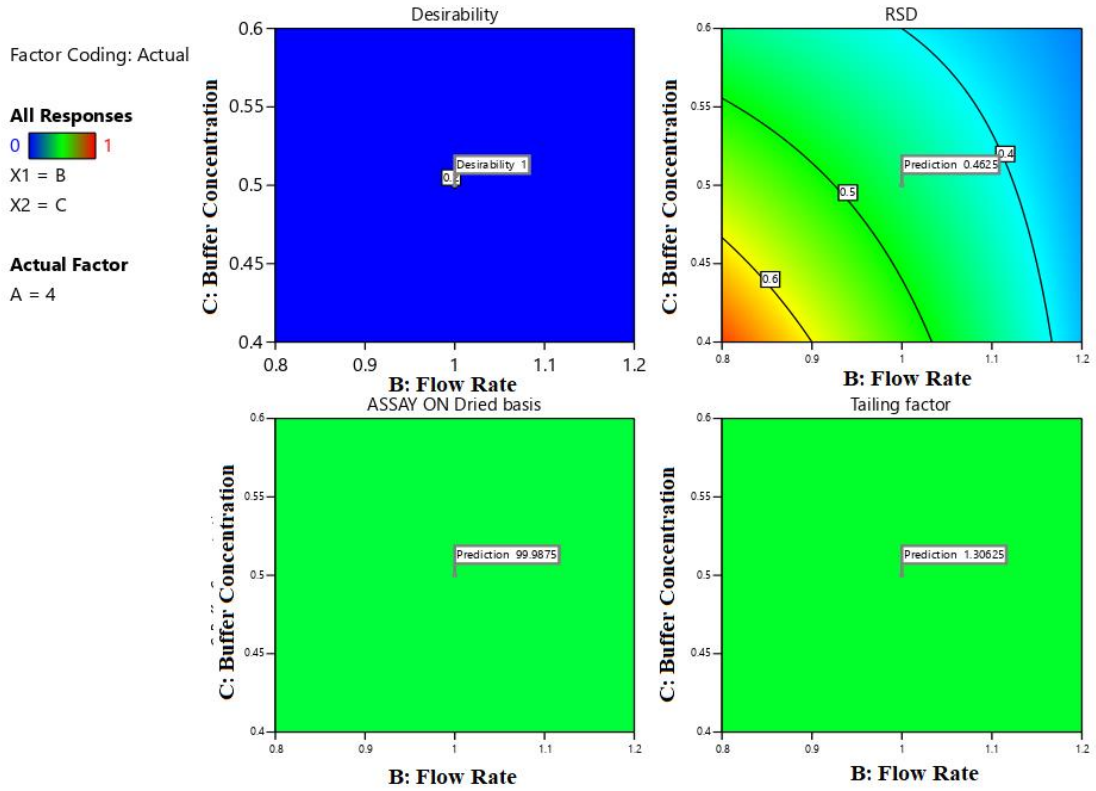

Figure 5. Desirability plot

Development of Analytical Method

A method for estimating the concentration of TFN and its impurities was developed on RP-HPLC. The separation of TFN from its impurities was achieved on a YMC-Triart Hybrid silica-based C18 column with specific dimensions: 125 cm length, 4.0 mm i.d, and 5.0 µm particle size. To separate the compounds, a mobile phase consisting of a 65:35 v/v mixture of buffer and ACN was used. The buffer contained 0.5% Triethylamine and had its pH adjusted to 4.0 (± 0.05) using diluted orthophosphoric acid. Isocratic elution was employed with a 1.0 ml/min flow rate at ambient conditions. Detection of the compounds was performed at a wavelength of 210 nm. A 10 µl sample was injected for analysis, and the total runtime for the procedure was 20 minutes.

Method Validation

System suitability

A standard solution with a concentration of 50 µg/ml was prepared and subjected to the proposed method to evaluate system suitability during tests for specificity. In the first injection of the standard solution, it was observed that the theoretical plates count exceeded 2000 for the parameter being tested. The tailing factor for the TFN peak fell within the range of 1.2 to 1.3 in the first injection of the standard solution for the parameter under examination. The RSD% for the TFN peak area, determined from six replicate injections of the standard solution conducted throughout the run, was found to be 0.62% for the system suitability. The results are tabulated in (Table 1).

Table 1. System suitability parameters

Peak	Retention time (R_T)	Theoritical Plates (N)	HEPT	Tailing Factor	RSD% (n=6)
TFN	5.61	5950	40.728	1.24	0.62

Specificity

There are no interfering peaks in the blank sample at R_T of the analyte. In the standard solution, the TFN peak is uniform, and there are no peaks overlapping with it. This investigation demonstrates that the TFN peak is effectively separated from any known specified impurities. (Figure 6). No interference is observed in the blank at the R_T of the TFN peak. (Table 2). The method was employed on both bulk and spiked samples. The variance in assay values between the sample solution and the spiked sample is -0.3% (Table 3). Hence, the method exhibits selectivity in accurately determining the assay of the TFN drug substance.

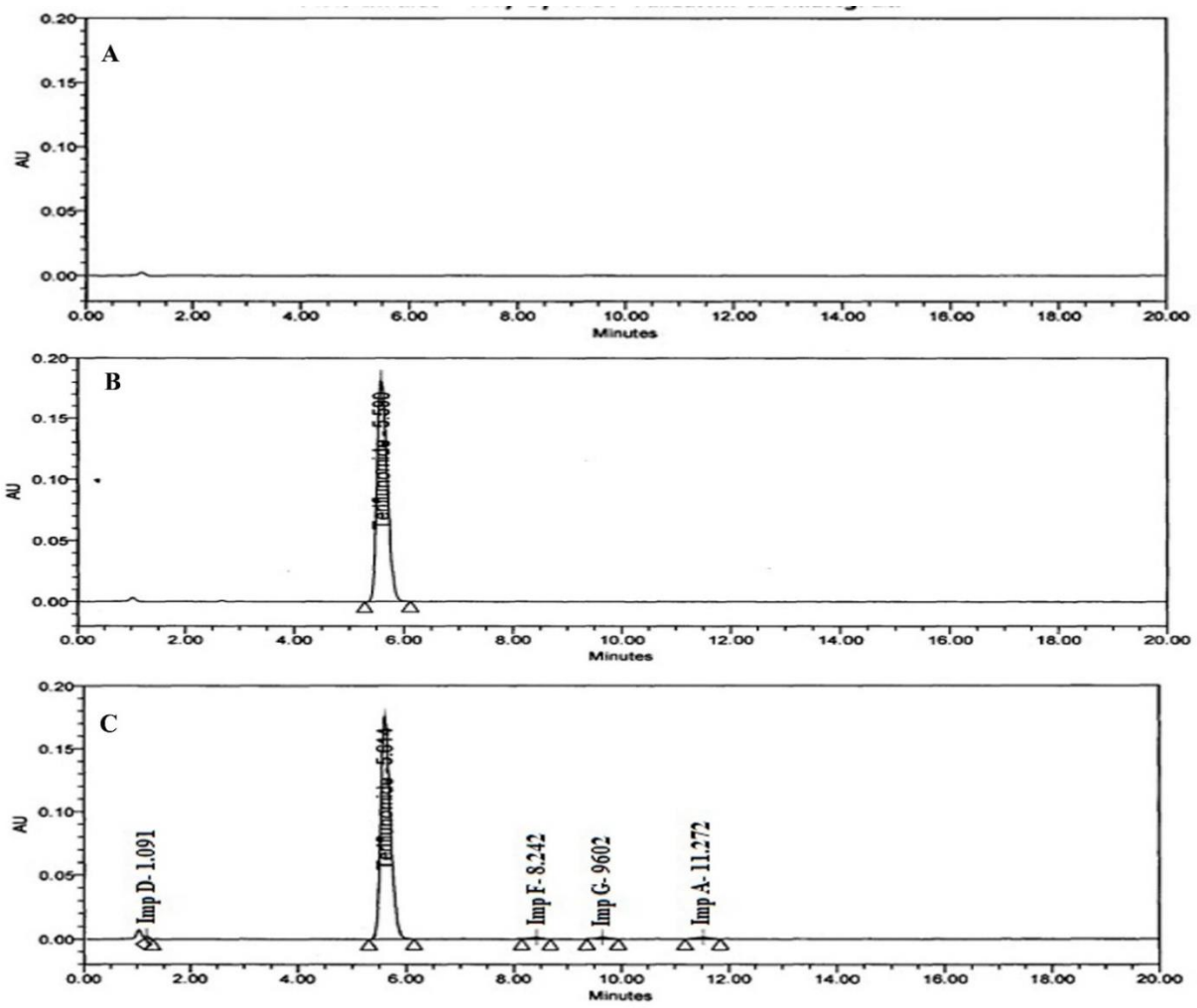

Figure 6. Chromatograms of A) Blank B) Standard solution C) Spiked solution

Table 2. Summary of R_T, R_T ratio for TFN and specified impurities

Peak name	R_T (min.)	R_T ratio
Impurity-D	1.09	0.19
TFN	5.60	1.00
Impurity-F	8.24	1.47
Impurity-G	9.60	1.71
Impurity-A	11.27	2.01

Table 3. Assay values (w/w%) for TFN sample and spiked sample

S.No.	Preparations	% Average Assay of TFN(w/w%)on driedbasis	Difference of assay values between unspiked and spiked samples
1	Sample Solution	99.9	-0.3
2	Spiked sample Solution	100.2	

Precision

System precision analysis was conducted, revealing a RSD% of 0.62% for the TFN peak area at the working concentration. The method precision assessment was carried out, resulting in an average assay of 100.1% w/w and a RSD% of 0.46 for six TFN preparations at the 100% working concentration. Various analysts evaluated intermediate precision by employing on different days., and it showed a bias of -0.1 in the determination of TFN assay content. Results are summarized in Table 4.

Table 4. Precision at intermediate level

Sample ID	Analyst (1) /Day (1) / TFN (w/w%)	Analyst (2) /Day (2) TFN (w/w%)	Bias
S-1	100.5	99.9	0.6
S-2	100.7	100.5	0.2
S-3	99.7	100.1	-0.4
S-4	99.5	100.0	-0.5
S-5	100.1	100.1	0.0
S-6	100.1	100.6	-0.5
Avg. (w/w%)	**100.1**	**100.2**	**-0.1**
SD	**0.45**	**0.28**	
RSD%	**0.46**	**0.28**	
Over all RSD% (12 preparations)	**0.37**		

Linearity

The obtained results for linearity are presented in Table 5, which displays the best-fit line for the average peak area in relation to the TFN concentration. The linearity results for TFN within the defined concentration range are deemed acceptable, as they exhibit a correlation coefficient (R) exceeding 0.999.

Table 5. Linearity Table for TFN

Level	Concentration (µg/ml)	Average peak area
70.0%	35	1530109
80.0%	40	1740125
90.0%	45	1951691
100.0%	50	2177901
110.0%	55	2414204
120.0%	60	2613053
Slope		**43790.52**
Intercept		**-8821.9**
Correlation coefficient (R)		**0.9997**
R^2		**0.9995**

Accuracy

The percentage recovery values are obtained for TFN in the range of 98.8% to 101.2%. The RSD% values for TFN recoveries are in the range of 0.20% to 1.13%. The percentage recovery results obtained for TFN is listed in Table 6.

Table 6. Accuracy test

Level	Theoretical concentration (µg / ml)	Measured concentration (µg / ml)	Recovery %	Average	RSD %
80% Level	40.6	40.1	98.8	99.8	1.13
	40.0	40.4	101.0		
	40.6	40.4	99.5		
100% Level	50.2	50.6	100.8	100.6	0.20
	50.2	50.5	100.6		
	50.5	50.7	100.4		
120% Level	60.5	60.9	100.7	100.7	0.50
	60.2	60.9	101.2		
	60.5	60.6	100.2		

Range

The method's range was established based on the data from linearity and accuracy assessments. The method's range spans from 30 to 60 µg/ml.

Robustness

The method robustness was confirmed by intentionally altering the method parameters. The TFN peak's tailing factor fell within the range of 1.1 to 1.4, and the number of theoretical plates was in the range of 4258 to 6272 in the standard solution. The RSD% ranged from 0.20% to 0.75% for six replicate injections of the standard solution. Significant changes in the assay value were not observed when examining robustness parameters, including variations in flow rate, mobile phase composition, buffer strength, injection volume, and buffer pH. During the robustness study, the retention time (R_T) of the TFN peak fluctuated between 4.64 minutes and 6.19 minutes, and the assay values on a dried basis ranged from 99.4% w/w to 100.8% w/w (Table 7).

Table 7. The system suitability data for robustness study

Validation Parameter (Robustness)	Retention Time	Average assay (% w/w)
1) Actual condition	5.77	100.6
2) Mobile phase ratio 66:34	6.11	100.5
3) Mobile phase ratio 64:36	4.64	100.8
4) Buffer pH : 4.1	5.36	99.8
5) Buffer pH : 3.9	5.28	100.8
6) 0.6% Triethylamine Buffer	5.01	100.1
7) 0.4% Triethylamine Buffer	4.81	99.7
8) Flow rate: 1.1 ml/min	5.10	99.7
9) Flow rate: 0.9 ml/min	6.19	99.9
10) Injection volume: 5 µl	5.78	100.4
11) Injection volume: 15 µl	5.76	99.4

Solution Stability

There is no substantial change in the assay observed for both the standard and sample solutions over a 48-hour period when stored at room temperature. The assay values exhibited a minimal variation, with a RSD of 1.24% for the standard solution and for the sample solution with RSD of 1.32%. The results consistently fell within the acceptable range, RSD values below 2%, demonstrating high precision and reliability of the method. According to the provided data, it can be concluded that both the standard and sample solutions remain stable for up to 48 hours at room temperature.

Conclusion

A rapid HPLC method was systematically developed for the estmation of TFN alongside its production related impurities. The optimization process involved careful adjustment of the mobile phase and chromatography specifications to achieve optimal peak parameters. The resulting method was extensively validated, confirming its sensitivity, precision, linearity across the studied concentration range, accuracy, and robustness in detecting TFN in the presence of its production related impurities. Utilizing Total Factorial Design as a QbD tool allowed for a comprehensive exploration of various factors' effects on the responses. This analysis affirmed that the TFN peak was effectively separated from other specified impurities. The developed analytical method was rigorously validated following the ICH guidelines, establishing its selectivity in determining the assay of TFN drug substance.

ACKNOWLEDGEMENTS

The authors are thankful to the management of vignan institute of pharmaceutical technology, Visakhapatnam for providing facilities for performing experimental work.

AUTHOR CONTRIBUTIONS

Concept: H.K.T., G.N.C., B.R.B.K.; Design: H.K.T., G.N.C., B.R.B.K.; Control: S.M., S.R.Y.; Sources: J.K.B., H.K.T.; Materials: J.K.B., S.R.Y.; Data Collection and/ or Processing: V.D., J.K.B., S.R.Y.; Analysis and/ or Interpretation: V.D., H.K.T., B.R.B.K.; Literature Review: S.M., H.K.T., B.R.B.K.; Manuscript Writing: B.R.B.K.; Critical Review: V.D., H.K.T., G.N.C., S.R.Y., B.R.B.K.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 975-992, 2024
Doi: 10.33483/jfpau.1417289

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IN SILICO TRIAL APPROACHES BETWEEN PHYTOCHEMICAL COMPOSITION OF *VERBENA OFFICINALIS AND* LIVER CANCER TARGETS

VERBENA OFFICINALIS'İN FİTOKİMYASAL BİLEŞİMİ İLE KARACİĞER KANSERİ HEDEFLERİ ARASINDAKİ İN SİLİKO DENEME YAKLAŞIMLARI

Hatice AKKAYA[1]*, Aydın OZMALDAR[2]

[1]Health Sciences University, Faculty of Pharmacy, Department of Biochemistry, 34668, Istanbul, Türkiye
[2]Krosgen Biotechnology, Department of IT, 34750, Istanbul, Türkiye

ABSTRACT

Objective: *The abundance of bioactive metabolites in Verbena officinalis explains the biological benefits and folkloric use of the plant. Liver cancer is an extremely heterogeneous malignant disease compared to other defined tumors. To explore the potential therapeutic value of bioactive metabolites in Verbena officinalis, this study aimed to filter secondary metabolites, conduct ADME-Tox assessments, perform drug similarity tests, and analyze with molecular dynamic simulations. The objective was to evaluate how potential drug candidates derived from Verbena officinalis behave in biological systems and assess their potential toxicity risks.*
Material and Method: *Ligands selected from the ADME assay were utilized in in silico molecular docking studies against Glucose-6-phosphate dehydrogenase enzyme in the oxidative part of the pentose phosphate pathway, which is crucial for liver diseases. These studies were conducted using Autodock Vina embedded in Chimera 1.16. Molecular dynamics simulations were performed with the AMBER16.*
Result and Discussion: *When the ADME test results were evaluated, 88 secondary metabolites were identified as ligands. Among all the ligands evaluated against Glucose-6-phosphate dehydrogenase enzyme, which is the key enzyme of the pentose phosphate pathway, quercetin flavonoid was determined to be the most active ligand with a docking score of -8.1 kcal/mol and binding energy of -118.51 kcal/mol. A molecular dynamics simulation performed for 300 nanoseconds confirmed that quercetin can remain stable in its microenvironment. The activity of this metabolite is worthy of further testing in vitro and in vivo as it may highlight a therapeutic modality within the pentose phosphate pathway.*
Keywords: *In silico, liver cancer, pentose phosphate pathway, toxicitiy, Verbena officinalis*

ÖZ

Amaç: *Verbena officinalis bitkisinde bulunan biyoaktif metabolitlerin bolluğu, bitkinin biyolojik faydalarını ve halk arasındaki kullanımını açıklar. Karaciğer kanseri, diğer tanımlanmış tümörlere kıyasla son derece heterojen kötü huylu bir hastalıktır. Verbena officinalis'teki biyoaktif metabolitlerin potansiyel terapötik değerini keşfetmek için, bu çalışma ikincil metabolitleri filtrelemeyi, ADME-Tox değerlendirmeleri yapmayı, ilaç benzerlik testleri gerçekleştirmeyi ve moleküler dinamik simülasyonları ile analiz etmeyi amaçlamıştır. Hedef, Verbena officinalis'ten elde edilen potansiyel ilaç adaylarının biyolojik sistemlerde nasıl davrandığını değerlendirmek,*

* **Corresponding Author / Sorumlu Yazar:** Hatice Akkaya
e-mail / e-posta: hatice.akkaya@sbu.edu.tr, **Phone / Tel.:** +902167778777

Submitted / Gönderilme : 09.01.2024
Accepted / Kabul : 11.07.2024
Published / Yayınlanma : 10.09.2024

potansiyel toksisite risklerini değerlendirmekti.
Gereç ve Yöntem: *ADME testinden seçilen ligandlar, karaciğer hastalıkları için önemli olan pentoz fosfat yolunun oksidatif kısmındaki Glukoz-6-fosfat dehidrogenaz enzimi için in siliko moleküler bağlanma çalışmalarında kullanıldı. Bu çalışmalar Chimera 1.16'ya gömülü Autodock Vina kullanılarak gerçekleştirildi. Moleküler dinamik simülasyonları AMBER16 programı ile gerçekleştirildi.*
Sonuç ve Tartışma: *ADME test sonuçları değerlendirildiğinde, 88 sekonder metabolit ligand olarak belirlendi. Pentoz fosfat yolunun anahtar enzimi olan Glukoz-6-fosfat dehidrogenaz enzimine karşı değerlendirilen tüm ligandlar arasında, kuersetin flavonoidi, -8.1 kcal/mol bağlanma skoru ve -118.51 kcal/mol bağlanma enerjisi ile en etkin ligand olarak belirlendi. 300 nanosaniye boyunca yapılan moleküler dinamik simülasyonu ise quercetinin bulunduğu mikroçevrede stabil olarak kalabildiğini doğruladı. Bu metabolitin aktivitesi, pentoz fosfat yolu içinde terapötik bir modaliteyi ortaya koyabileceği için in vitro ve in vivo testlerle daha ileri incelenmeye değerdir.*
Anahtar Kelimeler: *İn siliko, karaciğer kanseri, pentoz fosfat yolu, toksisite, Verbena officinalis*

INTRODUCTION

Computer-aided drug discovery (CADD) plays a pivotal role in identifying and optimizing hit compounds, thus advancing them through the drug discovery pipeline [1]. Its interdisciplinary essence, incorporating chemoinformatics, bioinformatics, molecular modeling, and data mining, has notably contributed to the endeavors of drug discovery. The integration of artificial intelligence, especially machine learning and deep learning, has propelled CADD's progress in recent years [2]. Despite encountering challenges and occasional disillusionments stemming from misuse and inflated expectations, CADD remains an indispensable tool in modern drug discovery initiatives [3].

Moreover, in the realm of drug discovery, *Verbena officinalis (V. officinalis)* is the main species in the genus *Verbena* of the *Verbenaceae* family [4]. This family has more than a thousand species, consisting of trees, shrubs, as well as herbaceous plants [5]. A monograph on "*Vervain herb*" was published in the European Pharmacopoeia (6th Edition) in 2008 regarding this plant, which is known as a traditional medicinal raw material. The main groups of secondary metabolites of *V. officinalis* herb, which has a rich chemical composition, consist of iridoids, phenylpropanoid glycosides, flavonoids, phenolic acids, terpenoids, carbohydrates, sterols, fatty acids and essential oils [6]. Therapeutic practices using *V. officinalis* plant extracts, which are used in traditional medicine as well as traditional Chinese medicine, are supported by scientific evidence [7,8]. It is known that *V. officinalis* plant helps in the treatment of urinary tract disorders, has supportive properties in the treatment of menstrual disorders [9,10], nervous system disorders [11] malaria and rheumatism.

In addition to being an antimicrobial and secretolytic raw material, it has also been stated to be an anti-inflammatory and antibacterial agent in skin diseases [10]. It has been stated that *V. officinalis* inhibits the mechanism or execution of neuronal apoptosis [12], increases serotonin, norepinephrine and dopamine levels in nerve terminals [13], its essential oil stimulates apoptosis through caspase-3 activation [14], and its flavonoids and polyphenols have been reported to have a gastroprotective effect [15]. In addition, it has various biological and pharmacological activities such as analgesic [16], antioxidant [17], hepatoprotective [18], antinephrosis [19], antiprostatitis [20]. In another study, it was stated that the anticonvulsant effect of *V. officinalis* flavonoids and phenolic acid residues probably occurs through activation of the GABAA receptor [11]. Pentose phosphate pathway (PPP), which is the important pathway for ribonucleotide synthesis, is the most important source of NADPH (reduced form of Nicotinamide Adenine Dinucleotide Phosphate), which is of great importance for cellular functions such as fatty acid synthesis and scavenging of reactive oxygen species [21]. The pentose phosphate pathway (PPP), in which cancer cells extensively use glucose, branches from glycolysis and is crucial for cancer cell metabolism. In the oxidative phase of PPP, glucose is converted into glucose 6-phosphate (G6P), which is then oxidized to 6-phosphogluconolactone by the rate-limiting enzyme glucose 6-phosphate dehydrogenase (G6PD), producing NADPH. Another source of NADPH in PPP is the conversion of 6-phosphogluconate into ribose (ribulose) 5-phosphate by 6-phosphogluconate dehydrogenase (6PGD) [22]. Glucose-6-phosphate dehydrogenase (G6PD) plays a crucial role in liver cancer, especially hepatocellular carcinoma. Its high expression in hepatocellular carcinoma affects

energy metabolism and redox balance through the PPP, leading to changes in NADPH levels and increased oxidative stress, promoting cancer progression. Abnormal activation of G6PD enhances cell proliferation and survival in hepatocellular carcinoma, making it a potential diagnostic marker closely associated with patient prognosis [23,24]. Additionally, G6PD is important in other cancers like glioma, breast cancer, and multiple myeloma, affecting energy metabolism and redox homeostasis [25]. Elevated G6PD levels in these cancers stimulate cell proliferation by increasing NADPH production and reducing reactive oxygen species (ROS). Targeting G6PD and the PPP could be a promising therapeutic approach by disrupting cancer cell growth and survival through modulation of cellular redox balance.

By leveraging "*in silico*" methods such as molecular docking, ADME-Tox studies, and drug similarity analyses [26-29], this study aims to evaluate the potential of *V. officinalis* metabolites as drug candidates targeting liver cancer. Through the integration of CADD principles with pharmacological research on *V. officinalis*, this study represents a multidisciplinary effort to explore novel avenues in drug discovery and therapeutic interventions.

MATERIAL AND METHOD

Selection of Receptors and Ligands

Through a review of the literature [6,30], metabolites from *V. officinalis* were collected, and SMILES notations were extracted from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). The energy of the molecules was minimized using the Build Structure tool integrated into Chimera, and the resulting ligands were saved in Mol2 format for docking studies. The target protein G6PD (PDBID: 6E08) was retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/) [31,32]. After removing small molecules and water molecules from the 3D crystallographic protein structure, polar hydrogen atoms and charges were added, and the final protein structure was saved as a Mol2 file.

Computer-Based Analysis of Pharmacokinetics and Toxicity Tests

Drug similarity as well as oral bioavailability (Lipinski's rule of 5) [33] of the compounds selected as drug candidates were evaluated with SwissADME (http://www.swissadme.ch/), which is used especially to estimate pharmacokinetic properties. Ligands that comply with this rule have been studied in molecular docking. The ProTox-II server (http://tox.charite.de/protoc_II;) was used to provide an estimate of the primary toxicity properties and acute toxicity values of the secondary metabolite of *V. officinalis* that was most active based on docking results and to help establish its safety profile for oral administration [29].

Molecular Docking

The ligand molecule was prepared in PDBQT format and utilized with the AutoDock Vina command prompt [25]. The active site of the ligand bound to G6PD was determined by averaging the coordinates of the x, y, and z axes. A grid box search area measuring 25 × 25 × 25 Å was defined [32,34]. Following this, the ligands were positioned within the enzyme's active site and the resulting binding energy (score) between the ligands and targets was computed.

Molecular Dynamic Simulation

The molecular dynamics simulation of the G6PD-quercetin complex was performed with the AMBER16 package using the ff14SB force field for the protein [35], with a 2-femtosecond time step in a truncated octahedron box containing 20530 explicit TIP3P water molecules [36]. The molecular dynamics simulations were performed for a total of 300 nanoseconds (ns). The Antechamber program, part of AmberTools, was used to parameterize quercetin, and to assign partial charges, RESP fitting approach was used. Heavy atom-hydrogen bond distances were fixed with SHAKE algorithm, and the Langevin thermostat was employed to maintain the system's temperature at 310 K. To account for electrostatic interactions, the Particle Mesh Ewald method was used. Additionally, three sodium ions (Na^+) were introduced to neutralize the system's overall charge.

Software Used

Windows 10 Microsoft operating system was installed. SwissADME online tool was used for drug design and evaluation. Protox II was used to help establish a safety profile via oral routes [29]. The UCSF Chimera (1.16) program (https://www.cgl.ucsf.edu/chimera/download.html) was run for docking with AutoDock Vina [28]. Protein and chemical (ligand) structures were searched in Protein Data Bank (https://www.rcsb.org/) and PubChem, respectively. IGemDOCK V2.1 was used to calculate the binding energies of the ligands. For the interaction poses of the resulting complex structure, Plip-tool (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) [37] and ProteinsPlus web servers (https://proteins.plus/) were used. The AMBER16 program [38] was used for the molecular dynamics (MD) simulations, which were run on supercomputers at the TÜBİTAK ULAKBİM High Performance and Grid Computing Center (TRUBA).

RESULT AND DISCUSSION

In this study, the molecular interactions of phytochemicals contained in *V. officinalis*, whose anticoccidial [41] and antioxidant potentials [42] were reported in *in silico* studies, with G6PD in cancer treatment were evaluated. It is thought that quercetin, one of the phytochemicals evaluated according to its pharmacokinetics, pharmacodynamics, drug similarity, physiochemical properties, low binding affinity value and toxicological analysis, is physiologically active and can be considered as an oral drug. Cancer cells need to divide and grow rapidly, which requires the production of high amounts of NADPH. It can be said that reducing the effect of the pentose phosphate pathway by inhibiting G6PD activity is theoretically a strategy in the treatment of liver cancer and other types of cancer [27,43].

ADME is an approach to study the ADME properties of drugs using computer-based models and calculations, which plays an important role in drug development processes [44]. Lipinski's Rule of Five Drug Molecules is a set of guidelines stating that a drug candidate must possess four specific physical and chemical properties within acceptable limits for its oral bioavailability to be high [33]. The suitability of 109 compounds belonging to *V. officinalis* as drug candidates was obtained using the SwissADME server. According to the results, it was determined that the log P value of all 109 compounds was less than 5, and the molecular weight of 96 compounds was within the acceptable range (MW<500). The number of H-bond acceptors (≤10) and donors (≤5) falls within the acceptable range for 90 and 86 compounds, respectively. 83 compounds were identified in the topological polar surface area range (TPSA; <140). The number of rotatable bonds is within the acceptable range (≤10) for 100 compounds (Table 1). Considering these results, molecular docking studies were applied to investigate the anticancer activity in the next step. For liver cancer, 87 Verbane-based compounds (iridoids, flavonoids, phenolic acids, terpenoids, carbohydrates, sterols, fatty acids, essential oils) were used as ligands, and G6PD was used as the receptor (Table 2). While the binding energy of quercetin was 118.51 kcal/mol, its docking score was determined as -8.1 kcal/mol. According to all the tested ligands, it can be said that the structure that binds most effectively to G6PD is quercetin. In this way, the physicochemical properties of the most active structure quercetin determined were examined in more detail (Table 2). When the complex structure of quercetin and G6PD is examined, hydrophobic interactions with the amino acid tyrosine (Y480), hydrogen bonds with the amino acids arginine (Arg366) and aspartate (Asp394), and π-stacking structures with the amino acids tyrosine (Tyr374) and tryptophan (Trp482) are observed (Figure 1a,b).

In biological systems, molecular recognition is based on the principle of recognizing specific attractive interactions between two molecules [45]. These interactions may help quercetin and the G6PD complex fold correctly and enable the desired chemical reactions to occur. Additionally, understanding how interactions can be controlled at the molecular level may be important for potential drug design or biotechnological applications (Table 1, Figure 2). When looking at the interactions of the original crystal structure of G6PD with the nicotinamide adenine dinucleotide phosphate (NADP) ligand [46], it was observed that it formed H bonds and pi interactions with the same amino acids, but hydrophobic interactions were not observed. This indicates that the binding conditions of molecular interactions or molecular conformations have changed. Such changes can sometimes be associated with the emergence of a binding site where one molecule recognizes or binds to another, sometimes facilitating the binding

of a ligand to the active site of the protein or providing specificity in molecular recognition processes. In a related study [47], it was observed that in the different three-dimensional structure (2BH9) of the G6PD enzyme, quercetin forms four hydrogen bonds with Gly (38), Asp (42), Arg (72), and Arg (246) amino acids. These diverse binding interactions may impact ligand binding, conformation, and interaction mechanisms, leading to varied biological effects, inhibitor activities, or therapeutic properties. Consequently, the formation of distinct amino acid bonds between the same ligand and enzymes with different three-dimensional structures can result in diverse biological and pharmacological outcomes.

Table 1. List of pharmacokinetic properties of 109 metabolites of *Verbane officinalis*

	Compound	Physicochemical properties						Lipo-philicity	Water Solubility	Pharmaco-kinetics	Drug-likeness
		Molecular weight (gr/mol)	Number of rotatable bonds	Number of H-bond acceptors	Number of H-bond donors	Molar Refractivity	TPSA (Å²)	Log Po/w	LogS (ESOL)	GI absorbtion	Lipinski/violation
1	verbenalin	388.37	5	10	4	86.4	151.98	2.19	-0.97	Low	Yes; 0 violation
2	hastatoside	210.23	2	4	0	52.86	52.60	2.10	-1.40	High	Yes; 0 violation
3	7-hydroxy-dehydro-hastatoside	418.35	5	12	6	88.70	192.44	1.37	-0.63	Low	No; 2 violations: NorO>10, NHorOH>5
4	aucubin	346.33	4	9	**6**	77.15	149.07	1.44	0.18	Low	Yes; 1 violation: NHorOH>5
5	verbeofflin	240.21	5	5	1	57.42	57.42	1.71	-1.54	High	Yes; 0 violation
6	verbascoside	624.59	11	15	9	148.12	245.29	3.00	-2.87	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
7	2,4-diacetyl-O-verbascoside	708.66	15	17	7	167.90	257.43	3.06	-3.82	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
8	sover-bascoside	624.6	11	15	9	148.42	245.29	2.33	-4.18	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
9	4-acetyl-O-soverbas-coside	666.6	13	16	8	158.16	251.36	3.16	-3.35	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
10	3,4-diacetyl-O-soverbas-coside	708.66	15	17	7	167.9	257.43	3.00	-5.62	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
11	eukovoside	666.6	13	16	8	157.9	251.36	2.27	-4.61	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
12	campenoside II	490.5	8	10	4	120.82	151.98	3.26	-3.03	Low	Yes; 0 violation
13	betonyoside A	654.6	12	16	9	154.05	254.52	3.20	-2.49	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
14	cistanoside D	652.6	13	15	7	157.36	223.29	2.59	-3.32	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
15	Leucoscep-toside	638.6	12	15	8	152.89	234.29	2.78	-3.09	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
16	5,7,4'-Tri-hydroxy-8-nethoxy-flavone	300.26	2	6	3	80.48	100.13	2.25	-3.99	High	Yes; 0 violation
17	diosmetin	300.26	2	6	3	80.48	100.13	2.47	-4.06	High	Yes; 0 violation
18	artemetin	388.4	6	8	1	102.40	96.59	3.59	-4.44	High	Yes; 0 violation
19	quercetin	302.23	1	7	5	78.03	131.36	1.63	-3.16	High	Yes; 0 violation
20	kaempferol	286.24	1	6	4	76.01	111.13	1.7	-3.31	High	Yes; 0 violation
21	Luteolin	286.24	1	6	4	76.01	111.13	1.86	-3.71	High	Yes; 0 violation
22	luteolin 7-O-diglucuronide	638.5	7	18	10	141.33	303.57	1.16	-3.23	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5

Table 1 (*continue*). List of pharmacokinetic properties of 109 metabolites of *Verbane officinalis*

	Compound	Physicochemical properties						Lipo-phillicity	Water Solubility	Pharmaco-kinetics	Drug-likeness
		Molecular weight (gr/mol)	Number of rotatable bonds	Number of H-bond acceptors	Number of H-bond donors	Molar Refractivity	TPSA (Å²)	Log Po/w	LogS (ESOL)	GI absorbtion	Lipinski/violation
23	luteolin 7-O-glucuronide	462.4	4	12	7	108.74	207.35	1.55	-3.41	Low	No; 2 violations: NorO>10, NHorOH>5
24	luteolin 7-O-glucoside	448.4	4	11	7	108.13	190.28	1.83	-3.65	Low	No; 2 violations: NorO>10, NHorOH>5
25	6-hydroxy-luteolin glycoside	464.4	4	12	8	110.16	210.51	1.67	-3.51	Low	No; 2 violations: NorO>10, NHorOH>5
26	luteolin-7-O-rutinoside	610.5	7	16	10	140.52	269.43	2.81	-3.0	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
27	apigenin	270.24	1	5	3	73.99	90.90	1.89	-3.94	High	Yes; 0 violation
28	apigenin 7-O-diglucuronide	622.5	7	17	9	139.71	283.34	2.03	-3.36	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
29	apigenin 7-O-glucoside	432.18	4	10	6	106.11	170.05	2.17	-3.78	Low	Yes; 1 violation: NHorOH>5
30	isoramnetin	316.26	2	7	4	82.50	120.36	2	-3.89	High	Yes; 0 violation
31	pedalitin	316.26	2	7	4	82.50	120.36	1.25	-3.76	High	Yes; 0 violation
32	scutellarein	286.24	1	6	4	76.01	111.13	2.08	-3.79	High	Yes; 0 violation
33	scutellarein 7-O-glucuronide	462.4	4	12	7	108.74	207.35	1.11	-3.27	Low	No; 2 violations: NorO>10, NHorOH>5
34	scutellarein 7-O-glucoside	448.4	4	11	7	108.13	190.28	1.75	-3.05	Low	No; 2 violations: NorO>10, NHorOH>5
35	chlorogenic acid	354.31	5	9	6	83.50	164.75	0.96	-1.62	Low	Yes; 1 violation: NHorOH>5
36	ferulic acid	194.18	3	4	2	51.63	66.76	1.62	-2.11	High	Yes; 0 violation
37	protocatechuic acid	154.12	1	4	3	37.45	77.76	0.66	-1.86	High	Yes; 0 violation
38	4,5-O-dicaffeoyl-quinic acid	516.4	9	12	7	126.9	211.28	1.25	-3.65	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
39	1,5-dicaffeoyl-quinic acid	516.4	9	12	7	126.9	211.28	1.11	-3.65	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5
40	rosmarinic acid	360.3	7	8	5	91.40	144.52	1.17	-3.44	Low	Yes; 0 violation
41	carnosol	330.4	1	4	2	92.83	66.76	2.97	-4.77	High	Yes; 0 violation
42	carnosolic acid	348.4	2	5	4	96.59	97.99	2.33	-4.31	High	Yes; 0 violation
43	rosmanol	346.4	1	5	3	93.99	86.99	2.50	-4.25	High	Yes; 0 violation
44	isorosmanol	346.4	1	5	3	93.99	86.99	2.59	-4.25	High	Yes; 0 violation
45	ursolic acid	456.7	1	3	2	136.91	57.53	3.71	-7.23	Low	Yes; 1 violation: MLOGP>4.15
46	3-epiursolic acid	456.7	1	3	2	136.91	57.53	3.71	-7.23	Low	Yes; 1 violation: MLOGP>4.15
47	arabinose	150.13	0	5	4	29.77	90.15	-0.39	1.13	Low	Yes; 0 violation
48	galactose	180.16	1	6	5	35.74	110.38	0.24	1.15	Low	Yes; 0 violation
49	galacturonic acid	194.14	1	7	5	36.35	127.45	-0.19	0.50	Low	Yes; 0 violation
50	glucose	180.16	1	6	5	35.74	110.38	0.24	1.15	Low	Yes; 0 violation
51	mannose	180.16	1	6	5	35.74	110.38	0.24	1.15	Low	Yes; 0 violation
52	rhamnose	164.16	0	5	4	34.57	90.15	0.66	0.46	High	Yes; 0 violation
53	Xylose	150.13	0	5	4	29.77	90.15	-0.39	1.13	Low	Yes; 0 violation
54	stigmasterol	370.61	4	1	1	118.33	20.23	4.53	-6.67	Low	Yes; 1 violation: MLOGP>4.15
55	daucosterol	576.85	9	6	4	165.61	99.38	4.98	-7.70	Low	Yes; 1 violation: MW>500

Table 1 (*continue*). List of pharmacokinetic properties of 109 metabolites of *Verbane officinalis*

	Compound	Physicochemical properties						Lipo-phillicity	Water Solubility	Pharmaco-kinetics	Drug-likeness
		Molecular weight (gr/mol)	Number of rotatable bonds	Number of H-bond acceptors	Number of H-bond donors	Molar Refractivity	TPSA (Å²)	Log Po/w	LogS (ESOL)	GI absorbtion	Lipinski/violation
56	β-sitosterol	414.71	6	1	1	133.23	20.23	4.79	-7.90	Low	Yes; 1 violation: MLOGP>4.15
57	cornudentanone	378.5	15	5	0	107.58	69.67	4.08	-4.99	High	Yes; 0 violation
58	oleic acid	282.5	15	2	1	89.94	37.30	4.27	-5.41	High	Yes; 1 violation: MLOGP>4.15
59	3-epioleanolic acid	456.7	1	3	2	136.65	57.53	3.89	-7.32	Low	Yes; 1 violation: MLOGP>4.15
60	isobornyl formate	182.26	2	2	0	51.92	26.30	2.43	-3.55	High	Yes; 0 violation
61	citral (geranial)	152.23	4	1	0	49.44	17.07	2.47	-2.43	High	Yes; 0 violation
62	limonene	136.23	1	0	0	47.12	0.00	2.72	-3.50	Low	Yes; 0 violation
63	carvone	150.22	1	1	0	47.32	17.07	2.27	-2.41	High	Yes; 0 violation
64	1.8-cineole	154.25	0	1	0	47.12	9.23	2.58	-2.52	High	Yes; 0 violation
65	hepten-3-one	112.17	4	1	0	35.49	17.07	2.03	-1.47	High	Yes; 0 violation
66	α-terpineol	154.25	1	1	1	48.80	20.23	2.51	-2.87	High	Yes; 0 violation
67	anethole	148.20	2	1	0	47.83	9.23	2.55	-3.11	High	Yes; 0 violation
68	β-pinene	136.23	0	0	0	45.22	0.00	2.59	-3.31	Low	Yes; 1 violation: MLOGP>4.15
69	thymol	150.22	1	1	1	48.01	20.23	2.32	-3.19	High	Yes; 0 violation
70	methyl heptenone	126.20	3	1	0	40.30	17.07	2.23	-1.61	High	Yes; 0 violation
71	carvacrol	150.22	1	1	1	48.01	20.23	2.24	-3.31	High	Yes; 0 violation
72	trans-carveol	152.23	1	1	1	48.28	20.23	2.50	-2.68	High	Yes; 0 violation
73	isopiperitone	152.23	1	1	1	47.80	17.07	2.38	-2.51	High	Yes; 0 violation
74	α-pinene	136.23	0	0	0	45.22	0.00	2.63	-3.51	Low	Yes; 1 violation: MLOGP>4.15
75	piperitone	152.23	1	1	0	47.80	17.07	2.38	-2.51	High	Yes; 0 violation
76	cis-carveol	152.23	1	1	1	48.28	20.23	2.50	-2.68	High	Yes; 0 violation
77	terpinen-4-ol	154.25	1	1	1	48.80	20.23	2.51	2.78	High	Yes; 0 violation
78	β-phellandrene	136.23	1	0	0	47.12	0.00	2.65	-2.79	Low	Yes; 0 violation
79	geraniol	154.25	4	1	1	50.40	20.23	2.75	-2.78	High	Yes; 0 violation
80	β-terpineol	154.25	1	1	1	48.80	20.23	2.41	-2.32	High	Yes; 0 violation
81	sabinene	136.23	1	0	0	45.22	0.00	2.65	-2.57	Low	Yes; 1 violation: MLOGP>4.15
82	cinerone	150.22	2	1	0	47.32	17.07	2.39	-1.75	High	Yes; 0 violation
83	p-cymene	134.22	1	0	0	45.99	0.00	2.51	-3.63	Low	Yes; 1 violation: MLOGP>4.15
84	nerol	154.25	4	1	1	50.40	20.23	2.75	-2.78	High	Yes; 0 violation
85	linalol	154.25	4	1	1	50.44	20.23	2.70	-2.40	High	Yes; 0 violation
86	(E)-β-ocimene	136.23	3	0	0	48.76	0.00	2.80	-3.17	Low	Yes; 0 violation
87	borneol	154.25	0	1	1	46.60	20.23	2.29	-2.51	High	Yes; 0 violation
88	iso-pinocamphone	152.23	0	1	0	45.90	17.07	2.18	-2.21	High	Yes; 0 violation
89	o-cymene	134.22	1	0	0	45.99	0.00	2.43	-3.81	Low	Yes; 1 violation: MLOGP>4.15
90	γ-terpinene	136.23	1	0	0	47.12	0.00	2.73	-3.45	Low	Yes; 0 violation
91	caryophyllene oxide	220.35	0	1	1	68.27	12.53	3.15	-3.45	High	Yes; 0 violation
92	spathulenol	220.35	0	1	1	68.34	20.23	2.88	-3.17	High	Yes; 0 violation

Table 1 (*continue*). List of pharmacokinetic properties of 109 metabolites of *Verbane officinalis*

	Compound	Physicochemical properties						Lipo-phillicity	Water Solubility	Pharmaco-kinetics	Drug-likeness
		Molecular weight (gr/mol)	Number of rotatable bonds	Number of H-bond acceptors	Number of H-bond donors	Molar Refractivity	TPSA (Å²)	Log Po/w	LogS (ESOL)	GI absorbtion	Lipinski/violation
93	α-curcumane	202.33	4	0	0	69.55	0.00	3.50	-4.52	Low	Yes; 1 violation: MLOGP>4.15
94	β-caryophyllene	204.35	0	0	0	68.78	0.00	3.29	-3.87	Low	Yes; 1 violation: MLOGP>4.15
95	trans-nerolidol	222.37	7	1	1	74.00	20.23	3.64	-3.80	High	Yes; 0 violation
96	bicyclosesquiphellandrene	204.35	1	0	0	69.04	0.00	3.33	-4.00	Low	Yes; 1 violation: MLOGP>4.15
97	δ-cadinene	204.35	1	0	0	69.04	0.00	3.32	-3.43	Low	Yes; 1 violation: MLOGP>4.15
98	germacene D	204.35	1	0	0	70.68	0.00	3.32	-4.03	Low	Yes; 1 violation: MLOGP>4.15
99	α-muurolene	204.35	1	0	0	69.04	0.00	3.38	-3.61	Low	Yes; 1 violation: MLOGP>4.15
100	bicyclogermacrene	204.35	0	0	0	68.78	0.00	3.34	-3.72	Low	Yes; 1 violation: MLOGP>4.15
101	cis-muurola-4(14).5-diene	204.35	1	0	0	69.04	0.00	3.33	-4.00	Low	Yes; 1 violation: MLOGP>4.15
102	isocaryophylene oxide	220.35	0	1	1	68.27	12.53	3.15	-3.45	High	Yes; 0 violation
103	β-cedrene	204.35	0	0	0	66.88	0.00	3.18	-4.16	Low	Yes; 1 violation: MLOGP>4.15
104	α-copaene	204.35	1	0	0	67.14	0.00	3.40	-3.86	Low	Yes; 1 violation: MLOGP>4.15
105	β-elemene	204.35	3	0	0	70.42	0.00	3.37	-4.76	Low	Yes; 1 violation: MLOGP>4.15
106	β-cubenene	204.35	1	0	0	67.14	0.00	3.39	-4.01	Low	Yes; 1 violation: MLOGP>4.15
107	α-humulene	204.35	0	0	0	70.42	0.00	3.27	-3.97	Low	Yes; 1 violation: MLOGP>4.15
108	α-7-epi-selinene	204.35	1	0	0	68.78	0.00	3.31	-4.32	Low	Yes; 1 violation: MLOGP>4.15
109	isoledene	204.35	0	0	0	67.14	0.00	3.26	-3.67	Low	Yes; 1 violation: MLOGP>4.15

Table 2. Binding energies and affinities of 88 ligands selected from *Verbena officinalis* to the G6PDH protein structure

Group of metabolites	Compounds	Pubchem CID	Binding energy (kcal/mol)	Binding affinity (kcal/mol)
Iridoids				
	hastatoside	92043450	-105.7	-6.8
	verbeofflin	101875571	-99.33	-5.9
Flavonoids				
	5,7,4'-Trihydroxy-8-methoxyflavone	5322078	-109.45	-7.3
	diosmetin	5281612	-109.88	-8.1
	artemetin	5320351	-109.18	-7.0
	quercetin	5280343	-115.06	-8.1
	kaempferol	5280863	-105.61	-7.9
	luteolin	5280445	-113.43	-8.0
	apigenin	5280443	-99.62	-7.9
	isoramnetin	5280681	-115.09	-7.8
	pedalitin	31161	-106.91	-8.4
	scutellarein	5281697	-103.01	-8.1

Table 2 (*continue*). Binding energies and affinities of 88 ligands selected from *Verbena officinalis* to the G6PDH protein structure

Group of metabolites	Compounds	Pubchem CID	Binding energy (kcal/mol)	Binding affinity (kcal/mol)
Phenolic acids				
	ferulic acid	445858	-89.4	-5.7
	protocatechuic acid	72	-79.14	-6.1
Terpenoids				
Diterpenoids				
	carnosol	442009	-87	-7.3
	carnosolic acid	11566445	-95.84	-6.8
	rosmanol	13966122	-107.9	-7.4
	isorosmanol	13820511		
triterpenoids				
	ursolic acid	64945	-93.05	-7.3
	3-epiursolic acid	7163177	-92.56	-7.4
Carbohydrates				
	arabinose	439195	-76.72	-4.8
	galactose	6036	-88.74	-5.1
	galacturonic acid	439215	-88.81	-5.9
	glucose	5793	-89.43	-5.1
	mannose	18950	-89.38	-5.1
	rhamnose	25310	-89.39	-5.1
	xylose	135191	-76.76	-4.8
Sterols				
	stigmasterol	5280794	-84.63	-7.5
	β-sitosterol	222284	-87	-7.2
Fatty acids				
	cornudentanone	442735	-107.57	'-6.4
	oleic acid	445639	-97.4	-5.8
	3-epioleanolic acid	11869658	-84.79	-7.0
Essential oil				
Monoterpenoids				
	isobornyl formate	23623868	-63.99	-5.2
	citral (geranial)	638011	-69.47	-5.6
	limonene	22311	-59.84	-5.7
	carvone	7439	-71.11	-6.1
	1.8-cineole	2758	-44.6	-5.0
	hepten-3-one	520420	-59.21	-4.5
	α-terpineol	17100	-67.04	-5.7
	anethole	637563	-72.34	-5.6
	β-pinene	14896	-45.68	-4.7
	thymol	6989	-69.74	-5.8
	methyl heptenone	9862	-61.29	-5.1

Table 2 (*continue*). Binding energies and affinities of 88 ligands selected from *Verbena officinalis* to the G6PDH protein structure

Group of metabolites	Compounds	Pubchem CID	Binding energy (kcal/mol)	Binding affinity (kcal/mol)
	carvacrol	10364	-70.69	-6.3
	trans-carveol	94221	-64.7	-5.2
	isopiperitone	6987	-67.86	-5.9
	α-pinene	82227	-46.83	-4.7
	piperitone	6987	-67.86	-5.9
	cis-carveol	330573	-64.72	-5.2
	terpinen-4-ol	11230	-66.84	-5.4
	β-phellandrene	11142	-57.18	-5.8
	geraniol	637566	-63.19	-5.7
	β-terpineol	8748	-68.77	-5.5
	sabinene	18818	-51.84	-5.0
	cinerone	5373127	-70.66	-6.3
	p-cymene	7463	-59.45	-6.1
	nerol	643820	-67.54	-5.6
	linalol	6549	-63.08	-5.1
	(E)-β-ocimene	5281553	-56.55	-5.4
	borneol	64685	-47.57	-4.8
	iso-pinocamphone	84532	-53.62	-5.1
	o-cymene	10703	-57.57	-6.2
	γ-terpinene	7461	-58.9	-5.9
Sesquiterpenoids				
	caryophyllene oxide	1742210	-65.75	-5.8
	spathulenol	92231	-65.47	-5.8
	α-curcumane	92139	-74.51	-6.7
	β-caryophyllene	5281515	-58.27	-5.5
	trans-nerolidol	5284507	-71.01	-6.2
	bicyclosesquiphellandrene	521496	-69.8	-6.4
	δ-cadinene	441005	-64.75	-6.2
	β-bourbonene	62566	-62.14	-6.1
	allo-aromadendrene	42608158	-58.6	-5.7
	α-cubenene	442359	-68.22	-6.1
	γ-cadinene	6432404	-67.12	-6.4
	germacene D	5317570	-71.04	-6.1
	α-muurolene	12306047	-69.57	-6.4
	bicyclogermacrene	13894537	-60.4	-5.3
	cis-muurola-4(14).5-diene	51351709	-71.4	-6.4
	isocaryophylene oxide	1742211	-65.87	-5.8
	β-cedrene	11106485	-56.85	-6.1
	α-copaene	92042749	-58.92	-5.7
	β-elemene	6918391	-64.58	-5.9

Table 2 (*continue*). Binding energies and affinities of 88 ligands selected from *Verbena officinalis* to the G6PDH protein structure

Group of metabolites	Compounds	Pubchem CID	Binding energy (kcal/mol)	Binding affinity (kcal/mol)
	β-cubenene	93081	-63.08	-6.2
	α-humulene	5281520	-65.95	-5.7
	α-7-epi-selinene	91753195	-64.13	-6.0
	isoledene	530426	-60.38	-5.9

This list indicates the chemicals that comply with Lipinski's Rule of 5

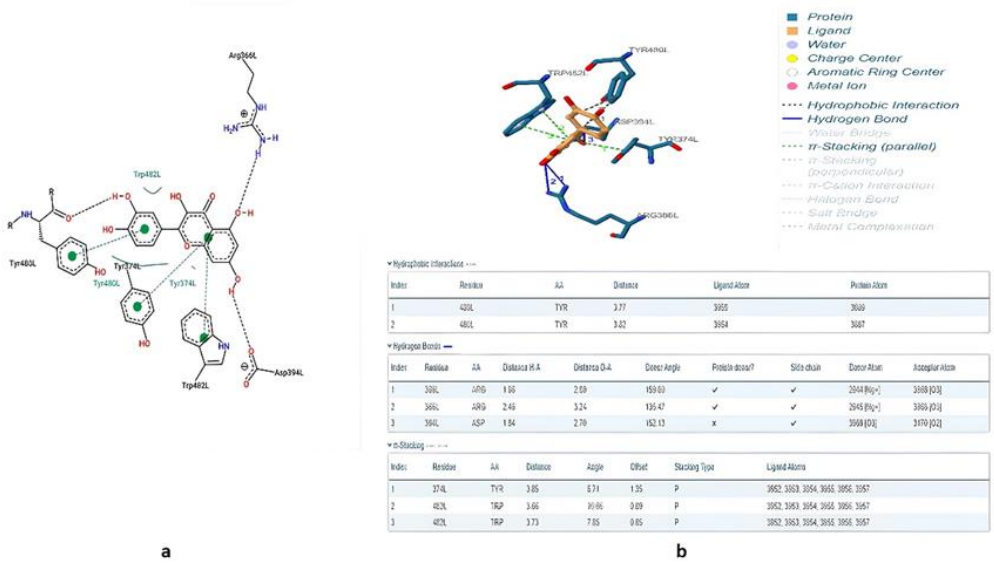

Figure 1. The 2D (a) and 3D (b) interaction poses of quercetin and G6PD complex

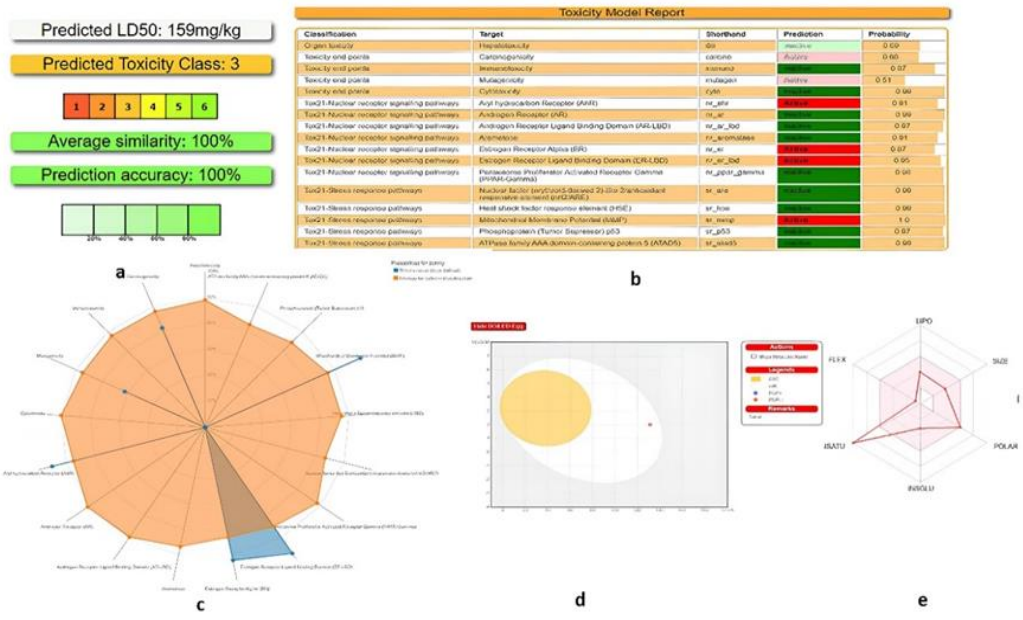

Figure 2. Toxicity results of quercetin calculated with Pro ToX-II (a, b), BOILED Egg model calculated with Swiss ADME (d) and radar graph for physicochemical properties of quercetin (e)

The Root Mean Square Deviation (RMSD), determines how much the atomic positions in a simulation deviate from a reference structure, typically the starting point [48]. During equilibration, the

RMSD typically increases as the system adjusts from its initial state. Once the simulation reaches equilibrium, the RMSD should fluctuate around a stable value, indicating the atoms are sampling their allowed conformations. This stable RMSD signifies the system is ready for the data collection phase of the MD simulation. The root mean square deviation plot of the overall simulation at 310 K is given in Figure 3. It can be seen from these plots that our system was equilibrated after 5.6 ns. Therefore, the first 5.6-nanosecond-long simulation is considered as equilibration period and excluded from further investigation. Moreover, the RMSD plot suggests minimal conformational change during the production period. The Root Mean Square Fluctuation (RMSF) values (Figure 4) per residue has shown that the solvent-exposed residues such as Ile353, Met384 and Asn403 contribute the most to the molecular motion throughout the simulation. After concluding the 300-ns-long MD simulation, a clustering analysis based on a "hierarchical agglomerative" approach was performed for post-processing of the trajectories. The cluster radii are set to 2.0, this cluster analysis in the G6PD-quercetin complex had produced three conformations and the percentage of the occurrences of each cluster were 84%, 10% and 6%. The most populated cluster, containing 84% of the data points, suggests that the G6PD-quercetin complex primarily adopts a single conformation during the simulation. The most populated cluster is shown in Figure 5. During the MD simulation, the quercetin molecule stays in the active site and interacts with several residues. For instance, the quercetin makes pi-stacking interactions with the Tyr374. Also, Asp394 is coordinated with one of the -OH group of quercetin, and backbone oxygen atom of Lys481 makes a hydrogen bond with another -OH group of quercetin. These interactions can be seen clearly in the most-populated cluster, which is shown in detail in Figure 6. The results have shown that the position of this quercetin molecule is very well defined, and the molecule is stable in this region. To prove their importance, these acidic and basic residues could be mutated to nonpolar amino acids, and further MD simulations could be done to check the activities in this state.

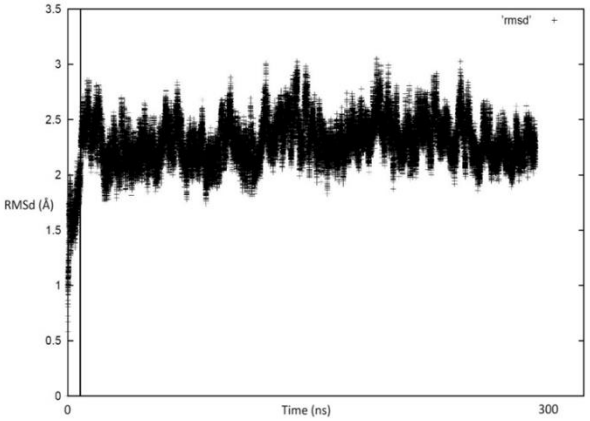

Figure 3. RMSD graph of the simulation. The left-side of the dash represents the equilibration time

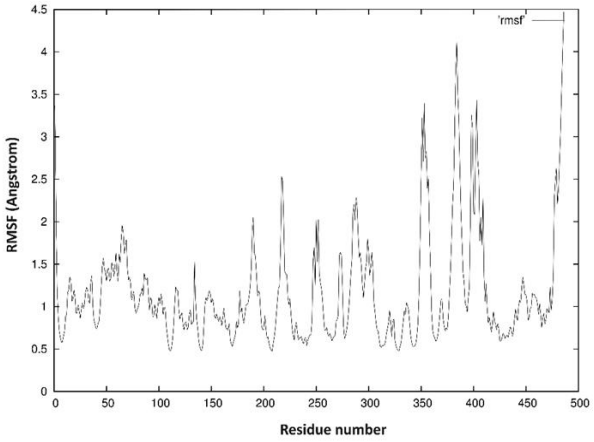

Figure 4. RMSF values at 310 K

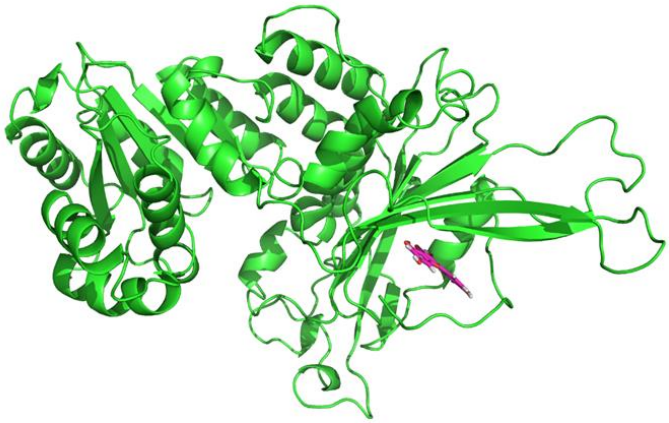

Figure 5. The most-populated cluster of the G6PD-quercetin complex

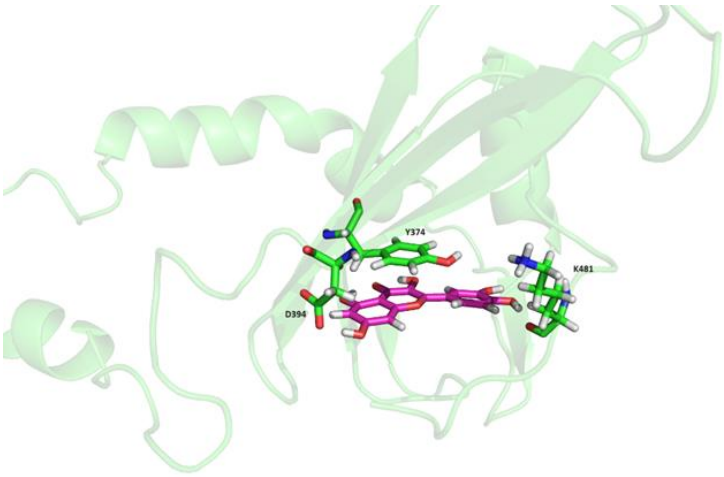

Figure 6. Close contacts between quercetin and its nearby residues in the most-populated cluster

ADME results have shown that quercetin complies with the limits set by the Lipinski rule and supports oral use and drug similarity. In the radar chart taken in SwissADME, the distribution of important physicochemical properties in our body has been created (Table 1, Figure 2d and e). In the pictures of BOILED Eggs, the white and yellow area respectively indicate passive absorption in the gastrointestinal (GI) tract and the ability to cross the blood-brain barrier (BBB). The outer grey area indicates molecules with low absorption and limited brain penetration (Figure 2d) [49]. According to the ADME profile, it has been stated that quercetin does not cross the blood brain barrier.

When the pink range was analysed (Figure 2e), lipophilicity (LIPO; iLOGP), size (MW), polarity (TPSA), solubility (INSOLU, log S), saturation (INSATU; carbon fraction in sp^3 hybridization was found to be 0) and flexibility (FLEX, rotatable bonds) are in the optimal range for quercetin. Deviation in saturation was observed. Molecules that are more saturated and have sp^3 hybridization are generally more water soluble and may be biologically effective. Nevertheless, a thorough examination of pharmacodynamics requires an all-encompassing viewpoint. The analysis results proved that the five properties were in the pink area and the compound fit into the group of drug-like compounds. Based on these properties, it can be said that the compound is not suitable for injectable administration due to its low flexibility [50].

In the BOILED Egg model, blue and red dots indicate P-glycoprotein (P-gp) substrates (PGP+), which is an ATP-dependent transmembrane protein that transports many drugs, and non-P-gp substrates (PGP−), respectively. While it is an advantage for a drug that is not a P-gp substrate to remain in the target cell for a longer time and interact less with other drugs, it is a disadvantage because the drug's tendency to remain in the body for a longer time will lead to toxicity [51]. Based on this, if the drug's

elimination from the body is to be accelerated, it may need to be designed as a P-gp substrate. Whether or not a compound is a P-gp substrate affects the efficacy and pharmacokinetics of the drug. These considerations need to be acknowledged throughout the drug development process.

Computational studies on absorption, distribution, metabolism, and excretion have reported that the ability of quercetin to combine with the plasma-protective protein varies between 85.36% and 99.82%. These studies also examined quercetin and their ADME properties, revealing that quercetin and 3′-methyl ether quercetin had 100% passive absorption, while other quercetin cells showed a lower absorption [41]. The fact that ADME, which has a very satisfactory predictive power in molecular design, states that quercetin does not cross the blood brain barrier may be due to the limited prediction of penetration of BOILED Egg [49,53]. The *in silico* interaction of geroprotective phytochemicals, including quercetin, with Sirtuin 1 was examined, and ADME results showed that quercetin could not cross the BBB and was toxic (class 3). However, animal studies have reported that quercetin can be tolerated at oral doses above the LD_{50} value [54] and is a safe nutritional supplement in mice [55]. For this reason, pharmacological toxicology studies on quercetin should be conducted.

In the Protox II analysis, the lethal dose 50 (LD_{50}) value of quercetin was estimated as 159 mg/kg and toxicity class 3. The toxicity model report of our study shows that quercetin is a carcinogenic and mutagenic structure, also has various interfering effects against Aryl Hydrocarbon Receptor (AhR), Estrogen Receptor Alpha (ER), Estrogen Receptor Ligand Binding Domain (ER-LBD) and Mitochondrial Membrane Potential (MMP) (Figure 2a-c).

It is extremely important to perform *in silico* toxicity analyses, examine drug candidates and perform risk assessments before clinical studies [56,57]. The fact that an antioxidant structure also has carcinogenic and mutagenic properties according to *in silico* toxicity analysis results may indicate that the compound may affect different cellular or molecular targets [58]. The effects of compounds are related to dose and prolonged exposure times. While a particular compound may have antioxidant effects at low doses, it may cause toxic effects at high doses [59]. The compounds are metabolized and biotransformed in the body. These processes can greatly affect the effects of the compound. For example, when quercetin is metabolized in the body, different products can be formed, some of which may be more toxic. The compound's targets and mechanisms of action can regulate a variety of biological responses. Binding of quercetin to targets such as AhR [60], ER [61], ER-LBD [62] and MMP [63] can affect different biological processes [64]. A study in 2010 emphasized the vulnerability of individuals with G6PD deficiency to oxidative stress and assessed the protective effects of the flavonoid quercetin against oxidative damage induced by H_2O_2. The research demonstrated that quercetin not only exhibits antioxidant properties but also offers cellular protection [43]. The investigations' findings in 2016, supported by numerous experiments, shed light on the mechanisms by which quercetin may protect against neurotoxicity, neuronal injury, and neurodegenerative diseases, offering potential therapeutic avenues for neurological disorders [65]. In a different 2018 research, quercetin's potential as an effective anticancer agent was highlighted. The study proposed that quercetin could influence O-GlcNAcylation, a process associated with cancer, warranting further investigation into its specific mechanisms for cancer treatment [66].

Moreover, recent findings have underscored the significant role of quercetin in combating liver cancer. Quercetin is shown to regulate intracellular processes, inhibiting the cell cycle and promoting apoptosis, which leads to the death of cancer cells. Consequently, this naturally occurring compound, found in plants, is being considered as a promising candidate for the development of novel anticancer drugs [58]. Molecular level analyses and predictions applied used the drug development process were applied to the phytochemical drug candidates in *V. officinalis*. Quercetin, the structure that computer-based analyses lead us to, has been shown to be effective in fighting liver cancer in *in vivo* experiments.

Within the scope of the study, in order to evaluate the potential of the new complex and direct it to clinical research; In addition to *in vitro* studies examining its effects on liver cancer cell lines in more detail, experiments can be conducted to evaluate the effects of the complex *in vivo* models. Optimization of the structure should also be provided to increase the effectiveness and safety of the drug.

AUTHOR CONTRIBUTIONS

Concept: H.A.; Design: H.A.; Control: H.A.; Sources: H.A.; Materials: H.A.; Data Collection and/or Processing: H.A.; Analysis and/or Interpretation: H.A., A.Ö; Literature Review: H.A.; Manuscript Writing: H.A., A.Ö.; Critical Review: H.A., A.Ö.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 993-1003, 2024
Doi: 10.33483/jfpau.1440531

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DESIGN AND EVALUATION OF PROPOLIS-LOADED BUCCAL PATCHES

PROPOLİS YÜKLÜ BUKKAL YAMALARIN TASARIMI VE DEĞERLENDİRİLMESİ

Ali ALAZZO[1]*, Amina Mudhafar AL-NIMA[1], Mohammed Nooraldeen AL-QATTAN[2]

[1]Mosul University, Faculty of Pharmacy, Department of Pharmaceutices, Mosul, Iraq
[2]Nineveh University, Faculty of Pharmacy, Department of Pharmaceutical science, Mosul, Iraq

ABSTRACT

Objective: *Propolis is highly recommended in aphthous stomatitis, a condition that requires a proper delivery tool to achieve an efficient treatment. For this indication, a buccal patch that provides prolonged mucosal contact and protection would be beneficial. Accordingly, in this study, we designed propolis-loaded buccal patches, composed of three polymers (alginate, carboxymethylcellulose, and polyvinylpyrrolidone) of complementary properties, and the proper combination of them to produce the patch of optimum properties.*
Material and Method: *Nine patches of different polymer ratios were prepared by casting method and evaluated by assessing their swelling, adhesion time and strength, and dissolution rate. Then the patches properties were correlated using artificial neural network analysis.*
Result and Discussion: *The results showed that all patches were smooth, translucent, and flexible with surface pH between 6.5 and 7.4. The correlation between polymer composition and measured properties was complex and non-linear. Therefore, an artificial neural network was used to analyze these properties and optimize them. The model of this analysis provides higher weights for favorable tensile and adhesion strengths while considering swelling, rigidity, and fast dissolution rate as unfavorable. According to summation analysis, the combination of CMC% and PVP% of 15.5 and 13, respectively, provides the best score of 3.5.*
Keywords: *Alginate patches, buccal patches, mucoadhesive polymers, propolis*

ÖZ

Amaç: *Etkili bir tedaviye ulaşmak için uygun bir uygulama aracı gerektiren bir durum olan aftöz stomatitte propolis kesinlikle önerilir. Bu endikasyon için uzun süreli mukozal temas ve koruma sağlayan bukkal yama faydalı olur. Buna göre bu çalışmada, tamamlayıcı özelliklere sahip üç polimerden (aljinat, karboksimetilselüloz ve polivinilpirolidon) oluşan propolis yüklü bukkal yamaları ve bunların optimum özelliklere sahip yamayı üretmek için uygun kombinasyonu tasarlanmıştır.*
Gereç ve Yöntem: *Döküm yöntemiyle farklı polimer oranlarına sahip dokuz yama hazırlanmış, şişme, yapışma süresi ve gücü ile çözünme hızı açısından değerlendirilmiştir. Sonra yamaların özellikleri yapay sinir ağı analizi kullanılarak ilişkilendirilmiştir.*
Sonuç ve Tartışma: *Sonuçlar, tüm yamaların pürüzsüz, yarı şeffaf ve esnek ile yüzey pH'ının 6,5 ile 7,4 arasında olduğunu göstermiştir. Polimer bileşimiyle ölçülen özellikler arasındaki korelasyon karmaşık ve doğrusal değildir. Bu nedenle bu özellikleri analiz ve optimize etmek için yapay sinir ağı kullanılmıştır. Bu analizin modeli, uygun çekme ve yapışma gücü için daha yüksek ağırlıklar*

* **Corresponding Author / Sorumlu Yazar:** Ali Alazzo
e-mail / e-posta: alialazzo@uomosul.edu.iq, **Phone / Tel.:** +9647517014655

Submitted / Gönderilme : 17.03.2024
Accepted / Kabul : 14.07.2024
Published / Yayınlanma : 10.09.2024

sağlarken şişme, sertlik ve hızlı çözünme oranını olumsuz olarak değerlendirir. Toplama analizine göre, sırasıyla 15,5 ve 13'lük CMC% ve PVP% kombinasyonu, en iyi 3,5 puan sağlar.
Anahtar Kelimeler: *Aljinat yamalar, bukkal yamalar, mukoadezif polimerleri, propolis*

INTRODUCTION

Propolis, a bee product, is a traditional remedy with many health benefits. It contains different flavonoids and cinnamic acid derivatives that have several biological effects, including anti-bacterial, anti-fungal, antiviral, anti-inflammatory, antioxidant, and immune modulation effect [1–3]. Propolis, therefore, has been used successfully for many therapeutic purposes, particularly in dentistry and oral health. For example, it was effectively applied in the treatment of aphthous stomatitis and other ulcerative disorders of the mouth cavity [4]. Interestingly, the clinical outcomes in these medical conditions are highly reliant on the modes of delivery with best outcomes being associated with prolonged duration of action and good mucosal protection. Thus, using a controlled strategy of good mucosal adhesion, such as buccal patches, for the administration of propolis is thought to be advantageous.

The concept of buccal drug delivery is gaining increased attention in pharmaceutical science for its local and systemic advantages. It is a readily accessible route, provides prolonged contact with the oral mucosa, and relatively rapid absorption with efficient avoidance of the first-pass effect [5,6]. Many studies, therefore, have attempted to formulate various buccal delivery systems such as tablets, patches, strips, and gels [7,8]. However, buccal patches have shown good flexibility, accurate dosing, and better toleration by patients than other formulations. Also, patches provide a good physical barrier for protecting the oral mucosa from irritation, which is beneficial in many cases, mainly aphthous and mouth ulceration [9]. However, a variety of factors affect proper buccal drug delivery such as saliva flow, shearing forces due to tongue movements, and loss of adhering vehicle. Thus, such factors should be considered in the design of such dosage forms [10].

Buccal vehicles need to possess enhanced and extended adhesive properties. Hence, different mucoadhesive polymers, natural and synthetic, should be evaluated in designing buccal patches such as sodium alginate, hydroxypropyl methylcellulose, and poly acrylic acid. Many of these mucoadhesive polymers, although possessing excellent adhesive properties, have a high swelling ratio and poor mechanical properties [9], which reduce the adhesion time of buccal patches. However, the proper combination of polymers may be helpful to overcome these limitations.

Therefore, in this study, we aim to investigate the effect of various combinations, of sodium alginate, carboxymethylcellulose, and polyvinylpyrrolidone, on swelling ratio, adhesion strength, adhesion time, and dissolution rate for the propolis buccal patches. Due to the possible complex interaction between the polymers, an artificial neural network was used to correlate the ratios of polymers in combination with the measured properties.

MATERIAL AND METHOD

Materials

Propolis samples were collected from hives of honeybees in Babylon City / Iraq. Sodium alginate (SAG), LR- moderate viscosity grade, was obtained from Thomas Baker, Mumbai, India. Carboxymethylcellulose sodium salt (CMC) and polyvinylpyrrolidone (PVP K-30) were procured from Quzhou Ebright Chemicals, China. Glycerin was purchased from Scharlab S.L., Spain. Ethanol was purchased from (Tedia Company, USA). All other used chemicals were obtained from BDH chemicals, UK**.**

Extraction of Propolis

The extraction procedure aimed to remove the waxy impurities, such as beeswax from the major active ingredients of propolis. Before starting the extraction, a frozen propolis was divided into small pieces using ordinary kitchen knife and ground into a fine powder using an electric grinder (Royal-Japan) for about 5 minutes at room temperature [2]. For extraction, 300 ml of 70% (v/v) ethanol were

mixed with 45g of the grounded propolis, shaked manually for 10 minutes, and left in a refrigerator for 3 days to ensure complete extraction. Then, the mixture was filtered, poured into glass Petri dishes, and allowed to dry at room temperature. The obtained propolis extract was then collected, weighed, and kept in a closed container in the refrigerator [3].

Formulation of Propolis-loaded Patches

The solvent casting method was used for the preparation of nine formulations of patches containing propolis (F1- F9) which were prepared by dissolving the calculated amounts of polymers in 25 ml distilled water. The dispersion of the three polymers was stirred using a magnetic stirrer (Fisher Scientific, Korea) for nearly 4 hours with the assistance of heat (50°C) at 350 rpm, and glycerin as plasticizer was also added under constant stirring. After cooling, the polymer dispersion was left overnight to allow air bubbles to be expelled. The 65 mg of propolis extract was weighed, dissolved in 1 ml of 90% ethanol, and added to the polymer dispersion under stirring (350 rpm, 1 hour) until a homogeneous mixture was formed. The final mixture was poured into glass Petri dishes having 9.4 cm diameter, which were kept in the oven at 35 ± 5°C for 24 hours. Finally, these dishes were wrapped with aluminum foil and stored at room temperature [7].

Evaluation of the General Properties of the Formulated Propolis-loaded Patches

All the measurements were carried out on three different pieces of each formulation; each piece had an area of 1.8 cm^2.

Thickness and Uniformity of Mass

Thickness was measured using a digital micrometer caliper (Ditron, China), while the mass measurements were performed by Equinox Analytical and Semi-Micro Balances from ADAM Scales and Balances [11].

Folding Endurance Test

Each formulated patch was repeatedly folded until breaking or folding up to 300 times, which is considered acceptable to reveal good patch properties [7].

Surface pH

Each patch's formulation was allowed to swell by keeping it in contact with 3-5 ml of distilled water for 1 hour at room temperature. The pH was measured by dipping the electrode into the swollen patch and allowing it to stand for 1 minute. The tests were carried out in triplicate, and average values were reported [12].

Evaluation of Mucoadhesive Strength

The adhesion strength of all the formulated patches (F1-F9) was evaluated by a texture analyzer (HD plus, Stable Micro System, Surrey, UK) using a surface of gelatin (6.67% w/v) covered with mucin solution (2% w/v) to represent the buccal mucosa surface [13]. Briefly, the propolis-loaded patch of size 1.8 cm^2 was fixed to the arm of the texture analyzer using cyanoacrylate adhesive (Hopson Chemical Industry Limited, China) and the exposed surface of the patch was kept in contact with the gelatin surface for 60 sec before starting the test for initial hydration and adhesion. The force, required to detach the patch from the gelatin surface, was considered to assess the adhesion strength [14].

***In vitro* Adhesion Time**

The *in vitro* adhesion time was measured for all the formulated patches (F1-F9) using a modified device consisting of a magnetic stirrer (Fisher Scientific, Korea) with a heating jacket supplied at about 37 ± 0.5°C. The medium was phosphate buffer, pH 6.8. A piece of silicone rubber (4 cm width and 1.5 cm length) was attached vertically to the inner surface of a 250 ml beaker. The formulated buccal patch was hydrated from one surface using a few drops of phosphate buffer, and attached to the silicone rubber. The magnetic stirrer was then started (125 rpm, 37.0±0.5°C), with the formulated patch completely immersed in the buffer. The time required for each patch to erode or separate from the silicone

rubber was recorded [15].

Swelling Study

A pre-weighed square-shaped glass slide (2×2) cm^2 was used to weigh a formulated propolis-loaded patch with a diameter of 1.3 cm (all formulations F1-F9 were tested). It was kept in a Petri dish covered with 50 ml of pH 6.8 phosphate buffer. After every 5 min, up to 30 min, the glass slide was removed and weighed using a stopwatch. The percentage of weight increment due to absorption of water and swelling of the propolis-loaded patch was recorded as a swelling percent [14].

Tensile Testing

The tensile properties of the formulated propolis-loaded patches were analyzed using a texture analyzer. The patches were cut into strips of fixed length using a template shaped like a dumbbell. Then, they were fixed on the texture analyzer and evaluated using a stretching speed of 2mm/sec. The elongation at break (%), tensile strength, and elastic modulus were calculated using the following equations [16].

$$\textbf{Percent of elongation at break} = (L_f - L_i)/L_i * 100 \quad \ldots\ldots\ldots (1)$$

$$\textbf{Tensile strength} = F/A \quad \ldots\ldots\ldots (2)$$

$$E = \sigma/\epsilon \quad \ldots\ldots (3)$$

Where **Lf** is the length of the specimen when it breaks, **Li** is the initial length of the specimen, **F** is the peak force at break, **A** is the cross-sectional area of the specimen, **E** is the elastic modulus, σ is the stress, ϵ is the strain.

***In vitro* Release Study**

The release of propolis was evaluated using the paddle dissolution apparatus. The propolis-loaded patch (1.8 cm^2) was applied to a glass disk and fixed at the bottom of the dissolution vessel. The experiment was performed using phosphate buffer (400 ml, pH 6.8) at 37 ± 0.5°C and a rotation speed of 50 rpm. At each time interval, samples of 3 ml were collected and replaced with phosphate buffer pH (6.8). Then the samples were filtered and measured spectrophotometrically at 278 nm [17].

Use of Artificial Intelligence to Find Correlations Between Observations and Settings and Training of Neural Networks

Using Matlab R2017b, an individual feed-forward neural network was trained to reproduce each of the individual physical measures. Each network is composed of the input layer, hidden layer, and output layer. For all networks, the input layer is composed of two neurons to accept the values of PVP% and CMC%, while the output layer is composed of a single neuron to provide the value of a particular physical measurement. The number of hidden neurons was varied to be 5, 6, 7, and 10 for the networks that reproduce force of adhesion, dissolution rate, rigidity-tensile strength, and water-alcohol swelling, respectively. The network was trained with a learning rate of 0.3. The activation functions for hidden and output neurons were Log-sigmoid and linear functions, respectively. The dataset used for training was randomly divided to include 90% of the data for training and 5% of data for each testing and validation. Due to the small dataset size (nine formulations), it was difficult to reproduce the same surface of correlation from the same neural network in repeated training runs. Therefore, to get a consistent surface of correlation, the training process was repeated 10 times and the average trained weights were used.

RESULT AND DISCUSSION

Good adhesion and mechanical properties are very important for buccal patches intended for mouth ulcers to provide long mucosal contact and good physical protection. Therefore, and because the properties of patches are a function of the type and concentration of their components, three polymers, namely SAG, CMC, and PVP were selected in this study to formulate propolis-loaded buccal patches as these polymers have complementary properties.

A natural polysaccharide, SAG, was used to build up the backbone of the patches. It is a safe, biocompatible, biodegradable, and hydrophilic polymer; however, its properties include low cell adhesion and mechanical strength [18,19]. On the other hand, CMC, an ether derivative of cellulose, has shown very good adhesion properties but its water solubility and swelling ratio is high and should be controlled for good buccal patch properties [11]. PVP is a synthetic polymer of good flexibility and mechanical strength. It has considerable hydrophobic groups and, therefore, a low swelling ratio, which helps it improve the patch's mechanical characteristics and regulate CMC's swelling ratio [7].

Formulation of Propolis-loaded Patches

Nine propolis-loaded patches of different polymer compositions are provided in Table 1. The physical dimensions of the prepared patches were consistent and had a narrow range of thickness (0.29 ± 0.03 mm) and mass (79.7 ± 8.5 mg) as shown in Table 2. All the patches showed the desired appearance of smoothness, translucency, flexibility, uniformity, and folding endurance (more than 300 times). The surface *p*H values for all the patches were between 6.5 and 7.4, which suggests that they had a good likelihood of causing no irritation to the buccal mucosa.

Table 1. Composition of the formulated propolis buccal patches with their assigned batch codes

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Propolis (mg)	65	65	65	65	65	65	65	65	65
***SAG (mg)**	1000	1000	1000	1000	1000	1000	1000	1000	1000
***CMC (mg)**	50	150	250	50	150	250	50	150	250
***PVP (mg)**	50	50	50	250	250	250	400	400	400
Glycerin (mg)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25

*SAG: sodium alginate, *CMC: carboxymethylcellulose, *PVP: polyvinylpyrrolidone

The physical measurements of the nine propolis-loaded patches are provided in Table 2. Principally, patches of low PVP content showed a higher force of adhesion compared to other patches. The first three patches (F1-F3) of low PVP composition showed higher force and time of adhesion relative to the other patches. The composition of 150 mg of CMC showed the highest force of adhesion of all patches.

Table 2. The general properties and mechanical measurements of the formulated propolis-loaded patches

	Thickness (mm)	**Mass (mg)**	**pH**	**Force of adhesion (N)**	**Adhesion time (min)**	**Tensile strength (g/mm^2)**	**% of elongation**	**Modulus of elasticity**
F1	0.30	83.1	7.4	0.81	117.5	55.5	83.0	56.2
F2	0.30	94.3	6.6	0.92	130.0	111.1	133.0	79.7
F3	0.32	85.7	7.4	0.84	145.0	130.2	92.0	125.5
F4	0.24	69.9	6.5	0.69	55.0	83.3	197.0	33.2
F5	0.29	73.0	7.1	0.72	140.0	137.9	200.0	64.5
F6	0.26	67.6	7.3	0.66	70.0	179.4	180.0	95.3
F7	0.32	79.5	7.2	0.78	30.0	52.1	158.0	24.3
F8	0.30	85.3	7.0	0.84	25.0	100.0	155.0	61.6
F9	0.31	78.8	7.4	0.77	25.0	86.0	88.0	84.6

The adhesion is a function of the interaction and entanglement of hydrophilic polymer chains with mucosal mucus [20]. Considering the fact that the carbonyl group in PVP is a strong proton acceptor that can easily interact with other polymers and forms complexes [21], then the interaction of PVP with CMC and SAG would possibly lower the mobility and flexibility of these polymers and so their entanglement with mucin. These results were in line the data reported by Patel et.al [22], who studied

the effect of PVP on the physicochemical properties of chitosan buccal patches. They found that the increasing the concentration of PVP produces patches with less adhesion force and time. A similar effect of PVP was also reported with buccal patches composed of Carbopol 934 and Eudragit-100 [23].

Regarding the mechanical properties, the tensile strength was increased with increasing concentration of CMC, except for F9, which had a high concentration of PVP. Also, F5 and F6 of intermediate PVP concentration showed the highest tensile strength. Similarly, the highest elongation was reported with F4 - F6 having intermediate concentrations of PVP. It was clear that the elasticity modulus was directly proportional to the concentration of CMC and inversely proportional to the concentration of PVP. These parameters determined the softness, hardness, and flexibility of the patches and are directly related to the intermolecular forces between the polymer's network [24]. The findings suggest that the introduction of PVP resulted in a noticeable increase in the elasticity of the patches. Conversely, the incorporation of CMC led to a transformation of the patches into a stiffer material with a higher modulus of elasticity.

The effect of the polymers on the swelling of the patches is shown in Figure 1. The highest swelling index was observed at F5, which had an intermediate concentration of PVP and CMC. It can be noted that the first three patches, F1-F3, with low PVP concentration and low elasticity, presented a low swelling index as these patches showed a high degree of erosion during the swelling study. Also, the swelling of high PVP patches, F8 and F9, significantly increased after 20 min, which is more likely related to the high elasticity of these patches that maintains the patches intact and able to accommodate more water without erosions over the swelling study in contrast to the low PVP patches of low elasticity and high degree of erosion during the swelling study. This effect of PVP is useful to provide good physical protection for the mouth ulcer; however, with a shorter adhesion time. These patches, with high PVP patches, have matrices of more polymers' interaction or cross-linked points and less free polymer chains, which would result in weak adhesion properties.

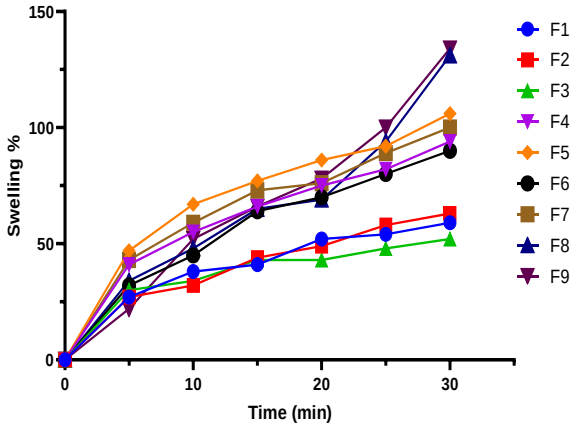

Figure 1. Swelling index of the formulated propolis-loaded patches

Furthermore, the release of propolis from different patches is shown in Figure 2. The patches with high PVP concentration achieved a slower release than the others. The release of drugs from buccal patches is function to several factors. One key factor is the swelling rate, the hydrophilic polymers such as PVP, CMC, and SAG facilitate the wettability, water absorption, and then swelling, which allow the loaded drug to dissolve and diffuse out. However, this can be regulated by the polymers entanglement and crosslinking of the polymers, which affect the relaxation and, consequently, the swelling of the patches. Another key factor that should be considered is the susceptibility of the patch matrix to erosion [25,26].

Therefore, the slow release of propolis obtained with high PVP patches is likely due to the tightly crosslinked polymers of these patches and the slower erosion rate as suggested by the mechanical measurements. In contrast, the patches with loosely bound polymers, such as low PVP patches, were readily eroded, allowing the easy release of propolis.

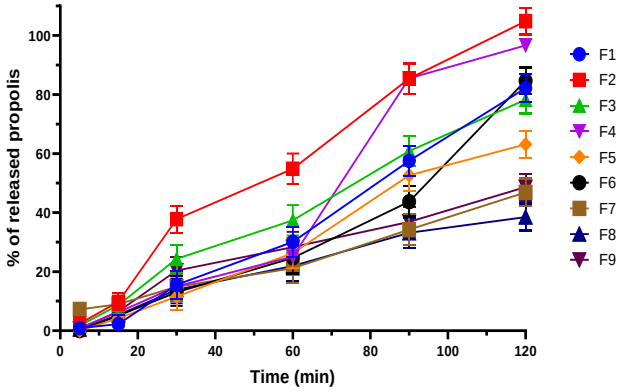

Figure 2. The percentage of propolis released from different patches

Correlation of Patch Composition with Experimentally Measured Physical Values Using Neural Networks

Correlations between composites and physicochemical measures are valuable in optimizing pharmaceutical formulations. This optimization is a multi-objective problem, where multiple properties need to be simultaneously adjusted. Accordingly, neural networks can perform well in such tasks to find linear as well as non-linear correlations that can be used to predict, characterize, and optimize pharmaceutical formulations [27]. Neural networks have found application in formulations of solid, liquid, and other dosage forms [28]. The applications include optimizing stability, loading power, dissolution, particle size, drug release, etc. during the formulation of different pharmaceutical dosage forms [29]. Recently, the neural network approach has been applied in the formulation of topical patches and hydrogel to predict drug release[30], viscosity, and sol-gel transition time [20].

The correlation between patch polymer composition and physically measured properties was thought to be complex and non-linear due to the possible intermolecular interactions [31,32]. Therefore, a neural network was trained to simulate the correlation between patch composition and each of the force of adhesion, tensile strength, rigidity, dissolution rate as well as swelling in water. The trained neural networks were used to visualize correlation surfaces for experimental data and provide predictions for an optimum formulation.

As illustrated in Figure 3, the correlation surface for tensile strength shows that the PVP% of 15 provides the best measure which is improved by increasing CMC%. While rigidity correlates with CMC%; however, it decreases as PVP% is increased. The patch swelling after 30 min was increased mainly by increasing PVP% and the highest release rate of 1.45 was expected to occur at PVP% and CMC % of 28.5 and 19, respectively. For tensile strength, the optimum value of 185 is expected to occur at PVP% and CMC% of 17.3 and 14.4, respectively. For dissolution rate after 60 min, the ratio of CMC to PVP of 0.6 provides the highest release of 29% propolis content of patches compared to other ratios.

This could indicate the formation of a specific intermolecular arrangement at this composite ratio that has a lower interaction with propolis extract. In order to incorporate all the observed correlations in guiding future formula design, a summation graph was calculated, Figure 4. The graph was obtained by normalizing z values of individual graphs to be between 0 and 1 before making a summation of matrices using the following model.

Summation = 2TS + 3AS - 1S - 1R - 1DR

Where TS is the tensile strength, AS is the adhesion force, S is the swelling in water, R is the rigidity of the patches, and DR is the dissolution rate.

The model provides higher weights for favorable tensile and adhesion strengths while considering water swelling, rigidity, and dissolution rate as unfavorable. According to the summation graph, the combination of CMC% and PVP% of 15.5 and 13, respectively, provides the best score of 3.5 on the graph.

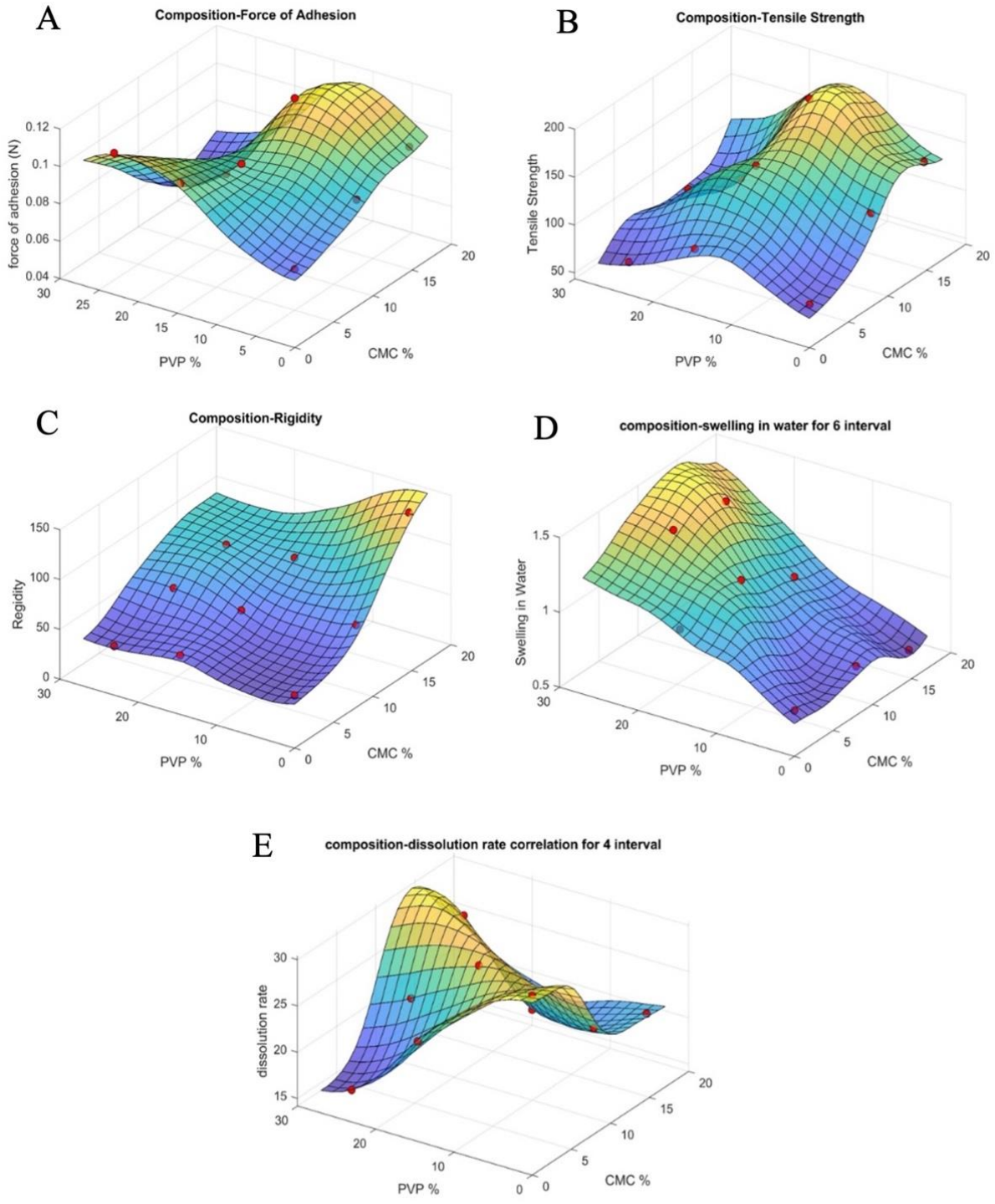

Figure 3. The predicted correlations between patch composition (only percentages of CMC and PVP are shown) and (A) the force of adhesion, (B) the tensile strength, (C) the rigidity of patches, (D) the swelling in water, and (E) the dissolution rate. The red dots represent the experimentally measured values used to train the neural network which in turn was used to construct the correlation surface

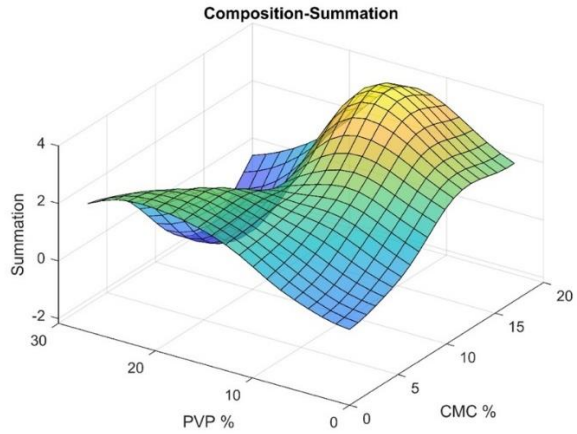

Figure 4. The predicted surface of correlation to find optimum formulation composition. The surface represents the summation of wanted properties (tensile strength, force of adhesion) minus the summation of unwanted properties (water swelling, dissolution rate, rigidity)

In summary, in this research we examined in this research the impact of different polymer combinations, including SAG, CMC, and PVP, on the physical characteristics of propolis-loaded patches. Overall, our findings indicate that higher CMC concentration improved adhesion properties, while PVP resulted in more flexible and durable patches. However, the relationship between polymer ratios and measured properties was complex and not easily assessed using conventional analysis tools. Consequently, we utilized neural network analysis to optimize the patches we prepared. The analysis revealed that a combination of 15.5% CMC and 13% PVP achieved the highest score of 3.5. Therefore, this particular combination offers the optimum adhesion and mechanical protection, as well as prolonged release of propolis.

ACKNOWLEDGEMENTS

The authors express their gratitudes to the College of Pharmacy/ University of Mosul for their assistance and for providing access to the laboratory services.

AUTHOR CONTRIBUTIONS

Concept: A.A., A.M.; Design: A.A., A.M., M.N.; Control: A.A.; Sources: A.A., A.M., M.N.; Materials: A.A., A.M., M.N.; Data Collection and/or Processing: A.A., A.M., M.N.; Analysis and/or Interpretation: A.A., A.M., M.N.; Literature Review: A.A., A.M., M.N.; Manuscript Writing: A.A., A.M., M.N.; Critical Review: A.A., A.M., M.N.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1004-1010, 2024
Doi: 10.33483/jfpau.1496909

ORIGINAL ARTICLE / ÖZGÜN MAKALE

MOLECULAR ANALYSIS OF THE MCR-1 GENE IN *PSEUDOMONAS AERUGINOSA* AND *ACINETOBACTER BAUMANII* STRAINS

PSEUDOMONAS AERUGINOSA VE ACINETOBACTER BAUMANII SUŞLARINDA MCR-1 GENİNİN MOLEKÜLER ANALİZİ

Ömer AKGÜL[1]*

[1]Van Yüzüncü Yıl University Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 65080, Van, Türkiye

ABSTRACT

Objective: *The emergence of antibiotic resistance in Pseudomonas aeruginosa and Acinetobacter baumanii isolates poses serious risks to public health. Our study aimed to investigate the presence of colistin resistance and mcr-1 gene positivity in these isolates.*
Material and Method: *Culture, biochemical tests, antibiotic susceptibility tests, and molecular tests were used to isolate and identify P. aeruginosa and A. baumanii strains.*
Result and Discussion: *A sum of 156 clinical isolates of Pseudomonas aeruginosa (n = 89) and Acinetobacter baumannii (n = 67) were obtained using the 550 clinical samples collected in one year from the largest hospital in Van, Turkey. The results of antibiotic susceptibility tests showed that approximately 82.8% of P. aeruginosa and 94.6% of A. baumannii strains were multidrug-resistant (MDR). Colistin resistance was detected in 11.23% (10/89) of P. aeruginosa isolates and 11.94% (8/67) of A. baumannii isolates using agar dilution and microdilution methods. Out of the 18 colistin-resistant isolates, the mcr-1 gene was detected in three P. aeruginosa and two A. baumannii strains. The detection of plasmid-mediated colistin resistance in P. aeruginosa and A. baumannii is of great concern due to the high potential for colistin resistance to spread in clinical settings. Understanding the unique circumstances of worldwide colistin resistance can be facilitated by promoting the creation of quick processes for identifying colistin resistance profiles and putting them into practice in hospital laboratories. Colistin and carbapenem treatment are two effective ways to treat emerging resistant super-microbes and slow down the emergence of resistance.*
Keywords: *Acinetobacter baumannii, antibiotic resistance, mcr-1, Pseudomonas aeruginosa*

ÖZ

Amaç: *Pseudomonas aeruginosa ve Acinetobacter baumanii izolatları içerisinde birçok antibiyotiğe direnç meydana gelmesiyle birlikte halk sağlığı açısından ciddi riskler ortaya çıkmıştır. Çalışmamızın amacı bu suşlar içerisinde kolistin direnci varlığı ile mcr-1 geni pozitifliğinin araştırılmasını hedeflemektedir.*
Gereç ve Yöntem: *P. aeruginosa ve A. baumanii suşlarının izolasyonu ve identifikasyonu amacıyla farklı yöntemler kullanılmıştır. Bunlar sırasıyla kültür, biyokimyasal testler, antibiyotik duyarlılık testleri ve moleküler testlerdir.*
Sonuç ve Tartışma: *Türkiye'nin Van kentindeki en büyük hastaneden bir yılda toplanan 550 klinik örnekten tam olarak 156 klinik P. aeruginosa (n = 89) ve A. baumannii (n = 67) izolatı elde edildi. Yapılan antibiyotik duyarlılık testleri araştırma sonuçlarına göre yaklaşık %82.8 P. aeruginosa ve*

* **Corresponding Author / Sorumlu Yazar:** Ömer Akgül
e-mail / e-posta: o.akgul@yyu.edu.tr, **Phone / Tel.:** +905077062438

Submitted / Gönderilme : 06.06.2024
Accepted / Kabul : 14.07.2024
Published / Yayınlanma : 10.09.2024

*%94.6 A. baumannii suşlarının MDR olduğu görüldü. Agar dilüsyon ve mikro dilüsyon yöntemleri kullanılarak P. aeruginosa izolatlarının %11.23'sinde (10/89) ve A. baumannii izolatlarının %11.94'unda (8/67) kolistin direnci keşfedildi. Kolistine dirençli 18 izolat arasında mcr-1 geni, üç P. aeruginosa ve iki A. baumannii suşunda tespit edildi. P. aeruginosa ve A. baumannii'da plazmit aracılı kolistin direncinin meydana gelmesi, kolistin direncinin klinik alanlarda yayılma eğiliminin yüksek oranda olması nedeniyle oldukça önemlidir. Küresel kolistin direncinin özel durumunun anlaşılması için kolistin direnç profillerinin tespitine yönelik hızlı prosedürlerin geliştirilmesi ve bu prosedürlerin hastane laboratuvarlarında uygulanması teşvik edilmelidir. Kolistin ve karbapenemin kombinasyonu ile oluşturulacak terapinin uygulanması, direnç gelişimi sürecinin hızının azaltılmasına ve ortaya çıkan bu dirençli süper mikropların tedavisinde yardımcı ola*bilir.

Anahtar Kelimeler: *Acinetobacter baumannii, antibiyotik direnci, mcr-1, Pseudomonas aeruginosa*

INTRODUCTION

Due to the absence of new antibiotic discoveries, drug-resistant (MDR) Gram-negative bacteria have become more common. These bacteria include members of the Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. It is critical that this matter be handled as soon as possible [1]. Following the addition of carbapenem-resistant *P. aeruginosa* and *A. baumannii* to the list of major killer infections in urgent need of antibiotics and novel modes of action, the World Health Organization (WHO) recognized growing concerns around MDR in February 2017 [2,3].

P. aeruginosa and *A. baumannii* are classified as ESKAPE pathogens, along with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Enterobacter* spp. These pathogens are responsible for nosocomial infections, including bacteremia, skin infections, urinary tract infections, soft tissue infections, and ventilator-associated infections [4,5]. It can also lead to severe health issues in individuals who are already ill or have weakened immune systems [4,6,7]. The mentioned pathogens are found to be resistant to most significant antibiotic types, such as carbapenems, beta-lactams, fluroquinolones, and aminoglycosides, making appropriate treatment difficult [8]. This presents significant challenges for physicians treating infections caused by MDR *P. aeruginosa* and *A. baumannii* strains. As a result, the WHO has included colistin in the group of "antibiotics of last resort" that can be used to treat these newly discovered super-microbes [9].

Colistin, a member of the polymyxin family of antibiotics, is often referred to as polymyxin E. Although it was developed in the 1950s, its usage was outlawed in a number of nations because of its detrimental effects on human health, especially renal function. Nevertheless, after a considerable amount of time, colistin was once again utilized in clinical practice to treat infections brought on by the carbapenem-resistant strains of *A. baumannii*, *P. aeruginosa*, and Enterobacteriaceae [10,11].

Many mechanisms contribute to colonistin resistance in Gram-negative bacteria. The most frequent way that Gram-negative bacteria become resistant to colistin is by genetic mutations [12-14]. Resistance resulting from differentiation in the lipo-polysaccharide layer and phospho-ethanolamine transferase binding to the phosphate group in the lipid A structure, produced by the two-component regulatory systems PhoP-PhoQ and PmrA-PmrB, is a major issue [15-17]. Although PhoP/PhoQ is responsible for colistin resistance in *K. pneumoniae* and *P. aeruginosa*, in *A. baumannii*, the PmrA/PmrB component mediates the mechanism of colistin resistance [18,19].

Following the first report by Lui et al. [23] on the plasmid-mediated *mcr-1* gene, numerous bacterial species have been the subject of research [20-27]. These species include *K. pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella*, *Cronobacter sakazakaii*, *Moraxella*, *Kluyvera*, *Shigella sonnei*, and *Citrobacter*. The plasmid-mediated *mcr-1* gene in MDR *P. aeruginosa* and *A. baumannii* is currently the subject of scant global data. Since our region lacks information on these two MDR bacterial species, our goal was to find the *mcr-1* gene in them.

MATERIAL AND METHOD

Sample Collection and Microbiological Analysis

From January to December 2022, the microbiology laboratories of Van Training and Research

Hospital obtained 156 clinical isolates of *A. baumannii* (n = 67) and *P. aeruginosa* (n = 89) from various samples, including blood, urine, ulcer swabs, and respiratory secretions. The samples were then transferred to the Microbiology laboratory at Van Yüzüncü Yıl University's Faculty of Pharmacy in a cold chain environment for further analysis. Prior to inoculation, the media underwent incubation in an autoclave to ensure sterility. The sterility of the media was confirmed by transparent plates without any microbial contamination. The samples were inoculated on MacConkey agar, Cystein Lactose Electrolyte Deficient (CLED) agar, chocolate agar, and blood agar media. The plates were then incubated at 37°C for 24 hours. All isolates were identified as *P. aeruginosa* and *A. baumannii* based on colony morphology, Gram staining results, and the API-10S system (bioMérieux, France). The bacterial isolates were preserved in Luria-Bertani broth medium (Oxoid, UK) supplemented with 30% glycerol and stored at -20°C until further analysis.

Antibiotic Susceptibility Tests

The Kirby-Bauer disk diffusion method was used to test the antibiotic susceptibility of all isolates. This was performed on Müller-Hinton agar (MHA) plates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [28]. To determine the MDR status of *P. aeruginosa* and *A. baumannii* isolates, Aztreonam, amikacin, ciprofloxacin, levofloxacin, cefepime, cefotaxime, imipenem, and piperacillin/tazobactam were used. The results were compared with CLSI practice guidelines. Colistin resistance was demonstrated through agar dilution and liquid medium micro-dilution methods using colistin sulfate powder (Sigma-Aldrich) [29]. The MIC results were interpreted according to the European Guidelines for Antimicrobial Susceptibility Testing (EUCAST) [30]. *E. coli* NCTC 13846 (*mcr-1* positive) strain was used as a positive control for sensitivity analysis of the tests.

DNA Extraction and Molecular Analysis

For molecular analysis, all colistin-resistant strains confirmed by phenotypic analysis were subjected to standard alkaline lysis method for plasmid DNA extraction [31]. The quantity of extracted DNA was measured using a micro volume spectrometer (Colibri, Titertek Berthold) and its quality was evaluated on a 1.5% agarose gel stained with ethidium bromide.

The *mcr-1* gene was amplified using gene-specific primers (MCR1-F: 5'-CGGTCAGTCCGTTTGTTC-3'; MCR1-R: 5'-CTTGGTCGGTCGGTCTGTAGGG-3') through conventional PCR, resulting in a 309 bp target gene region [32]. The protocol for DNA amplification of bacteria was carried out using the May Taq™ DNA Polymerase (Bioline, Bio-21105) kit. The Polymerase Chain Reaction (PCR) was performed using a set of chemical solutions and substances. The reaction mixture contained 10µl of 5x MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl2), 5µl of template DNA, 1µl of each primer (20uM), 1µl of MyTaq DNA polymerase, and 8µl of nuclease-free water, respectively, calculated as 25µl of the final solution. The PCR cycling conditions were as follows: denaturation at 94°C for 3 minutes (1 cycle), denaturation at 94°C for 30 minutes (25 cycles), annealing at 52°C for 30 seconds, initial extension at 72°C for 1 minute, and final extension cycle at 72°C for 10 minutes. The amplicon sizes were accurately analyzed using the HyperLadderTM marker (50 Base Pair, Bioline, USA). To visualize the bacterial amplicon products accurately, we operated a Thermo EC300XL2 electrophoresis device at 100 Volts on a 1.5% agarose gel for 1 hour. The Bio-Print-ST4 (Vilber Lourmant, France) was used to visualize the amplicons.

Our study was authorized by the Van Training and Research Hospital's clinical research ethics committee (decision dated 25/01/2018 and numbered 2018/02) to evaluate the accuracy of blood collection in patients.

RESULT AND DISCUSSION

Clinicians throughout the world are experiencing a great deal of comfort with the reintroduction of colistin as the newest treatment option for illnesses brought on by Gram-negative bacteria that is resistant to drugs. Colistin effectively treats illnesses brought on by MDR bacteria when taken either by alone or in conjunction with other antibiotics [33]. Colistin is not only used in humans but also in animals

for conditioning and in agriculture to increase yields. This usage has led to a significant rise in the incidence and prevalence of Gram-negative bacteria that are resistant to colistin [34].

A total of 156 clinical isolates were obtained from 550 clinical samples collected in one year from the largest hospital in Van, Turkey. Of these, 89 were identified as *P. aeruginosa* and 67 were identified as *A. baumannii* using API-10S test strips for microbiological identification of bacteria. Blood (43.6%), urine (27.1%), respiratory secretions (21.8%), and ulcer swab specimens (7.5%) were the sources of several *A. baumannii* isolates. In a similar manner, isolates of *P. aeruginosa* were obtained from blood samples (7.9%), urine (55%), wounds (25.8%), and stools (11.3%). The findings of the antibiotic susceptibility test showed that the strains of *P. aeruginosa* and *A. baumannii* exhibited a significant degree of multidrug resistance. Antibiotic susceptibility test results revealed that 94.6% of *A. baumannii* strains and 82.8% of *P. aeruginosa* strains were multidrug-resistant (MDR). Imipenem (41.2%) and cefotaxime (44.8%) showed the lowest resistance among *A. baumannii* isolates, whereas aztreonam (80.5%) and piperacillin/tazobactam (73.4%) showed the highest resistance. Antibiotic resistance to amikacin (92.3%) and aztreonam (85.8%) was highest in *P. aeruginosa* isolates, while antibiotic resistance to cefepime (27.8%) and imipenem (29.4%) was lowest. Table 1 provides a detailed list of all tested antibiotic percentages.

Table 1. Analysis of antibiotic resistance of *P. aeruginosa* and *A. baumanii strains*

Antibiotics	***P. aeruginosa***		***A. baumanii***	
	S (%)	**R (%)**	**S (%)**	**R (%)**
Aztreonam	14.2	85.8	19.5	80.5
Amikacin	7.7	92.3	43.3	56.7
Ciprofloxacin	23.9	76.1	27.8	72.2
Levofloxacin	43.2	56.8	29	71
Cefepime	72.2	27.8	33.8	66.2
Cefotaxime	20.6	79.4	55.2	44.8
Imipenem	70.6	29.4	58.8	41.2
Piperaciline/Tazobactam	20.6	79.4	26.6	73.4

Eighteen of the 67 (11.94%) *A. baumannii* isolates and ten (11.23%) *P. aeruginosa* isolates were among the eighteen colistin-resistant isolates discovered by the investigation. For isolates of *A. baumannii*, the MIC values varied between 8 and 16 μg/ml, while for isolates of *P. aeruginosa*, they ranged from 8 to 64 μg/ml. 43.6% of the isolates of *A. baumannii* were isolated from blood samples. India accounted for 45% of the reported *A. baumanniii* isolates, the bulk of which were recovered from wound swabs [35]. Our results are in line with the analysis of the same study, which found that 7% (7/100) of the isolates of *A. baumannii* were resistant to colistin. According to Oikonomou et al. [36], the MIC of colistin-resistant *A. baumannii* strains varied from 16 to 64 μg/ml, and the rate of colistin-resistant *A. baumanii* was 7% (86/1228). A different investigation discovered that 57% (12/21) of the isolates of *A. baumannii* were resistant to colistin, with MIC values ranging from 4 to >128 μg/ml [37]. The colistin-resistant *P. aeruginosa* isolates in our investigation had MIC values ranging from 8 to 64 μg/ml. This is not the same as the rate that Snesrud et al. [38] reported. Lescat et al. [37] reported 41.1% (7/17) of colistin-resistant *P. aeruginosa* isolates with MICs ranging from 4 to 128 μg/ml, which is in contradiction to our findings regarding colistin resistance and MIC.

Out of the eighteen isolates that were resistant to colistin, we found the *mcr-1* gene in three strains of *P. aeruginosa* and two strains of *A. baumannii*. The PCR picture of the *mcr-1* gene region is displayed in Figure 1. The *mcr-1* gene test results for the remaining 13 colistin-resistant isolates, however, were negative. This is the first report of *mcr-1* presence in *A. baumannii* and *P. aeruginosa*, as far as we are aware. However, prior research has demonstrated that these two clinically significant pathogenic bacteria have distinct mechanisms behind their colistin resistance. Chromosome mutations are the main cause of colistin resistance in *A. baumannii*. These mutations can be linked to changes in the outer membrane (such as those in pmr, lpx, lpsB, lptD, and vacJ) or unrelated to them (such as increased cell osmotic sensitivity and efflux pump activation) [39]. In the meantime, the main mechanisms linked to

the emergence of colistin resistance in *P. aeruginosa* are alterations in two-component regulatory systems [18]. It was recently discovered that *P. aeruginosa* harbors the *mcr-5* gene, which is encoded chromosomally. On the other hand, no cases of colistin resistance resulting from *mcr-1* gene mediated by plasmids have been recorded [38].

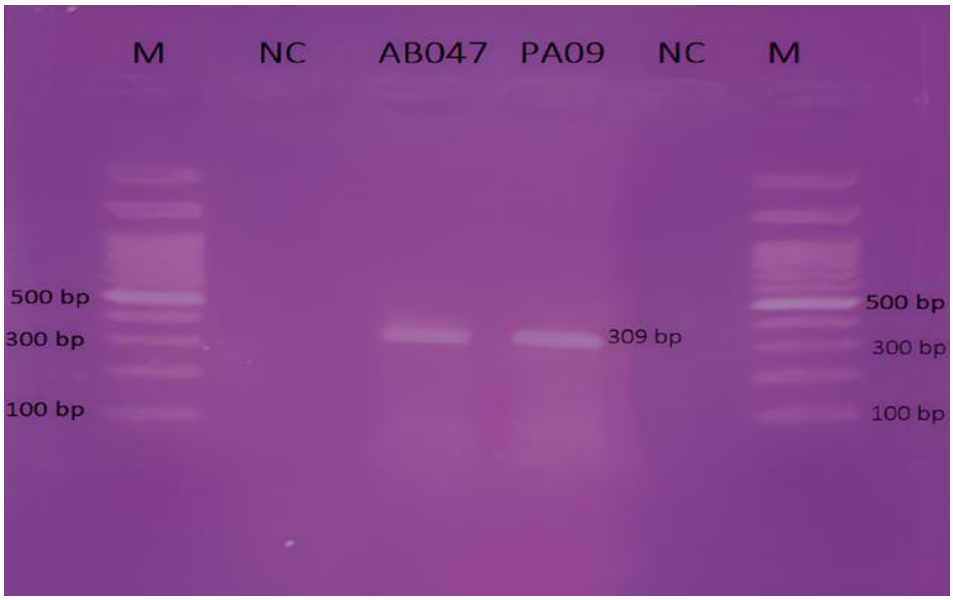

Figure 1. 309bp amplicon image of *P. aeruginosa* and *A. baumanii* strains obtained by PCR. M: 100bp marker; NC: Negative control; AB047: A. *baumanii* isolate; PA09: *P. aeruginosa* isolate

This is the first study to document the existence and frequency of the plasmid-mediated *mcr-1* gene in *A. baumannii* and *P. aeruginosa* isolated from different clinical samples in the Eastern Turkish province of Van. The only thing we looked at in these two infections was the presence of the *mcr-1* gene. As a result, we were unable to look into the processes underlying the remaining resistant strains' colistin resistance. Our results point to the necessity of additional experimental methods to identify plasmid-mediated colistin resistance in these two newly discovered pathogenic bacteria. Because of the great potential for resistance to spread in clinical settings, the presence of plasmid-mediated colistin resistance in *P. aeruginosa* and *A. baumannii* is noteworthy. To stop the spread of resistance, it is essential to carefully assess and set criteria for the usage of this medication used as a last-resort treatment. Understanding the unique circumstances of worldwide colistin resistance can be facilitated by promoting the creation of quick processes for identifying colistin resistance profiles and putting them into practice in hospital laboratories. Colistin and carbapenem treatment are two effective ways to treat emerging resistant super-microbes and slow down the emergence of resistance.

AUTHOR CONTRIBUTIONS

Conception: Ö.A.; Design: Ö.A.; Control: Ö.A.; Sources: Ö.A.; Materials: Ö.A.; Data Collection and/or Processing: Ö.A.; Analysis and/or Interpretation: Ö.A.; Literature Review: Ö.A.; Manuscript Writing: Ö.A.; Critical Review: Ö.A.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Our study was authorized by the Van Training and Research Hospital's clinical research ethics committee (decision dated 25/01/2018 and numbered 2018/02) to evaluate the accuracy of blood

collection in patients.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1011-1020, 2024
Doi: 10.33483/jfpau.1460173

ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARATIVE EVALUATION OF ARTIFICIAL INTELLIGENCE AND DRUG INTERACTION TOOLS: A PERSPECTIVE WITH THE EXAMPLE OF CLOPIDOGREL

YAPAY ZEKA VE İLAÇ ETKİLEŞİM ARAÇLARININ KARŞILAŞTIRMALI DEĞERLENDİRİLMESİ: KLOPİDOGREL ÖRNEĞİ İLE BİR BAKIŞ AÇISI

Zinnet Şevval AKSOYALP[1]*, Betül Rabia ERDOĞAN[1]

[1]Izmir Katip Celebi University, Faculty of Pharmacy, Department of Pharmacology, 35620, Izmir, Türkiye

ABSTRACT

Objective: *The study aims to compare the ability of free artificial intelligence (AI) chatbots to detect drug interactions with freely available drug interaction tools, using clopidogrel as an example.*
Material and Method: *The Lexicomp database was used as a reference to determine drug interactions with clopidogrel. ChatGPT-3.5 AI and Bing AI were selected as the free AI chatbots. Medscape Drug Interaction Checker, DrugBank Drug Interaction Checker and Epocrates Interaction Check were selected as free drug interaction tools. Accuracy score and comprehensiveness score were calculated for each drug interaction tool and AI chatbots. The kappa coefficient was calculated to assess inter-source agreement for interaction severity.*
Result and Discussion: *The results most similar to those of Lexicomp were obtained from the DrugBank and the ChatGPT-3.5 AI chatbot. The ChatGPT-3.5 AI chatbot performed best, with 69 correct results and an accuracy score of 307. ChatGPT-3.5 AI has the highest overall score of 387 points for accuracy and comprehensiveness. In addition, the highest kappa coefficient with Lexicomp was found for ChatGPT-3.5 AI chatbot (0.201, fair agreement). However, some of the results obtained by ChatGPT-3.5 AI need to be improved as they are incorrect/inadequate. Therefore, information obtained using AI tools should not be used as a reference for clinical applications by healthcare professionals and patients should not change their treatment without consulting doctor.*
Keywords: *Artificial intelligence, clopidogrel, drug interactions, patient safety, pharmaceutical databases*

ÖZ

Amaç: *Çalışmanın amacı, klopidogrel örneğini kullanarak ücretsiz yapay zekâ (AI) sohbet robotlarının ilaç etkileşimlerini saptama yeteneklerini ücretsiz olarak erişilebilen ilaç etkileşim araçları ile karşılaştırmaktır.*
Gereç ve Yöntem: *Klopidogrel ile ilaç etkileşimlerini belirlemek için referans veri tabanı olarak Lexicomp kullanılmıştır. Ücretsiz yapay zekâ sohbet robotları olarak ChatGPT-3.5 AI ve Bing AI, ücretsiz ilaç etkileşim araçları olarak ise Medscape Drug Interaction Checker, DrugBank Drug Interaction Checker ve Epocrates Interaction Check seçilmiştir. Her bir ilaç etkileşim aracı ve yapay zekâ sohbet robotu için doğruluk puanı ve kapsamlılık puanı hesaplanmıştır. Etkileşim şiddeti açısından kaynaklar arası uyumu değerlendirmek için kappa katsayısı hesaplanmıştır.*
Sonuç ve Tartışma: *Lexicomp veri tabanına en benzer sonuçlar Drugbank ve ChatGPT-3.5 AI*

* **Corresponding Author / Sorumlu Yazar:** Zinnet Şevval Aksoyalp
e-mail / e-posta: zinnetsevval.aksoyalp@ikcu.edu.tr, **Phone / Tel.:** +902323293535

Submitted / Gönderilme : 28.03.2024
Accepted / Kabul : 17.07.2024
Published / Yayınlanma : 10.09.2024

sohbet robotundan elde edilmiştir. ChatGPT-3.5 AI sohbet robotunun 69 doğru sonuç ve 307 doğruluk puanı ile en yüksek sonuçlara sahip olduğu bulunmuştur. Doğruluk ve kapsamlılık açısından 387 puanla en yüksek toplam puan ChatGPT-3.5 AI sohbet robotu sonuçları ile elde edilmiştir. Ayrıca Lexicomp ile en yüksek kappa skoru (0.201, orta düzey uyum) ChatGPT-3.5 AI chatbot için bulunmuştur. Ancak ChatGPT-3.5 AI ile elde edilen sonuçlardan bazılarının yanlış/yetersiz bulunması nedeniyle iyileştirilmesine ihtiyaç vardır. Sonuç olarak yapay zekâ araçlarından yararlanılarak elde edilen bilgiler sağlık profesyonelleri tarafından klinik uygulamalar için referans olarak kullanılmamalı ve hastalar doktora danışmadan tedavilerini değiştirmemelidir.

Anahtar Kelimeler: *Hasta güvenliği, ilaç etkileşimleri, ilaç veri tabanları, klopidogrel, yapay zekâ*

INTRODUCTION

Clopidogrel is an irreversible P2Y12 adenosine diphosphate receptor antagonist used to inhibit platelet activation and aggregation in patients with coronary artery disease, peripheral vascular disease, and cerebrovascular disease. These patients are often prescribed clopidogrel along with antihypertensive, antihyperlipidemic, and antidiabetic medications to reduce the risk of cardiovascular events [1]. Clopidogrel is a prodrug and is dependent on hepatic cytochrome P450 (CYP) metabolism for conversion to its active metabolite [2]; the therapeutic effect of clopidogrel may be increased or decreased by co-administration with other drugs that affect the CYP enzyme. The clinical outcomes of potential drug-drug interactions can be crucial in patients with coronary artery disease. Studies on the interaction of clopidogrel with other drugs have been conducted based on reviewing previous studies [1,3,4]. A paper on the evaluation of drug interactions with clopidogrel focused on studies of the interaction between clopidogrel with atorvastatin and omeprazole [1]. The interaction of clopidogrel with other drugs that induce and inhibit the CYP enzyme is also briefly discussed [1]. In another paper, the researchers reviewed studies of different drugs that interact with clopidogrel, including statins and proton pump inhibitors [3]. Several pharmacoepidemiological studies have been conducted to evaluate drug interactions with clopidogrel [5-9]; however, to our knowledge, studies have yet to compare these interactions using drug-drug interaction databases. Therefore, this study examined drug-drug interaction databases to introduce an assessment of the use of AIs in clinical practice, using the example of clopidogrel's interaction with other drugs.

Several databases and resources are available to help healthcare professionals and patients check for interactions in clinical practice and daily life. Some databases are free-accesible (Drugs.com, Epocrates, Medscape, RxList, and WebMD), and some are subscription-based (Lexicomp, Micromedex, and PEPID). However, these databases may vary in their information about drug-drug interactions [10-12]. The accessibility of these databases and the diversity of their content complicates the decision-making process for health professionals. There is a need to identify the most comprehensive, reliable, and freely accessible drug interaction database. Recently, there has been growing interest in using artificial intelligence (AI) to detect drug interactions [13-15]. As the use of AI chatbots becomes widespread, they are also becoming an increasingly popular source of information in healthcare. ChatGPT, Google Bard (now known as Gemine), and Microsoft Bing are the most popular AI chatbots. ChatGPT has both free version (ChatGPT-3.5) and commercial version (ChatGPT-4). This study aims to evaluate possible drug interactions with clopidogrel using both freely available drug interaction tools (Medscape, DrugBank, and Epocrates) and AI chatbots (ChatGPT-3.5 and Bing) and to compare the results obtained to test the capability of free AI chatbots.

MATERIAL AND METHOD

Data Collection

This study was conducted from April 25 to May 10, 2023. The list of drug interactions with clopidogrel was accessed from the UpToDate database [16], and a total of 84 cases of interaction with clopidogrel were identified. Interactions between clopidogrel and dietary supplements, nutrients, vitamins, and herbs, and repeated drug interactions were excluded from the analysis and were shown in yellow boxes in Figure 1. In addition, interactions of two drug classes (antiplatelet properties drugs and

calcium channel blockers) with clopidogrel were identified in the UpToDate database. Four drugs from antiplatelet properties drugs and two drugs from calcium channel blockers were selected and these drugs were shown in blue boxes in Figure 1. A total of 78 drugs were included in this study (Table 1).

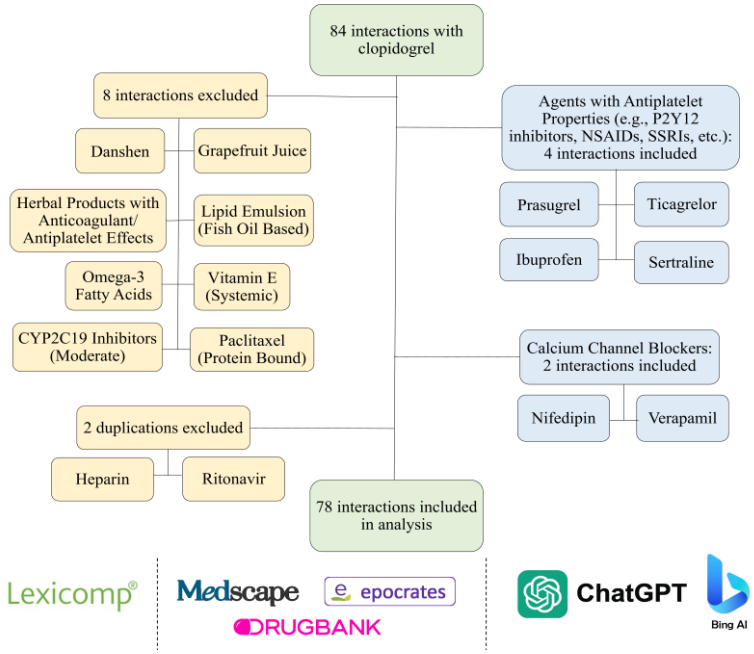

Figure 1. Flowchart for the inclusion/exclusion of drugs that interact with clopidogrel. Eighty-four cases of interaction with clopidogrel were identified in the UpToDate database. Interactions between clopidogrel and dietary supplements, nutrients, vitamins, and herbs, and repeated drug interactions were excluded from the analysis and were shown in yellow boxes. For the interactions of two drug classes (antiplatelet properties drugs and calcium channel blockers) with clopidogrel, selected drugs were shown in blue boxes. The interactions of clopidogrel with 78 selected medications were analyzed using Lexicomp, Medscape, Drugbank, Epocrates, ChatGPT-3.5 AI and Bing AI

Table 1. A list of 78 drugs that interact with clopidogrel was retrieved from the UpToDate database and included in the study

Abrocitinib	Cladribine	Enzalutamide	Lansoprazole	Pentoxifylline	Sodium Zirconium Cyclosilicate
Acalabrutinib	Cobicistat	Epoprostenol	Lecanemab	Pioglitazone	Talazoparib
Acetylsalicylic Acid	Collagenase	Erythromycin	Limaprost	Pirtobrutinib	Ticagrelor
Alpelisib	Dabigatran Etexilate	Esomeprazole	Morphine	Prasugrel	Tipranavir
Alteplase	Dabrafenib	Etravirine	Nifedipine	Repaglinide	Topotecan
Amodiaquine	Daprodustat	Fentanyl	Nirmatrelvir and Ritonavir	Rifampin	Treprostinil
Apixaban	Dasabuvir	Fluconazole	Obinutuzumab	Ritonavir	Tucatinib
Bemiparin	Dasatinib	Heparin	Omeprazole	Rivaroxaban	Ubrogepant
Berotralstat	Deoxycholic Acid	Ibritumomab Tiuxetan	Ozanimod	Rosuvastatin	Urokinase
Bupropion	Desloratadine	Ibrutinib	Paclitaxel	Selexipag	Verapamil
Cangrelor	Diamorphine	Icosapent Ethyl	Pantoprazole	Selumetinib	Vonoprazan
Caplacizumab	Edoxaban	Inotersen	Pazopanib	Sertraline	Warfarin
Cephalothin	Enoxaparin	Ibuprofen	Pentosan Polysulfate Sodium	Sibutramine	Zanubrutinib

Lexicomp (Wolters Kluwer, USA), a subscription-based drug interaction screening tool, was selected as the reference database and accessed through the library of Izmir Katip Celebi University. Firstly, drug interactions with clopidogrel were screened by the Lexicomp database. Then, three free software programs, Medscape (WebMD, USA), DrugBank (University of Alberta, USA), and Epocrates (Epocrates Inc., USA), were used to check the capability of ChatGPT-3.5 AI and Bing AI. These databases were chosen because they are available to the public free of charge. In this study, we focused on the free-accesible version of chatbots, which people more widely use due to their free access, so we included ChatGPT-3.5 AI and Microsoft Bing AI. Google Bard was excluded as it was used for testing during the study period. Free accounts were created on ChatGPT-3.5 AI and Bing AI and interacted with the AI-based language model to collect data. As both free AI chatbots have a maximum question limit of 24 hours, the drug interaction screening with AI chatbots was completed between 8 and 10 May 2023. A new conversation was started for each drug interaction question. Five questions were prepared based on information from Lexicomp, the reference database for drug interactions. Each pair of drug interactions was searched with five questions, and the answers were recorded for further analysis. The following questions were used:

"Can I take clopidogrel and X together?"
''What happens if I take clopidogrel and X together?''
''How should I take clopidogrel and X together?''
"What is the risk rating of interaction between clopidogrel and X?" and
"What is the severity of interaction between clopidogrel and X?".

In Lexicomp, the severity of the interaction is categorized as major, moderate, and minor. The results of the free drug interaction checkers and AI chatbots are also standardized as in Lexicomp. Drugs that were unavailable to these tools and AI chatbots were assumed not to interact. The true positive (TP), true negative (TN), false positive (FP), and false negative (FN) values of these databases and the AI chatbots assessing clopidogrel interactions were determined. A drug interaction defined as a major/moderate interaction in Lexicomp is defined as a TP if it is also a major/moderate interaction in other databases and is defined as an FN if it is a minor/no interaction in other databases. On the other hand, a minor interaction identified in Lexicomp is defined as TN if it is a minor/no interaction in other databases, while it is defined as FP if it is a major/moderate in other databases [11].

Statistical Analysis

The data were analyzed using descriptive statistics. The ability of the database to accurately detect major/moderate drug interactions is defined as the sensitivity, while the ability to ignore minor interactions is defined as the specificity [10]. Positive predictive value (PPV) is the probability that an interaction detected by the database is a significant interaction. The probability that interactions not detected by the database are insignificant is reported as negative predictive value (NPV). The sensitivity, specificity, PPV and NPV of these databases were calculated using the following equations [11]:

$$\text{Sensitivity} = TP / (TP + FN)$$
$$\text{Specificity} = TN / (FP + TN)$$
$$PPV = TP / (TP + FP)$$
$$NPV = TN / (TN + FN)$$

The accuracy score was calculated by multiplying the sum of the sensitivity, specificity, PPV and NPV values by 100, and the maximum accuracy score was 400 [10].

The results of drug interaction checkers and AI chatbots were also evaluated for severity level, onset, mechanism, risk rating, management, discussion, documentation level, references, clinical manifestations, and related drugs. Subsequently, the comprehensiveness score was calculated by multiplying the number of these items found in drug interaction checkers and AI chatbots by 13.4 [10]. As ten items are to be assessed, the maximum comprehensiveness score is 134.

In addition, kappa (κ) coefficients were used to assess the consistency of severity between the four drug-drug interaction tools and the two AI chatbots using SPSS (version 29.0, IBM, USA). To harmonize the severity of the interactions classified as major, moderate and minor in the Lexicomp

results with the results from other databases, minor and no interaction results were combined and scored as no interaction. Fleiss' kappa coefficient was calculated to assess the agreement between all drug-drug interaction tools and AI chatbots in terms of the severity of drug interactions with clopidogrel. Cohen's kappa coefficient was calculated to pairwise compare drug interaction tools and AI chatbots. A kappa coefficient of 1 indicates perfect agreement, -1 indicates perfect disagreement, and 0 indicates agreement that would be expected by chance. Kappa coefficient < 0.0 indicates poor agreement, 0.0 - 0.2 slight agreement, 0.21 - 0.40 fair agreement, 0.41 - 0.60 moderate agreement, 0.61 - 0.80 substantial agreement, and 0.81 - 1.00 near perfect agreement [17]. The calculated *p*-value for kappa coefficients is less than 0.05, meaning that the agreement between tools and AI chatbots is unlikely to be due to chance.

RESULT AND DISCUSSION

This study included 78 cases of interaction with clopidogrel; however, some drugs were unavailable in drug interaction checkers tools and AI chatbots (n = 12 for MedScape, n = 1 for Drugbank, n = 9 for Epocrates, and n = 54 for Bing AI) except ChatGPT-3.5 AI. These drugs were assumed to have no interaction with clopidogrel.

Lexicomp Drug Interactions Module, a reference database, has identified 20 major, 53 moderate, and five minor drug interactions with clopidogrel (Table 2). Among the free-accesible databases, Drugbank showed similar results to those of Lexicomp. The DrugBank found 13 major, 49 moderate and six minor interactions with clopidogrel and no interactions with ten other drugs. Epocrates identified 17 major, 35 moderate and two minor interactions with clopidogrel. However, Epocrates did not find interactions with 24 drugs. On the other hand, more than half of these clopidogrel-drug interactions were not found in the MedScape. Interestingly, ChatGPT-3.5 AI found that clopidogrel interacted with all but eight of the drugs that were identified in the UpToDate database. However, some moderate risk interactions in Lexicomp were identified as major interactions in ChatGPT-3.5 AI. In contrast, the Bing AI chatbot found interactions only for 15 drugs.

Table 2. Standardization of the interaction severity ratings of the drug interaction tools and AI chatbots as major, moderate, and minor

Lexicomp		**MedScape**		**Drugbank**		**Epocrates**		**ChatGPT-3.5 AI**		**Bing AI**	
Major	20	Serious/Use alternative Contraindicated	13	Major	13	Avoid/Use alternative	17	High	27	Severe	9
Moderate	53	Monitor closely	23	Moderate	49	Monitor/Modify treatment	35	Moderate	41	Moderate	6
Minor	5	Minor	0	Minor	6	Caution advised	2	Mild	2	Minor	0
		No interaction found	42	No interaction found	10	No interaction found	24	No known interaction	8	No interaction found	63

The highest number of correct answers (TP plus TN) was received by ChatGPT-3.5 AI chatbot (n = 69) and Drugbank (n = 64). However, some of the results were misinterpreted by the AI chatbot, such as the fact that clopidogrel is a prodrug. One of the most common interactions with clopidogrel is with CYP2C19 inhibitors. As clopidogrel is a prodrug, co-administration of clopidogrel with CYP2C19 inhibitors reduces serum levels of the active metabolite of clopidogrel. ChatGPT-3.5 AI and Bing AI chatbot accurately describe the interaction of clopidogrel with proton pump inhibitors, which are CYP2C19 inhibitors, suggesting that it leads to decreased plasma levels of the active metabolite. On the other hand, ChatGPT-3.5 AI chatbot incorrectly explains the interaction of fluconazole, nifedipine, and ritonavir (enzyme inhibitors) [1,18,19] and rifampin (enzyme inducer) [20] with clopidogrel, ignoring that clopidogrel is a prodrug. A similar incorrect result was found for erythromycin (enzyme inhibitor) [1] in the Bing AI.

The highest number of incorrect answers (FN plus FP) was received by the Bing AI chatbot (n = 60) and MedScape (n = 39). A reliable drug-drug interaction checker should be sensitive to detect significant interactions and specific to ignore insignificant interactions. The ChatGPT-3.5 AI chatbot

was the most sensitive, while the Bing AI chatbot was the least sensitive. Among the free drug interaction checkers, the DrugBank had the highest level of sensitivity, while the MedScape had the lowest level of sensitivity. Epocrates was found to have the highest specificity of all the databases, while the Bing AI chatbot was found to have the lowest specificity.

The positive predictive values of Drugbank, Medscape and ChatGPT-3.5 AI were comparable and higher, demonstrating their ability to identify significant interactions. On the other hand, both Drugbank and ChatGPT-3.5 AI showed high negative predictive value, further supporting their reliability. The most accurate database was the ChatGPT-3.5 AI chatbot, with 307 out of 400 accuracy points. On the other hand, Bing AI chatbot was the least accurate database, with 197 points (Table 3). Among the free drug interaction checkers, the most accurate database was Drugbank, with 299, and the least accurate was MedScape, with 244 points (Table 3).

Table 3. Accuracy score of drug interaction checkers and AI chatbots for assessing clopidogrel interactions with 78 drugs

	TP	FN	TN	FP	Sensitivity	Specificity	PPV	NPV	Accuracy Score*
MedScape	35	38	4	1	0.48	0.90	0.97	0.10	244
Drugbank	60	12	4	2	0.83	0.94	0.97	0.25	299
Epocrates	48	25	1	4	0.66	0.98	0.92	0.04	260
ChatGPT-3.5 AI	67	7	2	2	0.91	0.97	0.97	0.22	307
Bing AI	14	59	4	1	0.19	0.78	0.93	0.06	197

*Maximum accuracy score is 400. Abbreviation; FN, false negative; FP, false positive; TN, true negative; TP, true positive; PPV, positive predictive value; NPV, negative predictive value

The Bing AI and DrugBank are superior to other checkers in providing references for drug interactions with clopidogrel. On the other hand, ChatGPT-3.5 AI has cited the Lexicomp and Epocrates databases for some interactions, but the results are inconsistent (Table 4). Epocrates, ChatGPT-3.5 AI and Bing AI offer management of drug interactions with clopidogrel. The Bing AI chatbot had the highest comprehensiveness score of 107 points (Table 4). On the other hand, the most comprehensive free drug interaction checker was Drugbank, with 80 comprehensiveness scores. The total score was obtained by summing the accuracy and comprehensiveness scores [10]. ChatGPT-3.5 AI had the highest total score, with 387. Among the free interaction checkers, DrugBank had the highest score, with 379 (Table 5).

Fleiss' kappa coefficients were evaluated for interaction severity agreement between drug-drug interaction tools and AI chatbots for clopidogrel and drug interaction pairs (Table 6). The overall Fleiss kappa coefficient was 0.043 (slight agreement; $p = 0.040$). The kappa coefficient was 0.106 (slight agreement; $p < 0.001$) for interaction pairs with the major severity drug interaction category. Pairwise agreement for the severity of interactions between drug interactive tools and/or AI chatbots was assessed using Cohen's kappa coefficient (Table 7). The highest kappa coefficients were found between Epocrates and Medscape ($\kappa = 0.390$; $p < 0.01$) and Lexicomp and ChatGPT-3.5 ($\kappa = 0.201$; $p = 0.020$), both showing fair agreement.

Table 4. Comprehensiveness score of drug interaction checkers and AI chatbots for assessing clopidogrel interactions with 78 drugs

	Risk rating	Onset	Mechanism	Severity level	Management	Discussion	DL	Ref	CM	Related drugs	CS*
MedScape	Yes	No	Yes	Yes	No	No	No	No	Yes	No	54
Drugbank	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	No	80
Epocrates	Yes	No	Yes	Yes	Yes	No	No	No	Yes	No	67
ChatGPT-3.5 AI	Yes	No	Yes	Yes	Yes	Yes	No	No	Yes	No	80
Bing AI	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	107

*Maximum comprehensiveness score is 134. Abbreviation; CM, clinical manifestations, CS, comprehensiveness score, DL, documentation level, Ref, references

Table 5. Total score of drug interaction checkers and AI chatbots for assessing clopidogrel interactions with 78 drugs

	Accuracy Score	Comprehensiveness Score	Total Score*
MedScape	244	54	298
Drugbank	299	80	379
Epocrates	260	67	327
ChatGPT-3.5 AI	307	80	387
Bing AI	197	107	304

*Maximum total score (accuracy + comprehensiveness) = 400 + 134 = 534

Table 6. Fleiss' kappa coefficients for the interaction severity agreements of four drug interaction tools and two AI chatbots for clopidogrel and drug interaction pairs

	Kappa coefficient	*p*-value	Strength of agreement
Major	0.106***	< 0.001	Slight
Moderate	0.000	0.990	Slight
No interaction	0.045	0.127	Slight
Overall	0.043*	0.040	Slight

In drug interactions reported as minor or not applicable, it was assumed that there were no drug interactions. *$p < 0.05$, ***$p < 0.001$

Table 7. Cohen's kappa coefficients for the interaction severity agreements of between drug interaction tools and/or AI chatbots for clopidogrel and drug interaction pairs

	Lexicomp	MedScape	Drugbank	Epocrates	ChatGPT-3.5 AI	Bing AI
Lexicomp	-	0.095	0.108	0.004	0.201*	0.009
Medscape	0.095	-	0.071	0.390***	0.090	0.118
DrugBank	0.108	0.071	-	0.101	-0.007	0.030
Epocrates	0.004	0.390***	0.101	-	0.047	0.007
ChatGPT-3.5 AI	0.201*	0.090	-0.007	0.047	-	-0.037
Bing AI	0.009	0.118	0.030	0.007	-0.037	-

In drug interactions reported as minor or not applicable, it was assumed that there were no drug interactions. *$p < 0.05$, ***$p < 0.001$

An increasing number of medicines are coming onto the market, and many new interactions are reported that may interfere with treatment or cause adverse effects [21]. The identification of predictable and preventable drug-drug interactions is essential for patient safety, and healthcare professionals often use drug-drug interaction tools to help them identify all the possible interactions between medicines. There are many drug-drug interaction tools available, but the results from these databases are not always consistent, and several studies have been carried out to compare these databases [10-12,22-29]. Although the drug-drug interaction tools and the drugs compared in these studies were different, the conclusion was that subscription-based tools may be more useful than free tools and that health professionals should use at least two different resources to assess drug interactions. Most of these studies showed that Lexicomp had the highest score among subscription-based drug interaction tools [11,12,26], so we chose Lexicomp as our reference source. Artificial Intelligence (AI) is revolutionizing many fields, including health [30]. In recent years, AI applications in healthcare have been in the spotlight, and the potential for AI tools to provide information about drug use among the public is growing [31,32]. Our study focused on clopidogrel, a drug with significant drug-drug interactions, and compared the performance of drug-drug interaction databases and AI chatbots in identifying these interactions.

Our research, along with two previous studies [13,14], has shed light on the potential of AI chatbots in detecting drug interactions. In the first study, the results of another previous study evaluating 40 drug-drug interactions [10] were compared with the free-accesible version of ChatGPT [14]. ChatGPT is partially effective in detecting drug interactions. Although the information provided by ChatGPT is sometimes insufficient, it may be helpful in detecting drug interactions [14]. Similarly, the ChatGPT-3.5 AI chatbot provided useful information about clopidogrel-drug interactions in our study.

The other study searched ChatGPT-3.5 AI, ChatGPT-4 AI, Microsoft Bing AI and Google Bard for interactions of five drugs selected from the class of SGLT-2 inhibitors and macrolides with the 51 most prescribed drugs [13]. These results were compared with those from Micromedex and Drugs.com [13]. In contrast to our study, it was found that Bing AI had the highest accuracy and specificity [13]. In our research, ChatGPT-3.5 AI and DrugBank had the highest accuracy scores. These resources may be free and reliable for identifying drug interactions with clopidogrel. Although Bing AI has the highest comprehensive score, it appears inadequate as it only includes a few drugs. DrugBank and ChatGPT-3.5 AI had the highest comprehensiveness scores, except for Bing AI. As a result, our study shows that ChatGPT-3.5 AI and DrugBank are the most comprehensive and accurate drug interaction detection tools available to healthcare professionals and patients. On the other hand, the compatibility of Lexicomp-ChatGPT-3.5 AI chatbot results for interaction severity was found to be fair. The fact that clopidogrel is a prodrug leads to different results for interactions with drugs that induce or inhibit CYP3A4/A5, CYP2C19, CYP2C9 and CYP1A2, which are involved in the metabolism of clopidogrel [1]. The interaction between the CYP2C19 inhibitors, proton pump inhibitors [1], and clopidogrel was correctly explained by ChatGPT-3.5 AI. However, the interaction with the CYP3A4/A5 inhibitors nifedipine, fluconazole, ritonavir [1] and the CYP3A4/A5 inducer rifampin [1] was incorrectly explained. Even though ChatGPT-3.5 AI achieved the highest overall (accuracy + comprehensiveness) score, a major weakness of ChatGPT-3.5 AI is that it ignores the fact that clopidogrel is a prodrug in these interactions. In addition, our study has some limitations. One limitation is that the data included in the ChatGPT-3.5 AI is from before September 2021 and may not include the most recent medical data. More accurate results can be obtained with ChatGPT-4 AI, but its cost limits its use.

In conclusion, the main findings of our research are as follows: (a) Drugbank Drug Interaction Checker among free-accesible databases and ChatGPT-3.5 AI among AI chatbots provide the results most similar to Lexicomp, (b) More than half of the drugs are not available in Bing AI chatbot, (c) The ChatGPT-3.5 AI chatbot and the Drugbank database have the highest number of correct answers and accuracy score for drug interactions, (d) The Bing AI chatbot performs excellently on the comprehensiveness score, but the inaccessibility of 80% of medications limits this result, (e) Finally, ChatGPT-3.5 AI performed best when considering the overall score.

ChatGP T-3.5 AI shows promise in predicting drug interactions with clopidogrel but may lead to incorrect conclusions with CYP enzyme inhibitors/inducers, ignoring the properties of the drug compound. ChatGPT-3.5 AI can provide general information about drug therapy but cannot give specific medical advice or recommendations. Also, ChatGPT-3.5 AI emphasizes that other medications or supplements should be avoided without the advice of a healthcare professional while using clopidogrel. The AI chatbots recommend being aware of bleeding symptoms when taking clopidogrel and taking the medications at different times of the day to reduce the risk of bleeding. The AI chatbots also warn that the questioner should consult a healthcare professional, regardless of whether there is data on drug interactions, which is beneficial for patient safety. In the future, AI chatbots could become even more useful by improving their ability to predict the interactions between drugs accurately. However, patients should always consult their healthcare provider before changing or stopping taking their medicines.

AUTHOR CONTRIBUTIONS

Concept: Z.S.A.; Design: Z.S.A, B.R.E.; Control: Z.S.A, B.R.E.; Sources: Z.S.A.; Materials: - ; Data Collection and/or Processing: Z.S.A, B.R.E.; Analysis and/or Interpretation: Z.S.A.; Literature Review: Z.S.A, B.R.E.; Manuscript Writing: Z.S.A, B.R.E.; Critical Review: Z.S.A, B.R.E.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors state that ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1021-1032, 2024
Doi: 10.33483/jfpau.1454525

ORIGINAL ARTICLE / ÖZGÜN MAKALE

GREEN HPLC DETERMINATION OF PHENYTOIN AND METHOD VALIDATION

FENİTOİNİN YEŞİL HPLC TAYİNİ VE METOT VALİDASYONU

Ertuğrul Faruk ÖKMEN[1], Ebru ÇUBUK DEMİRALAY[1]*, İlkay KONÇE[2], Yaşar Doğan DALDAL[3]

[1]Süleyman Demirel University, Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences, 32200, Isparta, Türkiye
[2]Süleyman Demirel University, Faculty of Pharmacy, Department of Pharmaceutical Research and Development, 32200, Isparta, Türkiye
[3]Kahramanmaraş İstiklal University, Elbistan Vocational School of Health Services, Department of Pharmacy Services, 46300, Kahramanmaraş, Türkiye

ABSTRACT

Objective: *In this study, the chromatographic behavior of the antiepileptic drug phenytoin was determined by the green HPLC method. The optimization of the developed method was based on the capacity factor values of phenytoin in varying water-ethanol binary mixtures and the ethanol concentration in the mobile phase where the compound was analyzed.*
Material and Method: *Ethanol-water binary mixtures containing 35%, 40%, and 45% (v/v) ethanol were used in the optimization for the determination performed by the RPLC method. Retention times of the compound were determined with the Zorbax SB-CN (150x4.6 mm, 3.5 µm ID) column. Analyzes were performed at a constant flow rate (0.3 ml/min) and column temperature (37°C). The optimum condition for quantitative analysis was determined as an ethanol-water binary mixture containing 40% (v/v) ethanol with a pH of 6.5.*
Result and Discussion: *In this study, the hydrophobicity of phenytoin was calculated using the logk-φ relationship. The optimum condition was determined using the obtained chromatographic data, and the quantitative determination of phenytoin in the commercial tablet formulation was made by the internal standard method. Under these conditions, excellent linearity (r>0.99) was obtained in the concentration range of 0.8-2.8 µg/ml. The detection limit of the developed method is 0.021 µg/ml; the limit of quantitation was calculated as 0.064 µg/ml. The recovery value of the method was determined as 99.61%. It was concluded that the parameters of precision, accuracy, and method robustness were appropriate for the validation procedures.*
Keywords: *Antiepileptic drugs, binary mixture, green chemistry, method optimization, RPLC*

ÖZ

Amaç: *Bu çalışmada antiepileptik ilaç fenitoinin kromatografik davranışı yeşil HPLC yöntemi ile belirlenmiştir. Geliştirilen yöntemin optimizasyonu, değişen su-etanol ikili karışımlarındaki fenitoinin kapasite faktörü değerlerine ve bileşiğin analiz edildiği mobil fazdaki etanol derişimine dayandırılmıştır.*
Gereç ve Yöntem: *RPLC yöntemiyle geliştirilen metot optimizasyonunda %35, %40 ve %45 (h/h) etanol içeren etanol-su ikili karışımları kullanılmıştır. Bileşiğin alıkonma süreleri Zorbax SB-CN*

* **Corresponding Author / Sorumlu Yazar:** Ebru Çubuk Demiralay
e-mail / e-posta: ebrucubuk@sdu.edu.tr, **Phone / Tel.:** +905354935327

Submitted / Gönderilme : 18.03.2024
Accepted / Kabul : 21.07.2024
Published / Yayınlanma : 10.09.2024

(150x4.6 mm, 3.5 µm ID) kolonu ile belirlenmiştir. Analizler sabit akış hızı (0.3 ml/dakika) ve kolon sıcaklığında (37°C) gerçekleştirilmiştir. Kantitatif analiz için optimum koşul, pH'ı 6.5 olan %40 (h/h) etanol içeren bir etanol-su ikili karışımı olarak belirlenmiştir.

Sonuç ve Tartışma: *Bu çalışmada logk-φ ilişkisini kullanılarak fenitoinin hidrofobisitesi hesaplanmıştır. Elde edilen kromatografik veriler kullanılarak optimum koşul belirlenmiş, ticari tablet formülasyonunda fenitoinin kantitatif tayini dahili standart yöntemiyle yapılmıştır. Bu koşullar altında 0.8-2.8 µg/ml derişim aralığında mükemmel doğrusallık (r>0.99) elde edilmiştir. Geliştirilen yöntemin tespit limiti 0.021 µg/ml; miktar belirleme sınırı 0.064 µg/ml olarak hesaplanmıştır. Yöntemin geri kazanım değeri %99.61 olarak belirlenmiştir. Kesinlik, doğruluk ve metot sağlamlığı parametrelerinin validasyon prosedürleri için uygun olduğu sonucuna varılmıştır.*

Anahtar Kelimeler: *Antiepileptik ilaçlar, ikili karışım, RPLC, yeşil kimya, yöntem optimizasyonu*

INTRODUCTION

Epilepsy is one of the neurological disorders that affects approximately 1% of the world's population. Antiepileptic (Anticonvulsants) drugs are a wide variety of pharmacological agents used in the treatment of epileptic seizures. Phenytoin has been used as an anticonvulsant drug since the late 1930s. It limits the spread of seizure discharges by blocking voltage-dependent sodium channels. A usage mediates this response- and voltage-dependent slowdown in the recovery rate of voltage-activated sodium channels from inactivation [1,2]. Phenytoin is a broadly effective ion channel blocker because it inhibits several sodium and calcium channels [3].

Phenytoin is a weakly basic compound with a dissociation constant (pK_a) value between 8.06 and 9.2. Slightly soluble in water, phenytoin is soluble in alkaline and most organic solvents. The poor solubility of phenytoin in water is due to the hydrophobic nature of the diphenyl structure at the C-5 position of hydantoin. (Figure 1) [4].

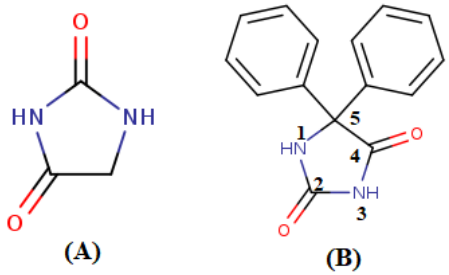

Figure 1. Structure of hydantoin (A) and phenytoin (B)

Analyzes performed by reversed-phase liquid chromatography (RPLC) method for the qualitative and quantitative analysis of the antiepileptic drug phenytoin are available in the literature [5-8]. RPLC is considered the most common technique used in many fields, including developing and analyzing drugs in quality control laboratories and analyzing active substances in biological fluids [9,10]. In most studies carried out with the RPLC method, significant amounts of organic solvents that can hurt the environment, produce large quantities of waste to be disposed of, and cause problems related to ecological impact continue to be used. In this method, methanol and acetonitrile are most commonly used. These solvents with high elution power have some problems in terms of safety and environment. This study aims to introduce a new chromatographic method using the less hazardous solvent ethanol, which is considered an alternative to methanol and acetonitrile. The most suitable green organic solvent for the green RPLC method is ethanol. Due to its lower vapor pressure, ethanol has less toxicity than methanol and acetonitrile, which have higher vapor pressures. In terms of selectivity, ethanol is in the same group as methanol, as in the classification of organic solvents. The main disadvantage of ethanol is that increasing column temperature creates high pressure in the HPLC system due to its higher viscosity compared to acetonitrile and methanol [11,12].

In RPLC optimization, the best separation conditions must be provided. For this purpose, various approaches have been developed to predict the retention behavior of the analyte. Thus, mobile phase polarity, pH, and column temperature values are used in the analysis of ionizable and neutral compounds without the need for trial and error [13-15]. Among these three parameters, the change in the organic solvent content in the mobile phase also causes changes in the degree of ionization and retention of the compound. Achieving the desired separation is very important and is often possible at certain pH values. For this reason, mobile phase pH is important in the determination of ionizable compounds [16,17].

According to the solvophobic theory, the retention will be a function of the chromatographic behavior of the analyte determined in the mobile phase [16]. Using the equations for estimation of retention in chromatography ensures the performance of the experiment in a shorter time and the selectivity of the compounds [14,18]. The relationship between the percentage of the organic solvent in the mobile phase and the capacity factor value is given in Equation 1.

$$\log k = log k_w - S\varphi \qquad (1)$$

φ is the volume fraction of the organic modifier in the binary mixture. The S value is a factor associated with the solvent strength of the organic modifier. As seen in Equation 1, the k value of the compounds shows a linear relationship depending on the volume percentage of organic solvent in the mobile phase. In the determined linear function, S can be calculated with the slope value, and capacity factor (k_w) values of the compounds in pure water can be calculated with the intercept value. The $logk_w$ value calculated in the RPLC method is a measure of hydrophobicity [19]. Although the S value depends on the solvent power of the pure organic modifier, it is not constant for analytes with different chemical structures. S values can range from -3.0 to -6.0. This value indicates that a given increase in organic modifier concentration causes large differences in retention [20]. Equation 1, also referred to as the linear solvent strength (LSS) model, is used to develop methods in RPLC [21]. It is also used to predict chromatographic separations in both isocratic and gradient studies [21,22].

This study focused on the ease of replacing conventional mobile phases with less toxic and greener solvents without altering the performance of the developed method. A green RPLC method was developed for the qualitative and quantitative analysis of phenytoin, an antiepileptic drug. With this developed method, the chromatographic analysis of phenytoin, a hydrophobic compound, was performed using less ethanol than the analyses made with toxic solvents. This study aimed to determine the effect of ethanol content and pH change in the mobile phase on the retention of phenytoin. The pH value of the mobile phase to be studied was chosen in the range of $pK_a \pm 1.5$ of the compound. For the quantitative determination of phenytoin, the k value should be in the range of 1-5 [9,19]. Selectivity factor (α) and separation factor (R_s) values were calculated to be able to separate with the selected internal standard dofetilide in the quantitative determination. In addition, the method was validated according to the International Conference on Harmonization (ICH) and Association of Official Analytical Chemists (AOAC) parameters [23,24] and then the quantitative determination in the pharmaceutical formulation was performed.

MATERIAL AND METHOD

Chemical Substances

The chemicals used for the developed method are of analytical purity and have not been subjected to any purification process. Phenytoin (5,5-diphenylhydantoin), internal standard dofetilide, and uracil were obtained from Sigma Aldrich (St. Louis, USA). Ethanol, ortho-phosphoric acid, sodium hydroxide, and potassium hydrogen phthalate (KHP) were purchased from Merck (Darmstadt, Germany).

Apparatus

The qualitative determination of phenytoin and its quantitative determination in drug formulation was made in an HPLC device. Shimadzu brand HPLC device consists of a UV detector (SPD-20A), pump (LC-20AD), column oven (CTO-20A), and degasser unit (DGU-20A3). The pH values of the prepared mobile phases were measured with a pH meter (Mettler Toledo, Switzerland) using an Ag/AgCl combined glass electrode. According to the International Union of Fundamental and Applied

Chemistry (IUPAC), potassium hydrogen phthalate was chosen as the reference standard for the calibration of the electrode in the ethanol-water mixture [25]. The ultrapure water used in the preparation of the mobile phase was obtained from the Direct Q3 (Millipore, Bedford, MA, USA) device.

Liquid Chromatographic Conditions

Analysis of phenytoin and selected internal standard dofetilide was performed on the Zorbax SB-CN (150x4.6mm, 3.5µm ID) column. The column temperature is 37°C and the flow rate is 0.3 ml/min. Compounds injected into the manual injection system in a volume of 20 µl were analyzed in triplicate and the relative standard deviation of the analysis was calculated below 1%. The wavelengths of the compounds analyzed in the UV detector were 230 nm for phenytoin, 215 nm for dofetilide, and 210 nm for uracil, respectively.

pH Measurement in the Mobile Phase

In a water-organic solvent mixture, pH was measured by IUPAC guidelines [25] considering the determined reference pH of the National Institute of Standards and Technology (NIST) buffer solutions in the hydroorganic mixture studied [26]. According to IUPAC rules, pH standardization was carried out with the primary standard reference solution (KHP, 0.05 mol/kg) used in ethanol-water binary mixtures [25].

Solutions Used and Preparation of Mobile Phase

Stock solution concentrations of phenytoin and selected internal standard dofetilide were prepared as 50 µg/ml. Compounds and mobile phases were stored in the refrigerator at +4°C unless used. Uracil solution prepared by dissolving it in a mobile phase medium was used to determine the capacity factors. The primary standard was prepared using the reference Potassium hydrogen phthalate compound in the mobile phase medium, where the electrode calibration solution was 0.05 mol/kg. For phenytoin analysis, ethanol-water binary mixtures containing 35%, 40%, and 45% (v/v) ethanol with pH adjusted to 5.0 and 6.5 were prepared as mobile phases. For the robustness test, the ethanol-water mixture containing 35% (v/v), 45% (v/v) ethanol, and mobile phases at different pH values (5.0 and 8.0) were prepared in the same way.

Preparation of Tablet Solution

To determine the active ingredient of phenytoin in tablet formulation, 10 tablets were crushed in a porcelain mortar, and tablet powder equivalent to 1 tablet was weighed and taken into a 100 ml balloon jug. Some mobile phase was added to it and it was dissolved in the ultrasonic bath and its volume was completed with the mobile phase. This solution was filtered through a blue band filter paper and diluted at different concentrations so that phenytoin was within the determined linear calibration range.

Method Robustness Test

The robustness of the developed method was evaluated by examining the changes in the amount of ethanol in the mobile phase (±5%, v/v), mobile phase pH (±1.5), and column temperature (25°C, 40°C) in the analysis of phenytoin.

RESULT AND DISCUSSION

In this study, the simultaneous effect of mobile phase pH and ethanol concentration was used for the retention of phenytoin. The analysis of this acidic and basic functional group-containing compound using the RPLC method is highly dependent on the degree of ionization of this compound and thus the pH of the mobile phase.

Since the analyzed phenytoin has moderate solubility and dofetilide has poor solubility [27], water-organic solvent binary mixtures are preferred in liquid chromatographic analyses with these compounds. In the study, water-soluble and polar organic solvent ethanol was preferred. Solvent polarity in the mobile phase affects chromatographic separation. The selection of more polar solvents increases the retention in the column in RPLC [9]. In these analyses made with polar solvent ethanol, classical

alkyl chain columns such as C18, and C8 were not preferred so that the interaction of the compounds with the column is not too much. For the analysis of compounds, a cyano column with a stable bond structure, which provides less interaction of analytes with the column, was preferred. Since the Zorbax SB-CN column (150x4.6 mm, 3.5 µm; Agilent) is nonpolar, the water-ethanol binary mixture was chosen as the mobile phase components.

The t_R values of phenytoin in mobile phases containing ethanol-water hydro-organic mixtures containing 35%, 40%, and 45% (v/v) ethanol at a constant column temperature of 37°C and a flow rate of 0.3 ml/min were determined by averaging the results of three replications. In addition, dead time (t_o) values were determined by using the type of uracil that was not retained in the column under each condition. The capacity factor values of the compound were calculated for each condition using the data obtained as a result of the qualitative analysis. The pH value of the mobile phase to be studied was chosen in the pK_a±1.5 range of phenytoin. Since there is no experimental data on the pK_a value of the compound, the Chemicalize program [28], which makes an estimation, was used to determine this physicochemical parameter. The pK_a value of the acidic nitrogen (Number 3 nitrogen) atom in the hydantoin ring in the chemical structure of the compound is around 8.0 [28]. For this, the pH values to be analyzed were chosen as pH 6.5 and pH 5.0. Since the limit pH value of the analyzed column is 8.0, the working pH value did not exceed 6.5. Since the peak symmetry and reproducibility of phenytoin will not be good at pH 8.0, no study has been carried out at pH where the pK_a value of the compound [9,19].

According to the LSS model, the linear relationship obtained when the logarithmic capacity factor (logk) values of phenytoin are plotted with the volume percentage of ethanol (φ, 35%, 40%, and 45%, v/v) in the mobile phase is given in Figure 2 for pH 5.0 and pH 6.5.

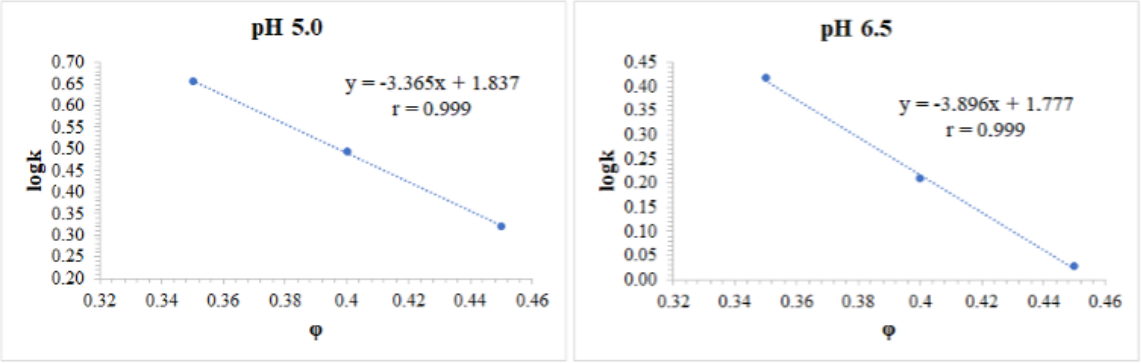

Figure 2. Graphs showing the φ-logk relationship at pH 5.0 and pH 6.5

The intercept value of the linear functions in these graphs gives the $logk_w$ value. According to this data, the k_w value of phenytoin at the studied pH values was calculated without any experiment. Accordingly, while the k_w value was 68.77 at pH 5.0, the k_w value was calculated as 59.83 at pH 6.5. The desired k value in chromatographic analyses is between 1-5. According to these calculated data, the calculated k values in the aqueous medium are very large. According to these values, it was concluded that the t_R values would also be very high, and under this condition, this compound could not be analyzed in a 100% water environment. In addition, the compound with an ionizable acidic functional group depends on the pH change, and the retention time in the column decreases as the pH value increases [16]. The k and k_w values obtained at two different pH values support this situation.

In the linear functions of the graphs given in Figure 2 (Equation 1), k values for phenytoin can be estimated when any percentage by volume value of ethanol outside the experimental study is substituted for the φ value. For ethanol-water binary mixtures containing 5%, 20%, and 70% (v/v) ethanol outside the experimental working range, the k values of the compound could also be calculated without any experiment (Table 1).

Table 1. k values calculated for phenytoin

Compound	pH 5.0			pH 6.5		
	5% (v/v)	20% (v/v)	70% (v/v)	5% (v/v)	20% (v/v)	70% (v/v)
Phenytoin	46.68	14.60	0.30	38.20	4.06	0.11

According to the data in Table 1, since the k value must be between 1-5, it is not possible to analyze phenytoin in studied binary mixtures containing 5%, 20%, and 70% (v/v) ethanol at pH 5.0. At pH 6.5, it is possible to determine phenytoin in binary mixtures containing only 20% (v/v) ethanol. In addition, since the ionized functional group in the structure of the compound is acidic, the k value of the compound will decrease as the pH value of the mobile phase increases. In addition, as the amount of ethanol in the environment increases, the interaction of the compound with the HPLC column will decrease and the k value will also decrease. The data in Table 1 supports these situations [9,19].

Hydrophobicity or lipophilicity is defined as a measure of a compound's tendency to "prefer" non-aqueous media over aqueous media. The logarithm of the partition coefficient, logP, and its logarithmic form, log k_w, are used as the hydrophobicity index in the RPLC method [22]. The relationships between logP values and RPLC retention parameters can be used to predict the hydrophobicity of the compound by estimating the log k_w values from the linear model using the k values in ethanol-water binary mixtures.

In this study, the logk_w (1.77-1.84) values calculated according to pH values, which differ slightly, are very close to the estimated logP (1.89) value of phenytoin [27]. logP > 0 indicates hydrophobic substances soluble in the lipid phase, and log P < 0 indicates polar compounds soluble in the water phase [22]. In this case, the determined and predicted hydrophobicity values show that the phenytoin compound is hydrophobic. In this study, the k values that can be estimated using the logk-φ relationship and the low solubility of phenytoin in water (logS -5.62) also support that the compound is hydrophobic [27].

Optimization of Chromatographic Analysis

For the qualitative and quantitative determination of phenytoin, which will be determined by the RPLC method, the capacity factor (k) value should be 1 and above. The determination of the compound with low solubility by the green RPLC method was carried out in a water-ethanol binary mixture. For this, three mobile phases containing 35%, 40%, and 45% (v/v) ethanol were prepared. Since phenytoin is an ionizable compound, it is affected by changing mobile phase pH values. In determining the pH value to be studied, the pH value of the compound in the range of $pK_a \pm 1.5$ is selected. The pK_a value of phenytoin is 8.33 [28]. Accordingly, the mobile phase pH value of the compound to be studied was chosen as 6.5. This is because the HPLC column Zorbax SB-CN (150x4,6mm, 3.5µm ID) where the determination will take place has a working pH range of 1-8, so the pH value 1.5 units beyond the pK_a value of the compound could not be reached. In a chromatographic analysis, it is desired to analyze a compound as quickly as possible. For this reason, it is important to determine the k value. In the ethanol-water binary mixture containing 40% ethanol adjusted to pH 6.5, a very symmetrical peak with a k value above 1 was obtained at 37 °C for phenytoin. At this pH, the compound is in molecular form.

In this study, the internal standard method was used for the quantitative determination of phenytoin in commercial tablet formulation. For this, different standards with UV properties and chromatographic separation from phenytoin were tried. In the separation for quantitative determination, to separate two compounds from each other, the k value must be 1 and above 1, the selectivity factor (α) value must be 1.15 and above, and the resolution factor (R_s) value must be 2 and above. As a result, dofetilide, an antiarrhythmic drug that meets these conditions, was chosen as the internal standard (IS). Dofetilide has poor solubility in water (logS -6.61) and is less hydrophobic than phenytoin (logP 0.79) [27]. Chromatographic parameter values calculated with the Purnell equation [19] under specified conditions are given in Table 2.

In this assay performed in triplicate, the first dofetilide (IS) was eluted from the HPLC column in 9.108 minutes. Phenytoin eluted from the column at 11.803 minutes. To calculate the k values of the compounds, the uracil solution given to the column was taken from the column in 4.502 minutes.

According to these data, it is seen that the k value calculated for both compounds is above 1. In addition, the calculated α and R_s values show that the compounds are separated from each other.

Table 2. Calculated chromatographic parameter values

Compounds	k_2	α	$k_2/(k_2+1)$	$\alpha - 1/\alpha$	$(1/4)\sqrt{N}$	R_s
Phenytoin/ Dofetilide (IS)	1.622	1.585	0.619	0.369	12.544	2.864

System Suitability Test

The tests performed to determine the suitability and effectiveness of the chromatographic system before the quantitative determination in a chromatographic separation is called the system suitability test (SST). The results obtained in these analyses performed according to the United States Pharmacopeia (USP) [29] are given in Table 3.

Table 3. SST results

Chromatographic parameter	Dofetilide (IS)	Phenytoin	Recommended values
Retention time	9.030	11.696	
Tailing factor	1.410	1.400	<2
Theoretical plate number	2464	2517	>2000
Capacity factor	1.045	1.649	>1
Selectivity factor		1.578	>1
Resolution factor		2.859	>2
RSD (%) of peak retention time	0.188	0.058	≤1
RSD (%) of peak area	0.059	0.057	≤1

Results from SST meet United States Pharmacopoeia requirements. These values seem to be suitable for the method developed for the quantitative determination of phenytoin. SST according to the AOAC guideline, RSD% of the t_R and the peak area of the phenytoin are below 2%. This indicates that the change in repeatable injections is small [24]. Spectral data versus retention time were taken from the HPLC device. Using these data, chromatograms were drawn in the Origin LabPro 2017 program. The standard mixture chromatogram showing the separation of the compounds is given in Figure 3. The two compounds were separated with good peak sharpness and symmetry in a total time of 13 min.

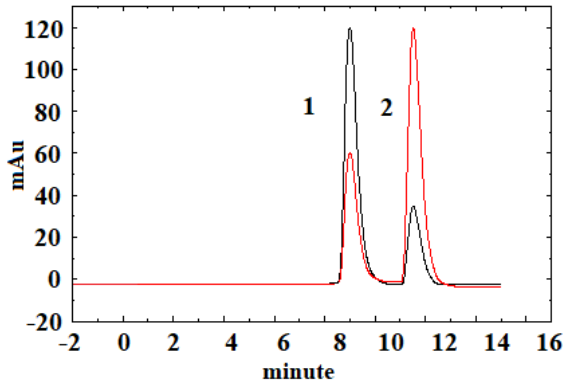

Figure 3. Standard mixture chromatogram showing separation of compounds.1) Dofetilide (IS) 2) Phenytoin – :215 nm; –:230 nm

Validation of the Green RPLC Method

Determination of Linear Range

The purpose of chemical analysis is quantitative analysis in which the amount of a substance in the sample is determined. Careful preparation of the sample is very important to accurately calculate the concentration of an unknown substance from the sample. Some of the samples may be lost with each preparation. However, there are some strategies to minimize sample loss. The internal standard (IS) method is widely used to deal with sample loss and still make accurate concentration measurements. The concentration of selected IS in this study was kept constant at 0.5 µg/ml throughout the study. The working range of the developed method was determined to be in the range of 0.8-2.8 µg/ml. A calibration graph was drawn using the peak area (mAu) ratio values obtained as a result of the analysis of solutions containing phenytoin at six concentration levels and IS at a fixed concentration, against varying phenytoin concentrations (µg/ml). According to the results, there is an excellent correlation (correlation coefficient (r)>0.999) between phenytoin concentration and peak area ratio. The regression equation is as follows:

$$y = 2.601x + 0.012 \qquad (2)$$

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated for phenytoin. LOD and LOQ values are 0.021 µg/ml and 0.064 µg/ml, respectively. The results obtained meet the acceptance criteria according to ICH and AOAC guidelines [23,24].

Precision Data of the Method

To determine the precision of the developed Green RPLC method, intraday (repeatability) and interday (reproducibility) studies were carried out. For this, phenytoin solutions containing a constant concentration of IS were prepared at two different concentrations within the linear working range determined in the calibration. Independent solutions prepared at 1.2 µg/ml and 2.4 µg/ml concentrations were analyzed three times a day. These prepared solutions were kept in a refrigerator at +4°C by cutting off contact with air. After the intraday analysis, the retention times and peak area values of the compound were recorded in the analysis performed on the 3rd day. Measured concentration and RSD% values calculated using the calibration function according to these data are given in Table 4.

Table 4. Intraday-interday data

Compounds	Theoretical concentration (µg/ml)	Intraday measured concentration mean (µg/ml)	RSD%	HorRat value	Interday measured concentration mean (µg/ml)	RSD %	HorRat value
Phenytoin	1.2	1.201	0.137	0.009	1.197	0.326	0.021
	2.4	2.401	0.178	0.013	2.396	0.413	0.039

According to ICH guidelines, the RSD% value should be 1% and below in intraday repeatability data, and the RSD% value should be 2% and below in interday repeatability data. The RSD% values calculated in these data are presented in Table IV. show that the precision of the developed method is high. Additionally, precision values are calculated from the Horwitz (HorRat) equation [30], which represents the empirical relationship between acceptable precision and the corresponding analyte concentration in the analyzed sample. HorRat value (Equation 3) was calculated by AOAC guidelines [24].

$$\text{HorRat} = \frac{RSD_R}{PRSD_R} \qquad (3)$$

$PRSD_R$ is the RSD value estimated from the Horwitz equation. This value is calculated with Equation 4.

$$PRSD_R = 2^{(1-0.5\log C)} \qquad (4)$$

RSD_R is the relative standard deviation under reproducibility conditions. C is the mass concentration expressed in the power of 10, i.e. 1 µg/g=10^{-6}. The precision is better than expected if the ratio is less than 1, and poorer if greater than 1 [30]. Reproducibility and reproducibility results for phenytoin also demonstrated compliance with the AOAC guidelines (Table 4).

Accuracy Data of the Method

For quantitative analysis, tablets containing phenytoin active ingredient (Eptandoin, Exeltis®, 100 mg Phenytoin) were prepared as stated in the material and method section. As a result of the HPLC analysis, when the peak area ratio values of the compounds were substituted in the calibration function, the amounts in the tablet samples for phenytoin were calculated. The data are presented in Table 5.

Table 5. The amount of phenytoin in the tablet sample and recovery results ([1]Five experiments)

Labeled claim (mg)	100
Amount found (mg)[1]	100.50
RSD %	0.88
Bias%	0.50
Recovery%	99.61
RSD %	0.65
Bias%	0.39

According to the data obtained from the table, an average value very close to the amount of 100 mg of phenytoin active ingredient in the tablet sample was obtained. The calculated RSD% value shows that the precision of the results is good, and the accuracy is high since the bias percentage value is below 1%.

A recovery study was carried out to express the accuracy of the developed method. For this purpose, the tablet sample prepared from the standard solution of phenytoin, not exceeding the calibration working range, was added. As a result of the HPLC analysis, when the peak area ratio values of the compounds in the additive sample were substituted in the calibration function, their amounts in the sample were calculated. Using these results, the recovery values of the method could be calculated (Table 5). According to the ICH guideline, the average % recovery should be between 95-105% [23]. This result shows that the accuracy of the method is high.

In this quantitative analysis study, excipients commonly used in tablets did not hurt the analysis results. Chromatograms showing the tablet sample and the tablet sample containing phenytoin spiked at a certain concentration for the recovery study are given in Figure 4. No interfering peaks were found in the chromatograms.

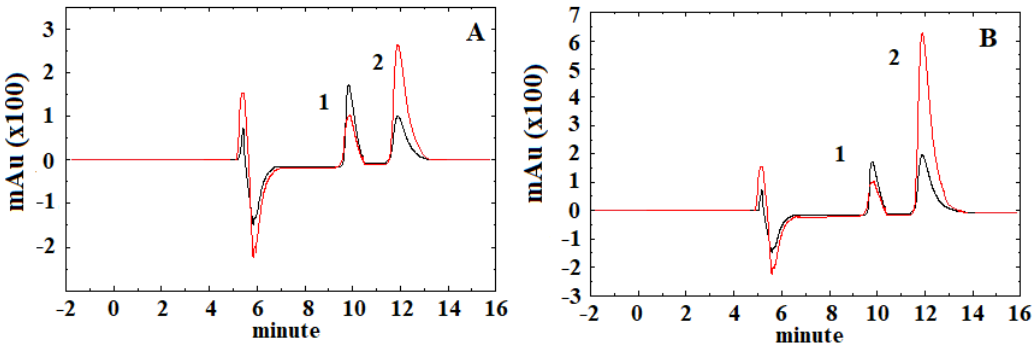

Figure 4. A) Tablet sample analysis (1-Dofetilide 0.5 µg/ml; 2-Phenytoin 1.2 µg/ml) B) Spiked sample analysis (1-Dofetilide 0.5 µg/ml; 2-Phenytoin 2.4 µg/ml)

Robustness Test

The robustness test shows the degree to which the method is affected when a small but significant change in the study parameters of the method is made. The smaller the effect of small changes in method parameters during routine processing on the analysis result, the more robust the method. In the optimized chromatographic condition, the changes in the amount of ethanol in the mobile phase, the pH change and the column temperature change, the tailing factor, and the peak area of phenytoin are given in Table 6.

Table 6. Robustness test results with different conditions

Parameter	Optimized condition	Used	Retention time (min)	Tailing factor	Peak area (mAu)
Mobile phase	**40:60 (v/v%) ethanol-water binary mixture**	45:60 (v/v%)	9.301	1.392	6725621
		35:65 (v/v%)	16.272	1.419	6649433
pH	**6.5**	8.0	9.322	1.407	6537047
		5.0	12.717	1.411	6419294
Column temperature	**37 ºC**	40 ºC	11.569	1.402	6581862
		25 ºC	13.243	1.410	6696332

According to these results, minor changes made in the repeatability of the results proved that the developed method was effective. There are many HPLC studies on the separation of phenytoin alone or simultaneously with different compounds in different samples. In these studies, qualitative and quantitative analysis of the compound was carried out using classical trial and error methods. The stationary phases used in the studies are generally C18 and the organic solvents used are common solvents in RPLC [31,35]. In the study conducted by Ayman et al., the micellar liquid chromatography method, known as green chromatography, was used. In this study, 10% (v/v) acetonitrile was used, although it was low compared to the amount of solvent used in RPLC [36].

Conclusion

In this study, the green RPLC method, an environmentally friendly method for the qualitative and quantitative analysis of the antiepileptic drug phenytoin, which is widely used in the treatment of epilepsy, one of the neurological disorders, was developed. This study, in which the optimum condition is determined depending on the pK_a value of phenytoin, is far from trial and error. First of all, the pH value measured in the ethanol-water binary mixtures prepared to determine the retention time of phenytoin was measured by pH standardization. This type of study is the first for this hydro-organic mixture. According to the LSS method, the $logk_w$ value, known as the hydrophobicity index of the compound, was calculated using the $logk\text{-}\varphi_{ethanol}$ linear relationship. Chromatographic conditions were determined for the analysis of phenytoin according to the suitability of the chromatographic parameters. This developed method is the first study in the literature with this optimization and suitability for green chemistry. In this study, an analytical procedure suitable for routine use was developed, and the method was validated for the determination of the amount of phenytoin in a single-active ingredient pharmaceutical dosage form. Method validation showed excellent results for linearity, precision, accuracy, limit of quantitation and limit of detection, and robustness parameters. This environmentally friendly analysis is a pioneering study for method optimization and validation without the need for any trial and error.

AUTHOR CONTRIBUTIONS

Concept: E.Ç.D., Y.D.D.; Design: E.Ç.D., Y.D.D.; Control: E.Ç.D., Y.D.D., İ.K.; Sources: E.Ç.D.; Materials: E.Ç.D., Y.D.D., E.F.Ö., İ.K.; Data Collection and/or Processing: E.Ç.D., E.F.Ö., İ.K.;

Analysis and/or Interpretation: E.Ç.D., Y.D.D., E.F.Ö.; Literature Review: E.Ç.D., Y.D.D., E.F.Ö., İ.K.; Manuscript Writing: E.Ç.D.; Critical Review: E.Ç.D., Y.D.D.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1033-1040, 2024
Doi: 10.33483/jfpau.1505825

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IL-6 LEVELS AND COGNITIVE COMPLICATIONS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS: A CROSS SECTIONAL STUDY

TİP 2 DİYABETLİ HASTALARDA IL-6 DÜZEYLERİ VE KOGNİTİF KOMPLİKASYONLAR: KESİTSEL BİR ÇALIŞMA

Hümeyra İVECEK[1], Abdüsselam ŞEKERCİ[2], Betül SÜMBÜL-ŞEKERCİ[3]*

[1]Bezmialem Vakıf University, Faculty of Pharmacy, 34093, Istanbul, Türkiye
[2]Bezmialem Vakıf University, Faculty of Medicine, Department of Internal Medicine, 34093, Istanbul, Türkiye
[3]Bezmialem Vakıf University, Faculty of Pharmacy, Department of Clinical Pharmacy, 34093, Istanbul, Türkiye

ABSTRACT

Objective: *It is important to monitor and manage macrovascular and microvascular complications, which are significant causes of mortality and morbidity in type 2 diabetes (T2DM). Diabetes-related cognitive impairment is an important complication that has recently attracted more attention, but its pathophysiology and clinical features are not known. The aim of this study was to evaluate the potential role of inflammation in diabetes-related cognitive impairment.*
Material and Method: *A total of 122 participants (67 patients with T2DM and 55 controls) took part in this cross-sectional observational clinical study. Cognitive performance was assessed using the Montreal Cognitive Assessment (MoCA) test. IL-6 levels were measured using the ELISA method on blood samples obtained from the participants.*
Result and Discussion: *Although IL-6 levels increased with diabetes and cognitive impairment, there was no significant difference between the groups ($p>0.05$). Except for attention and orientation, we observed significantly more impaired cognitive performance in T2DM patients. The MoCA total score was significantly correlated with age, education level, fasting glucose, HbA1c, and vitamin D levels ($p<0.05$). Our results found no evidence that IL-6 is involved in the pathophysiology of cognitive impairment in T2DM, but these findings the importance of cognitive screening in monitoring complications associated with diabetes.*
Keywords: *Cognitive impairment, inflammation, type 2 diabetes*

ÖZ

Amaç: *Tip 2 diyabette (T2DM) önemli mortalite ve morbidite nedenleri olan makrovasküler ve mikrovasküler komplikasyonların izlenmesi ve yönetilmesi önemlidir. Diyabetle ilişkili kognitif bozukluk son dönemler daha fazla dikkat çeken önemli bir komplikasyondur, ancak patofizyolojisi ve klinik özellikleri net olarak bilinmemektedir. Bu çalışmanın amacı diyabetle ilişkili kognitif bozuklukta inflamasyonun potansiyel rolünü değerlendirmektir.*
Gereç ve Yöntem: *Bu kesitsel gözlemsel klinik çalışmaya toplam 122 katılımcı (67 T2DM hastası ve 55 kontrol) dahil edilmiştir. Bilişsel performans Montreal Kognitif Değerlendirme (MoCA) testi kullanılarak değerlendirilmiştir. IL-6 düzeyleri katılımcılardan alınan kan örneklerinde ELISA yöntemi kullanılarak ölçülmüştür.*
Sonuç ve Tartışma: *IL-6 düzeyleri diyabet ve bilişsel bozuklukla birlikte artmasına rağmen, gruplar arasında anlamlı bir fark yoktu ($p>0.05$). Dikkat ve oryantasyon dışında, T2DM*

* **Corresponding Author / Sorumlu Yazar:** Betül Sümbül-Şekerci
e-mail / e-posta: bsekerci@bezmialem.edu.tr, **Phone / Tel.:** +902125232288

Submitted / Gönderilme : 27.06.2024
Accepted / Kabul : 22.07.2024
Published / Yayınlanma : 10.09.2024

hastalarında kognitif performansın anlamlı olarak daha fazla bozulduğunu gözlemledik. MoCA toplam skoru yaş, eğitim düzeyi, açlık glukozu, HbA1c ve D vitamini düzeyleri ile anlamlı korelasyon gösterdi ($p<0.05$) Sonuçlarımız IL-6'nın T2DM'de kognitif bozukluğun patofizyolojisinde rol oynadığına dair bir kanıt bulmadı dakat bu bulgular diyabetle ilişkili komplikasyonların izlenmesinde kognitif taramanın önemini vurgulamaktadır.
Anahtar Kelimeler: *İnflamasyon, kognitif bozulma, tip 2 diyabet*

INTRODUCTION

The incidence of Type 2 diabetes mellitus (T2DM) is expected to rise in the coming years due to lifestyle changes, insufficient physical activity, unhealthy diets, and other factors [1, 2]. This disease is particularly common in individuals over 65 and poses significant health challenges for the elderly [3]. An observational study conducted in 28 countries across Asia, Africa, South America, and Europe found that 53.2% of patients with type 2 diabetes mellitus (T2DM) have microvascular complications, and 27.2% have macrovascular complications [4]. As vascular complications are a significant cause of mortality and morbidity in diabetes, effective management of these complications is crucial. Recently, cognitive impairment has emerged as a complication of diabetes that, while attracting growing attention, remains less understood and researched compared to other complications [5].

Patients with T2DM are 1.5 to 2 times more likely to experience cognitive decline, impairment, or dementia compared to non-diabetics.[6]. This is a significant clinical interest, especially given the aging global population and the high prevalence of T2DM among the elderly. Impairments in attention, memory, processing speed, executive function, and general cognitive function have been observed in T2DM patients [7]. Prospective studies indicate that T2DM is a risk factor for dementia and mild cognitive impairment, with the risk of Alzheimer's disease being approximately twice as high in T2DM patients compared to non-diabetics [8,9].

The mechanisms behind cognitive decline in T2DM are not fully understood, but chronic systemic inflammation is thought to play a role [10]. Research into biomarkers to understand brain changes in T2DM patients is growing, with proinflammatory cytokines such as TNF, IL-1, IL-2, and IL-6 being found in excess in the brains of patients with T2DM and Alzheimer's Disease, suggesting inflammation's role in neuronal damage [11]. IL-6, an important inflammatory biomarker, is implicated in the development of insulin resistance and T2DM, where chronic inflammation can initiate pathological processes [12]. Low-grade inflammation in middle age, indicated by IL-6 levels, is linked to cognitive decline in later life [13]. More research is needed to evaluate the potential of inflammatory molecules as predictive and diagnostic biomarkers for diabetes-related cognitive dysfunction [11]. The aim of this study is to investigate the relationship between cognitive impairment and plasma IL-6 levels in patients with T2DM.

MATERIAL AND METHOD

Patients

In this cross-sectional, observational study, 122 patients participated, with 67 diagnosed with T2DM and 55 comprising the control group. Patients presenting to the Internal Medicine Clinic of of Bezmialem Vakif University Hospital were evaluated by an Internal Disease Specialist based on specific inclusion and exclusion criteria. Inclusion criteria required participants to be at least primary school graduates and aged between 30 and 65 years. In our study, the geriatric population over 65 years of age was not included due to the significant increase in the risk of dementia and low-grade inflammation in this age group. Exclusion criteria of both study groups included a neurological or psychiatric diagnosis, insulin therapy, hypothyroidism, vitamin B12 and folic acid deficiency, hypoglycemia-hyperglycemia attacks, and alcohol or drug addiction. All participants signed written informed consent, and approval was obtained from the Bezmialem Vakif University Clinical Research Ethics Committee (05.04.2023/07-2). The study started on 30.04.2023 and concluded on 30.04.2024.

Cognitive Assessment

The Montreal Cognitive Assessment (MoCA) test is a brief cognitive screening tool designed to assist in the detection of mild cognitive impairment. It assesses various cognitive domains, including short-term memory recall, visuospatial abilities, executive functions, attention, working memory, language and orientation. The MoCA test is widely used in clinical settings due to its sensitivity and specificity in identifying mild cognitive impairment and dementia. The test is quick to administer, takes about 10-15 minutes, and provides a total score out of 30 points, with higher scores indicating better cognitive function. A score of 26 or above is considered normal, while lower scores may indicate cognitive impairment [14]. According to a validation study conducted on the Turkish population scores of 21 and above are regarded as normal [15].

Laboratory

Blood samples were collected in the morning following an 8-hour fasting period. They were placed into gel biochemistry tubes and centrifuged at 3500xg for 10 minutes at room temperature. The resulting samples were stored at -80°C until analysis. After obtaining blood samples from the patients and completing their cognitive assessments, the samples were processed collectively. IL-6 levels were measured using the ELISA method with human ELISA kits based on the Sandwich-ELISA principle (Elabscience Cat.No.:E-EL-H6156). All steps of the ELISA experiments were carried out following the protocol provided by the kit manufacturer. The sample protocol can be found at https://www.elabscience.com/p-human_il_6_interleukin_6_elisa_kit-532924.html. Insulin levels, blood sugar levels, and hemogram values related to diabetes were routinely requested from the patients and accessed via the hospital information system.

Statistical Method

All statistical analyses were conducted using IBM SPSS 28.0 software. The Shapiro-Wilk test was used to assess normality. Continuous variables with a normal distribution were reported as mean ± standart deviation (SD), while continuous variables with a non-normal distribution were reported as median (Q1-Q3). For normally distributed variables, comparisons between the diabetes and control groups were made using the t-test, and for non-normally distributed variables, the Mann-Whitney U test was used. The categorical variables were expressed as numbers (percentages) and compared using the chi-square test. A significance level of ≤ 0.05 was considered statistically significant.

RESULT AND DISCUSSION

One hundred and twenty-two patients participated in our study. The mean age of the patients was 49.43 ± 7.10 years, and the mean years of education were 8.66 ± 3.78. Among the participants, 67 were diagnosed with T2DM, while 55 comprised the control group. Demographic and clinical data of the groups were compared in Table 1. Significant differences were observed between the groups in terms of fasting glucose, HbA1c, HOMA-IR, BMI, ALT, LDL, HDL, Mg, neutrophil count, and years of education ($p < 0.05$). Among patients with type 2 DM, 26 (38.8%) exhibited impaired cognition, while cognitive performance was preserved in 41 (61.2%) patients. In contrast, only 5 (9.1%) individuals in the control group had impaired cognition, indicating a significant difference between the two groups.

The cognitive performances of the groups were compared in 2 different models and presented in Table 2. Correction was made for age and education in Model 2. It was determined that cognitive performance in T2DM patients was significantly impaired compared to controls ($p<0.001$). Except for orientation and attention, T2DM patients exhibited poorer performance in all cognitive domains compared to controls.

IL-6 levels were compared between patients with T2DM and controls (Figure1). In the T2DM group, the IL level was 1.27 ± 0.83 while it was 1.18 ± 0.84 in the control group. There was no significant difference between the IL-6 levels of the groups ($p=0.569$). The relationship between IL-6 levels and MoCA total was examined, and no statistically significant difference was found between them ($p>0.05$,$r=0.058$). IL-6 levels were compared between Type 2 DM patients with and without impaired cognition. The IL-6 levels in Type 2 DM patients with impaired cognition were 1.35±0.82, whereas

those with normal cognition were 1.22±0.82. Despite an increase in IL-6 levels in T2DM patients with impaired cognition, there was no significant difference between the two groups (p=0.521). The IL-6 levels of T2DM patients with and without impaired cognition were compared with those of the control group in Figure 1. No significant difference was found among the groups (p=0.691).

The relationship between MoCA total score and other cognitive subdomain scores with clinical and demographic variables was examined. A significant association was found between MoCA total score and age, education, fasting glucose, HbA1c, and vitamin D ($p<0.05$). A significant relationship was observed between visual spatial functions and education, fasting glucose ($p<0.05$). Naming was significantly associated with education, fasting glucose, HbA1c, TSH, and Mg ($p<0.05$). Attention showed a significant relationship with age, education, eGFR, and hemoglobin ($p<0.05$). Language was significantly related to age, education, fasting glucose, B12, and vitamin D ($p<0.05$). Abstract thinking was significantly associated with education and TSH ($p<0.05$). Memory showed significant relationships with education, HbA1c, TSH, and folic acid ($p<0.05$). There was no significant relationship between orientation and clinical and demographic characteristics ($p<0.05$).

Table 1. Demographic and clinical data of the groups

Variables	T2DM (n=67)	Control (n=55)	p
Age(year)	50.48 ± 8.24	48.15 ± 5.21	0.060
Education(year)	5 (5-11)	11 (5-13)	**<0.001**
Gender, male (%)	35 (52%)	20 (36%)	0.080
Cognitive impairment, n (%)	26 (38.8 %)	5 (9,1%)	**<0.001**
BMI(kg/m²)	30.65±4.76	27.41± 3.92	**<0.001**
Fasting Glucose(mg/dl)	143 (122-177)	92 (89-95)	**<0.001**
HbA1c(%)	7.28 (6.45-8.10)	5.24 (5.11-5.49)	**<0.001**
Iron(µg/dl)	79.12 ± 36.06	91.67 ± 41.13	0.210
AST(U/L)	18.81 ± 5.44	18.89 ± 5.01	0.954
ALT(U/L)	27.49 ± 15.25	22.13 ± 10.50	**0.050**
HOMA-IR	2.83 ± 1.28	1.83 ± 0.84	**0.003**
LDL(mg/dl)	137.81 ± 46.29	124.13 ± 26.66	**0.050**
HDL(mg/dl)	48.44 ± 11.60	54.79 ± 14.07	**0.037**
TSH(mU/L)	2.68 ± 5.87	1.82 ± 1.00	0.326
Triglyceride (mg/dl)	157.15 ± 77.30	130.21 ± 71.39	0.073
B12(ng/l)	333.82 ± 147.60	337.28 ± 86.30	0.877
Folic Acid(µg/l)	8.01 ± 2.92	10.54 ± 14.78	0.279
Vitamin D(µg/l)	20.33 ± 10.91	23.86 ± 13.22	0.270
Ca(mg/dl)	9.55 ± 0.40	9.52 ± 0.42	0.814
Na(mmol/l)	139 (138-141)	140 (139-140)	0.210
K(mmol/l)	4.51 ± 0.56	4.33 ± 0.24	0.095
Mg(mg/dl)	1.87 ± 0.21	1.97 ± 0.15	**0.042**
Urea(mg/Dl)	29.96 ± 8.34	26.44 ± 7.03	0.092
eGFR(ml/min/1.73 m²)	94.37 ± 13.61	96.44 ± 12.09	0.472
Creatinine(mg/dl)	0.82 ± 0.14	0.77 ± 0.15	0.104
Neutrophil(10^3/ul)	4.41 ± 1.35	3.79 ± 1.20	**0.015**
Lymphocyte (10^3/ul)	2.70 ± 0.70	2.29 ± 0.55	**0.001**
Hemoglobin(g/l)	14.19 ± 1.62	13.73 ± 1.93	0.198
Platelet(10^3/ul)	259.29 ± 55.48	258.22 ± 83.67	0.937

For normally distributed variables, mean ± SD values are presented, while for non normally distributed variables, median (Q1-Q3) values are provided. The statistically significant ones are bolded T2DM,.type 2 diabetes; BMI, body mass index; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; LDL, Low-Density Lipoprotein; HDL,High-Density Lipoprotein, TSH, Thyroid-stimulating hormone; eGFR, Estimate Glomerular Filtration Rate

Table 2. Comparison of cognitive performances between groups

Cognitive domain	Model 1			Model 2		
	T2DM (n=67)	Control (n=55)	p	T2DM (n=67)	Control (n=55)	p
MoCA Total score	21.52±4.39	25.22±2.942	**<0.001**	22.10±3.36	24.44±3.41	**<0.001**
Visuospatial/Executive	3.58±1.11	4.29±0.99	**<0.001**	3.68±1.02	4.14±1.03	**0.017**
Naming	3 (2-3)	3 (3-3)	**<0.001**	2.49±0.51	2.80±0.52	**0.002**
Attention	5 (4-6)	6 (5-6)	0.065	4.82±1.26	4.90±1.29	0.741
Language	1 (0-2)	2 (1-3)	**<0.001**	1.44±0.94	1.88±0.96	**0.015**
Abstraction	1.28±0.75	1.65±0.55	**0.003**	1.33±0.67	1.58±0.68	**0.047**
Memory	2.46±1.39	3.31±1.35	**<0.001**	2.51±1.41	3.22±1.43	**0.009**
Orientation	6 (6-6)	6 (6-6)	0.227	5.83±3.76	5.88±0.38	0.451

Normally distributed continuous variables were presented as mean ±SD, non-normally distributed continuous variables were presented as median (Q1-Q3). Model 1 represents the T2DM and control groups, whereas correction has been made for age and education in Model 2. The statistically significant ones are bolded. T2DM,.type 2 diabetes; MoCA, The Montreal Cognitive Assessment

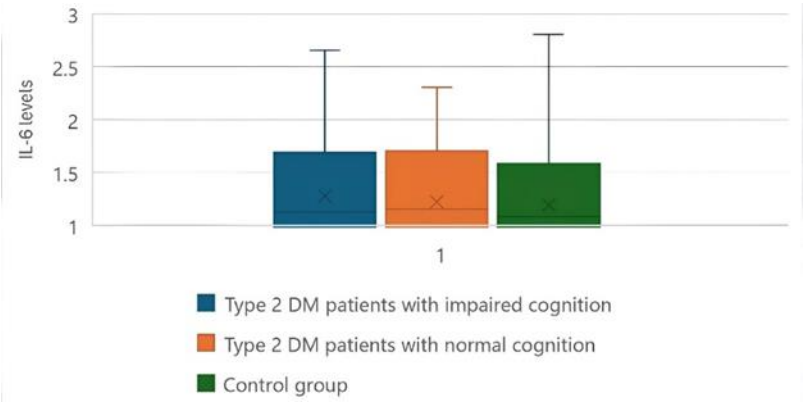

Figure 1. Comparison of IL-6 levels among groups
No significant difference was found between the groups (p=0.691)

This study aimed to investigate the relationship between cognitive impairment and IL-6 levels in middle-aged T2DM patients and to contribute to the literature on the potential role of inflammation in diabetes-related cognitive impairment. IL-6 levels of the groups were compared and no significant difference was found (p<0,05). Our study demonstrated that, except for attention and orientation, T2DM patients exhibited notably lower scores across other cognitive subdomains. We observed a marked cognitive impairment in T2DM patients compared to controls. We further explored the correlation between the Montreal Cognitive Assessment (MoCA) total score, cognitive subdomain scores, and various clinical and demographic variables. A significant association emerged between MoCA total score and age, education level, fasting glucose, HbA1c, and vitamin D levels (p<0.05).

The transition of T2DM from a metabolic phenomenon to one mediated by inflammation has been noted in previous studies [16]. The activation of the inflammation cascade and endothelial dysfunction are significant in both the development of diabetes and the pathophysiological mechanisms underlying diabetes-related complications [17]. The irregular elevation of inflammatory markers is believed to influence glycemic control and insulin sensitivity, contributing to the pathogenesis of T2DM. Although IL-6 typically exists at appropriate levels, its dysregulated production and prolonged exposure can lead to inflammation [18]. However, the role of IL-6 as an early biomarker for T2DM remains uncertain. IL-6 is a versatile inflammatory cytokine known for its multifaceted biological effects. In this study, we observed that IL-6 levels were increased in patients with type 2 diabetes but did not show a significant difference compared to controls. Various pathways have been proposed to explain the relationship between diabetes and cognitive complications. These include vascular system damage, protein

misfolding, and inflammation or oxidation, which can promote both neurodegenerative and vascular damage [19]. The presence of low-grade inflammation in middle-aged individuals has emerged as an independent risk factor for compromised cognitive function in later stages of life, with IL-6 being proposed as a potent biomarker for cognitive performance and decline [13]. Chronic inflammation and endothelial dysfunction caused by elevated IL-6 levels can affect cerebral blood flow and promote neurodegenerative processes, leading to cognitive decline. Inflammation has been highlighted as a key pathway that can impair cerebral vasoregulation, potentially leading to cognitive decline in individuals with diabetes [20]. Data from longitudinal studies indicate that elevated IL-6 levels may increase the risk of cognitive decline and dementia [21]. While some studies in the literature reported a relationship between IL-6 levels and cognitive functions [22, 23], others did not observe a significant relationship [24-26]. In our study, unlike some results, we did not find a statistically significant difference between IL-6 levels and MoCA total score and cognitive subdomain scores. In the Edinburgh Type 2 Diabetes Study, levels of three pro-inflammatory markers (IL-6, TNF-α, and CRP) were associated with poorer cognitive performance in 1,066 elderly adults with T2DM, most of whom had complications associated with T2DM identified [27]. Evidence is increasing in the general population suggesting that inflammatory markers can predict cognitive change. A prospective cohort study found that higher baseline levels of fibrinogen and IL-6 were associated with greater cognitive decline over a 10-year follow-up [27]. Cognitive impairment in diabetes is thought to be determined by multiple etiologies, some of which are specific to diabetes, and may be related to complex pathologies that vary between individuals [28]. When we consider our results alongside existing literature, it appears that research on the impact of IL-6 on cognitive impairment in diabetes is limited, and the findings appear to be inconsistent. Therefore, it is likely that in such a complex pathology, observing the impact of inflammation through a single biomarker, as in our study, can be challenging.

Given the burgeoning aging population worldwide and the heightened prevalence of T2DM among the elderly, understanding the disease's adverse effects on health and cognitive function holds significant clinical importance. Awareness of diabetes' other complications is better established compared to cognitive impairment [29]. Future studies aim to clearly elucidate the role of inflammation in cognitive impairment in diabetes, potentially investigating the role of anti-inflammatory treatments or newly developed drugs in preventing these complications. The smaller size of our study group compared to other large-scale studies is a limitation that restricts and undermines the reliability of the outcome. More research should be conducted in middle age to prevent the complications of T2DM and consider preventive treatment options. Also antihyperglycemic treatments have been found to change biomarkers linked to inflammation [30-32]. So, our study is limited by the lack of standardization of antihyperglycemic therapy in T2DM patients. Future research will explore the impact of antihyperglycemic therapy and other medications on these biomarkers, providing a more comprehensive understanding of their relationship.

In conclusion, we observed a significant cognitive impairment in patients with T2DM. This underscores the importance of cognitive screening in monitoring complications associated with diabetes. We observed that both diabetes and cognitive impairment increased IL-6 levels, but the increase was not significant. Our results found no evidence that IL-6 is involved in the pathophysiology of cognitive impairment in T2DM. Future studies with larger samples may capture significant evidence of the elevated levels of IL-6 that we observed. Identifying the cognitive complications and biomarkers of T2DM is crucial. Future studies in this area could provide a broader understanding of the pathology, leading to early detection and significant progress in the treatment of complications.

ACKNOWLEDGEMENTS

This research was supported by Tubitak 2209-A Research Project Support Programme for Undergraduate Students (grant numbers: 1919B012224507).

AUTHOR CONTRIBUTIONS

Concept: H.İ., B.S.Ş.; Design: H.İ., A.Ş., B.S.Ş.; Control: A.Ş., B.S.Ş.; Sources: H.İ.; Materials: - ; Data Collection and/or Processing: H.İ., A.Ş.; Analysis and/or Interpretation: H.İ., B.S.Ş.; Literature

Review: H.İ.; Manuscript Writing: H.İ.; Critical Review: A.Ş., B.S.Ş.; Other: -

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

ETHICS COMMITTEE APPROVAL

The studies involving human participants were reviewed and approved by Bezmialem Vakif University Clinical Research Ethics Committee (05.04.2023/07-2). The patients/participants provided their written informed consent to participate in this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1041-1049, 2024
Doi: 10.33483/jfpau.1484457

ORIGINAL ARTICLE / ÖZGÜN MAKALE

CHOLINESTERASE AND TYROSINASE INHIBITORY ACTIVITY OF SUBCRITICAL WATER AND MICROWAVE EXTRACTS OF *RAPHANUS SATIVUS* L. 'RED MEAT' RADIX

RAPHANUS SATIVUS L. 'RED MEAT' KÖKLERİNİN SUBKRİTİK SU VE MİKRODALGA EKSTRELERİNİN KOLİNESTERAZ VE TİROZİNAZ İNHİBİTÖR AKTİVİTESİ

Halil KOYU[1]*

[1]İzmir Katip Çelebi University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 35620, İzmir, Türkiye

ABSTRACT

Objective: *Cholinesterase and tyrosinase inhibitory activity potential of microwave-assisted and subcritical water extracts of Raphanus sativus L. Red Meat roots were investigated.*
Material and Method: *Total phenol, flavonoid and anthocyanin content of extracts from conventional solvent and advanced extraction systems were spectrophotometrically quantified. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities were investigated with the calculation of the rate of absorbance change with kinetic readings.*
Result and Discussion: *The subcritical water extract was found to provide highest acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities with an IC_{50} of 0.71 mg/ml, 2.13 mg/ml and 1.21 mg/ml, respectively. Consistent with the anaylsis of total phenol (27.57 mg GAE/g) and flavonoid (4.80 mg QE/g) contents, subcritical water extract of red meat radish can be considered as a potential source for products aiming enzyme inhibitory activity.*
Keywords: *Cholinesterase, microwave, Raphanus sativus, red meat radish, subcritical water, tyrosinase*

ÖZ

Amaç: *Raphanus sativus L. Red Meat köklerinin mikrodalga destekli ve subkritik su ekstrelerinin kolinesteraz ve tirozinaz inhibitör aktivite potansiyeli araştırılmıştır.*
Gereç ve Yöntem: *Geleneksel çözücü ve gelişmiş ekstraksiyon sistemlerinden elde edilen ekstrelerin toplam fenol, flavonoid ve antosiyanin içeriği spektrofotometrik olarak ölçülmüştür. Asetilkolinesteraz, bütirilkolinesteraz ve tirozinaz inhibitör aktiviteleri, kinetik okumalarla absorbans değişim hızının hesaplanmasıyla araştırılmıştır.*
Sonuç ve Tartışma: *Subkritik su ekstresinin sırasıyla 0.71 mg/ml, 2.13 mg/ml ve 1.21 mg/ml IC_{50} değerleri ile en yüksek asetilkolinesteraz, bütirilkolinesteraz ve tirozinaz inhibitör aktivitelerini sağladığı bulunmuştur. Toplam fenol (27.57 mg GAE/g) ve flavonoid (4.80 mg QE/g) içeriği analizi ile de tutarlı olarak, kırmızı etli turpun subkritik su ekstresi enzim inhibitör aktivite amacıyla üretilen ürünler için potansiyel bir kaynak olarak değerlendirilebilir.*
Anahtar Kelimeler: *Karpuz turpu, kolinesteraz, mikrodalga, Raphanus sativus, subkritik su, tirozinaz*

* **Corresponding Author / Sorumlu Yazar:** Halil Koyu
e-mail / e-posta: halil.koyu@ikcu.edu.tr, **Phone / Tel.:** +905368103405

Submitted / Gönderilme : 15.05.2024
Accepted / Kabul : 23.07.2024
Published / Yayınlanma : 10.09.2024

INTRODUCTION

The sustainable use of natural sources for medicinal purposes requires the search for appropriate plant materials and extraction systems. Identification of plant sources that carry bioactives with therapeutic potential and are also easy to grow, store, transport and mass produce can be an effective approach towards sustainability goals. On the extraction technology side, systems with reduced solvent, energy, raw material and time consumption are valuable for both environmental and economic benefits.

Raphanus sativus L. 'Red Meat' (Brassicaceae) is an agricultural radish cultivar which has an inner tissue with red color due to anthocyanins. This cultivar has been called red meat radish, watermelon radish and also Chinese radish. Radish is widely grown and consumed throughout the world, especially in East Asia. Several cultivars have been produced with a wide variety of shape and color. Various parts of radish such as roots, leaves, stems and seeds have been used for medicinal purposes with laxative, stimulant, digestive, stomachic and decongestant aims. The main constituents are phenolic acids, flavonoids (including anthocyanins) and isothiocyanates. Various preparations of *Raphanus sativus* have been shown to exhibit antioxidant, antimicrobial and anticarcinogenic activities [1-3]. Traditionally, radish has been used for food and medicinal purposes. Radish can be consumed as cooked or raw as meal, mainly in salads. For treatment purposes, radish has been used in home remedies against cough, rheumatoid arthritis and and gallstones; in addition to jaundice, liver disease and gastric disorders in Unani, Greeko-Arab, Indian folk medicine, and kidney stones in Yemenite folk medicine [4]. In traditional Chinese medicine, it was mainly used as digestive aid, and for the treatment of diarrhea, dysentery, and cough. Radish was benefited for carminative, diuretic, expectorant, stomachic purposes in Korean and Indian medicine [5].

Advanced extraction techniques have been valuable in obtaining plant bioactives, as they provide a more feasible process, particularly for production purposes. Microwave systems have been emphasised as improved technologies for the extraction of herbal material because they can better protect thermolabile compounds and offer greater yields of active compounds with less time, energy and solvent consumption. Without the need for adding organic solvents, polarity adjustment can be achieved through subcritical water extraction. Subcritical water is one of the green extraction methods that aims to reduce the amount of raw materials, energy and organic solvents used, while increasing selectivity and efficiency and leaving no harmful residues [6,7]. There have been studies investigating the effect of the subcritical water and microwave assisted extractions of phenolic rich plants targeting enzyme inhibitory activities. For the subcritical water extraction of black mulberry fruits, 100°C-60 min.-2 ml/min-150 bar conditions were found to have the highest total phenol (46.71 mg GAE/g), flavonoid (7.34 mg QE/g) and anthocyanin (1.05 mg Cya3GluE) contents with an IC_{50} of 1.48 mg/ml for tyrosinase inhibitory activity [8]. The subcritical water extraction of red cabbage leaves was studied in the range of 120-160°C, where optimum conditions were determined to be 160°C-30 min.-10 solvent:solid ratio-200 bar conditions with total phenol (89.34 mg GAE/g), flavonoid (13.13 mg QE/g), anthocyanin (1.03 mg Cya3GluE/g) contents and exhibiting acetylcholinesterase (IC_{50}: 0.058 mg/ml), butyrylcholinesterase (IC_{50}: 0.215 mg/ml) and tyrosinase (IC_{50}: 0.86 mg/ml) inhibitory activities [6]. In the microwave extraction of black mulberry fruits at 500 watt-10 min.-1:10 solid:solvent ratio conditions, nearly 2 times more potent extract (IC_{50}: 1.44 mg/ml) than conventional solvent extracts (orbital shaker extract IC_{50}: 2.81 mg/ml, ultrasonic bath extract IC_{50}: 2.61 mg/ml) for tyrosinase inhibitory activity was achieved with improved total phenol (21.05 mg GAE/g), flavonoid (2.62 mg QE/g) and anthocyanin (13.28 mg Cya3GluE/g) contents [9]. The optimum conditions for microwave extraction of red cabbage leaves were determined to be 800 watt-4 min.-50% ethanol conditions with total phenol (36.14 mg GAE/g), flavonoid (3.41 mg QE/g) and anthocyanin (10.38 mg Cya3GluE/g) contents, while providing the highest tyrosinase inhibitory activity (IC_{50}: 5.03 mg/ml). The highest butyrylcholinesterase inhibitory activity (IC_{50}: 0.48 mg/ml) was exhibited by 600 watt-12 min.-100% ethanol condition extract, while acetylcholinesterase inhibition was found to be below 50% inhibition at 6.25 mg/ml [7].

Phenolic compounds have been shown to be important among bioactive substances due to their capability to exert a wide range of activities. It has been demonstrated that plant phenolics and extracts have the ability to inhibit certain enzymes which were linked with diseases [10-12]. Acetylcholinesterase and butyrylcholinesterase enzymes were shown to have role in Alzheimer's and

Parkinson's diseases. Alzheimer's disease is a complex neurodegenerative illness, and the progress is affected by various parameters like age and genetics. The degeneration of cholinergic neurons has been pointed out as the cause of cognitive impairment due to reduced cholinergic transmission. Inhibition of cholinesterase enzymes has been a treatment option to overcome this issue. While acetylcholinesterase activity has been mainly targeted, mechanisms to inhibit butyrylcholinesterase also present importance due to its discovered activity in neuronal lesions in Alzheimer's disease [13]. Similar treatment has also been studied for the cognitive impairment in Parkinson's disease, where the use of cholinesterase inhibitors was shown to provide significant improvement in clinical trials [14]. The critical role of tyrosinase enzyme has been demonstrated in the formation of hyperpigmentation due to excess accumulation of produced melanin and melanogenesis in melanoma. Therefore, research on natural bioactives that can inhibit tyrosinase activity has long been conducted [10].

As a source of phenolic compounds, investigation of extracts from red meat radish roots targeting acetylcholinesterase, butyrylcholinesterase (associated with cognitive impairment in Alzheimer's and Parkinson's disease) and tyrosinase (associated with hyperpigmentation and melanoma) activity may reveal beneficial effects and its medicinal potential.

In this study, the research of enzyme inhibitory potential of a plant source which could be appointed as a good candidate for above listed properties, using advanced extraction systems as microwave assisted and subcritical water extraction was aimed.

MATERIAL AND METHOD

Plant Material

Fresh roots of red meat radish (*Raphanus sativus* L. 'Red Meat') were obtained from an agricultural production site in Bayindir Tokatbasi village, Izmir, Turkiye. The roots were cleaned from any residual dirt, white outermost parts were peeled off and red innertissue was sliced for drying. A controlled airflow drying cabinet was used for drying the roots. All of the dried roots were stored at -24 °C until the experiments. Prior to the extractions, the plant material was ground to obtain a homogeneous batch. A voucher specimen was kept at IZEF Herbarium located in Ege University, Türkiye (IZEF-6716).

Extractions

Conventional solvent extractions with 70% ethanol and water were performed with ultrasonic bath (Isolab, Germany). Before extractions, dried roots were homogenized with a blender (Isolab, Germany). In glass flasks, 5 g ground roots were mixed with 100 ml extraction solvents. 0.1% TFA (trifluoroacetic acid) was added to maintain a pH under 3 for anthocyanin's stability. Each round as 1 h was performed at 25°C in dark. After a total of three rounds, the filtrates were combined for evaporation with a rotary evaporator (Buchi, Switzerland). A vacuum concentrator system (Thermo Scientific, USA) and a lyophiliser (Labconco, USA) were used to totally dry the extracts. The samples were stored at -24°C during the experiments [7].

Microwave assisted extraction was performed with a closed vessel microwave system (Sineo, China). In each microwave vessel (2 vessels used) 2.5 g of ground dried roots were mixed with 50 mL extraction solvent and extracted under optimized conditions previously identified in an optimization study. The studied optimized conditions for extraction time, solvent composition and microwave power with the aim of higher bioactive content and enzyme inhibitory activity were as; 12 min-100% ethanol-600 watt, 4 min-50% ethanol-800 watt and 8 min-50% ethanol- 600 watt. Microwave assisted extractions were performed at 25°C. After the extractions, the filtrates were evaporated with the rotary evaporator. Complete dryness of the samples were ensured using the vacuum concentrator system and the lyophiliser. The samples were stored at -24°C during the experiments [7].

Subcritical water extraction was performed with a high pressure reactor system (Amar Equipments, India). The extractor was filled with 10g of ground roots and 100 ml of water. A previously determined optimized conditions of 160°C-30 min-10 solvent:solid ratio were used for subcritical water extraction. A batch extraction was performed at 200 bar. After the extraction, the filtrate was evaporated with the rotary evaporator. Complete dryness of the samples was ensured using the lyophiliser. The

samples were stored at -24°C during the experiments [6].

Analysis of Bioactive Content

Total phenol, flavonoid and anthocyanin contents of the extracts were determined with spectrophotometry (BMG Labtech, Germany). 96 well microplate methods based on Folin-Ciocalteu, aluminum chloride and pH differentiation methods were used, respectively. Gallic acid and quercetin (1-250 µg/ml) were used as reference standards to establish the calibration curves. Absorbance measurements were performed at 760 nm and 415 nm for total and flavonoid contents, while 520 nm and 700 nm for total anthocyanin content. The results of triplicate analysis for total phenol, flavonoid and anthocyanin contents were expressed as mg gallic acid equivalent (GAE)/g extract, mg quercetin equivalent (QE)/g extract and mg cyanidin-3-glucoside equivalent (Cya3GluE)/g extract [7]. Statistical significance (p<0.05) of the results were determined with one-way ANOVA and post hoc comparison Tukey's HSD Test using IBM SPSS Statistics 29.0 software.

Enzyme Inhibitory Activity

Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of the samples were determined with microplate assays. Previously developed and validated assays using inhibiton kinetic models (Michaelis-Menten, Lineweaver-Burk and Eadiee-Hofstee methods) were used. Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates and galantamine as reference drug for cholinesterase inhibitory activity, whereas levodopa and kojic acid were used for tyrosinase inhibitory activity, respectively. Enzyme inhibitory activities were determined with kinetic readings taken with 30 sec intervals during incubation period (10 min for cholinesterase, 5 min for tyrosinase). The activity results were calculated based on the rate of absorbance change during incubation period against blank. The results of triplicate analysis were acquired to calculate IC_{50} values by linear regression analysis using GraphPad Prism 5 software [7].

RESULT AND DISCUSSION

The preparation of the homogeneous dried batch of the plant material was carried out with ten parallel samples. Each sample was weighed during the drying process and the moisture content was calculated. An average dry weight percentage of 9.50%±0.61 with a relative standard deviation of 6.45% was observed. The moisture content of the samples was determined as 91.50%, which was in agreement with the literature data [15].

Conventional solvent extracts (70% ethanol and water), microwave assisted extracts (12 min-100% ethanol-600 watt, 4 min-50% ethanol-800 watt and 8 min-50% ethanol- 600 watt) and subcritical water extracts of red meat radish were evaluated for bioactive content and enzyme inhibitory activity (Table 1). Among the solvent extracts, 70% ethanol (0.1% TFA) provided higher total phenol (10.87 mg GAE/g), flavonoid (0.92 mg QE/g) and anthocyanin (3.71 mg Cya3GluE/g) contents compared to water extract. In accordance with literature data, higher bioactive content was observed with aqueous ethanol with respect to the phenolic compounds [16]. An 80% methanol extract of red radish pulps was determined with a total phenol content of 41.17 mg GAE/g dry weight [3], while in another study the total phenol content of watermelon radish was shown to be 6.27 mg GAE/g dry weight [17]. Lower levels of total phenol content (6.07 mg GAE/g dry weight) obtained with 70% ethanol extraction could be related to variations in *Raphanus sativus* cultivars. The difference for total anthocyanin content was outstanding with 2.3 times, where 70% ethanol was also previously determined with enhanced capability for anthocyanin extraction [7]. Regarding the microwave assisted extraction, the highest total phenol content (5.21 mg GAE/g) was obtained with 8 min-50% ethanol-600 watt conditions. 12 min-100% ethanol-600 watt conditions provided the highest total flavonoid content (0.67 mg QE/g) while 4 min-50% ethanol-800 watt was found to be superior for total anthocyanin content (1.54 mg Cya3GluE/g). The results were found to be consistent with previous findings, as simultaneous increase in both extraction time and ethanol concentration had a positive effect on total phenol content [7]. However, in another study contrary results were observed. In the study with the black mulberry fruits, lowering the ethanol conc. to 35% with an extraction time of 10 min allowed the extraction with the highest total

phenol content [9]. This situation may highlight the limited applicability of the direct use of different predefined optimisation parameters determined for subcritical water and microwave extractions, which can vary greatly due to the form of the studied plant material as fruit, leaf or root. The anthocyanin content of red meat radish (from USA) juice was quantified as 33.6 mg/100 g roots in a previous study [18]. The extraction of *Raphanus sativus* L. var. Crimson Gigant (from Mexico) was performed with 85% methanol (0.01% HCl), where the total anthocyanin content was determined as 17.36 mg Cya3GluE/g fresh weight [19]. In another study, the purified anthocyanin fraction of *Raphanus sativus* L. (from USA) was quantified as 154 mg Pel3GluE/100 g fresh weight for total anthocyanin content [20]. The total anthocyanin content of aqueous acetone (30:70) extract of red meat radish (from USA) was determined to be 53 mg Pel3GluE/100 g fresh weight [21]. Acidified hexane extract of red meat radish (a red flesh *Raphanus sativus* L. cultivar from China) was shown to yield 2.08 mg Pel3Glu/g fresh weight [22]. The research on methanol (0.1% HCl) extracts of twenty-four radish varieties from India exhibited a great variation in bioactive content; where anthocyanin content was found to vary as much as 36.16-fold (ranging from 0.46 to 16.6 mg Cya3GluE/100 g fresh weight) among the radishes tested [23]. The variations in anthocyanin content of radishes in different studies may be determined due to the extraction method applied, the quantification reference used (quantification based on cyanidin-3-glucoside or pelargonidin-3-glucoside), the use of radishes from different cultivars, genotypic and environmental factors [21-23]. Based on the crude extracts, 70% ethanol ultrasonic extraction outperformed both microwave and subcritical water extractions for total anthocyanin content. The microwave extraction (1.54 mg Cya3GluE/g extract, 0.23 mg Cya3GluE/g dry weight, 2.2 mg Cya3GluE/100 g fresh weight) was found with 2.4 times lower anthocyanin content compared to conventional solvent extraction (3.71 mg Cya3GluE/g extract, 2.07 mg Cya3GluE/g dry weight, 19 mg Cya3GluE/100 g fresh weight). With the studied extraction parameters, regarding the extraction of anthocyanins, selective and exhaustive extraction capability of the microwave and subcritical water extractions were determined to be limited. The highest values for the quantified bioactive contents were obtained with subcritical water extraction as 27.57 mg GAE/g and 4.80 mg QE/g for total phenol and flavonoid content, respectively. No anthocyanin content was able to be extracted with the studied parameters from red meat radish roots. Previous investigations on other anthocyanin rich sources as black mulberry fruits [8] and red cabbage leaves [6] had yielded 0.61 mg Cya3GluE/g and 1.03 mg Cya3GluE/g total anthocyanin content at 120°C-60 min-150 bar and 160°C-30 min-200 bar extractions, respectively. Therefore, 160°C can be considered as the overthreshold condition for the extraction of red meat radish anthocyanins, where reduced temperature values of around 120°C can be targeted for the optimization of subcritical water extraction of red meat radish roots in future studies. Both in the previous studies, increasing the temperature had a dramatically improving effect on total phenol and flavonoid contents of the extracts. Similar results were obtained with the subcritical water extraction of red meat radish, where 160°C-30 min-200 bar extract possessed 27.57 mg GAE/g and 4.80 mg QE/g total phenol and flavonoid contents, respectively. When the conventional solvent and advanced extraction systems (microwave and subcritical water) were compared, the subcritical water extract was found to provide the extract with the most rich bioactive content for total phenol and flavonoid contents, while the highest anthocyanin amounts were obtained with 70% ethanol extraction (Figure 1). The use of relatively high microwave power as 600 and 800 watts could be the reason for the limited ability for the extraction of red radish anthocyanins, where microwave extraction of red radish roots might better be more effective as studied with lower power values for anthocyanin extraction.

Subcritical water extract was found to provide an IC_{50} of 0.71 mg/ml and 2.13 mg/ml for acetylcholinesterase and butyrylcholinesterase inhibitory activity, respectively. IC_{50} values for cholinesterase inhibitory activity of conventional solvent and microwave assisted extracts could not be determined as 50% inhibition was not exceeded at the starting concentration of 1.50 mg/ml. For tyrosinase inhibitory activity, the strongest activity (IC_{50}: 1.21 mg/ml) was found in the subcritical water extract, similar to the cholinesterase inhibition results. Microwave II extract with the extraction conditions of 4 min-50% ethanol-800 watt provided the highest butyrylcholinesterase inhibitory activity among the microwave extracts studied. On the side of the conventional solvent extracts, IC_{50} of 70% ethanol extract for butyrylcholinesterase inhibitory activity was found to be 1.88 mg/ml. Compared with reference drugs as galantamin (IC_{50}: 0.23 µg/ml and 2.62 µg/ml) and kojic acid (IC_{50}: 4.82 µg/ml), all

extracts from conventional solvent, microwave assisted, and subcritical water systems possessed remarkably weaker activity in the crude form. Among the extracts, subcritical water was noted with greater potency both for cholinesterase and tyrosinase inhibitory activity. In previous studies similar results were observed as 160°C-30 min-10 solvent:solid ratio extract was found to possess the highest acetylcholinesterase and butyrylcholinesterase inhibitory activity in red cabbage extracts [6]. Tyrosinase inhibitory activity of different radish varieties were determined with an IC_{50} of 3.09 mg/ml and 9.62 mg/ml for juice and methanol extracts of Thai radish [24], and 42% inhibition at 2 mg/ml dose for *Raphanus sativus* var. *caudatus* [25], respectively. Tyrosinase inhibition by ethanol extract of *Raphanus sativus* var. *longipinnatus* was shown to be 42.85% at 1.67 mg/ml dose [26]. In comparison with the literature, both subcritical water (IC_{50}: 1.21 mg/ml), microwave assisted (IC_{50}: 2.20 mg/ml) and conventional solvent (IC_{50}: 1.88 mg/ml) extracts of red meat radish were found to have greater potency for tyrosinase inhibitory activity.

Table 1. Comparison of microwave and subcritical water extracts of red meat radish with conventional solvent extracts

Microwave and Subcritical Water Extracts vs Conventional Solvent Extracts	Temp (°C)	Time (min)	Solvent: solid ratio	Ethanol conc. (%)	Power (watt)	TPC* (mg GAE/g)	TFC* (mg QE/g)	TAC* (mg Cya3 GluE/g)	AchEInh (IC_{50}, mg/ml)	BuChEInh (IC_{50}, mg/ml)	TyroInh (IC_{50}, mg/ml)
Microwave I	25	12	20	100%	600	4.51±0.12^{a,b}	0.67±0.07^{a,b}	0.82±0.04^{a}	ND	ND	ND
Microwave II	25	4	20	50%	800	3.89±0.06^{a}	0.61±0.09^{a,b}	1.54±0.10^{b}	ND	ND	2.20±0.09^{a}
Microwave III	25	8	20	50%	600	5.21±0.38^{b}	0.23±0.02^{a}	1.08±0.10^{a}	ND	ND	ND
Subcritical Water	160	30	10	0%	NA	27.57±1.09^{c}	4.80±0.34^{c}	-	0.71±0.04	2.13±0.18	1.21±0.04^{b}
70% Ethanol (0.1% TFA)	25	180	60	70%	NA	10.87±0.10^{d}	0.92±0.01^{b}	3.71±0.17^{c}	ND	ND	1.88±0.16^{c}
Water (0.1% TFA)	25	180	60	0%	NA	8.83±0.07^{e}	0.46±0.01^{a,b}	1.62±0.01^{b}	ND	ND	ND

p<0.05 (Different letters in column indicate statistical significance); *: mg/g extract ± SD; NA: Not applied; -: Not detected
ND: Not determined due to 50% inhibition could not be exceeded at 1.50 mg/ml concentration; TPC: total phenol content; TFC: Total flavonoid content; TAC: total anthocyanin content; GAE: gallic acid equivalent; QE: quercetin equivalent; Cya3GluE: cyanidin-3-glucoside equivalent; AChEInh: acetylcholinesterase inhibitory activity; BuChEInh: butyrylcholinesterase inhibitory activity; TyroInh: tyrosinase inhibitory activity

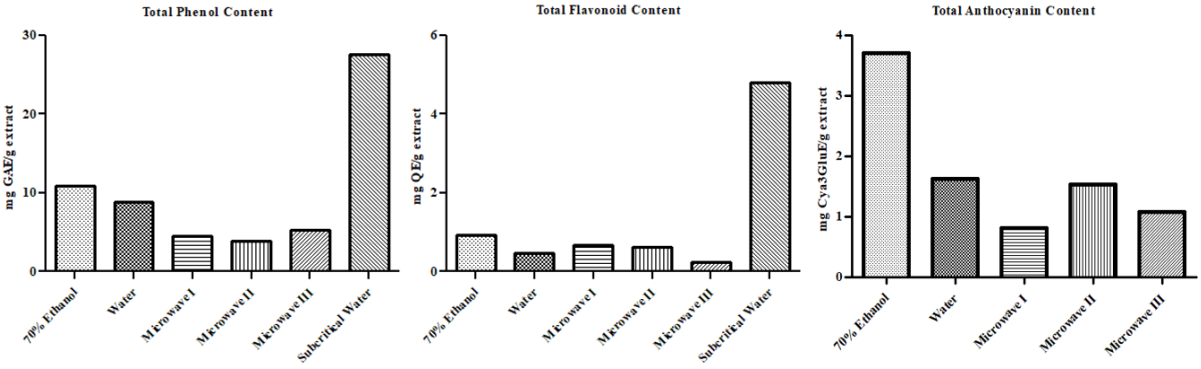

Figure 1. Comparison of bioactive content of red meat radish's conventional solvent, microwave assisted and subcritical water extracts (The statistical significance of the results are presented with Table 1)

Considering the content-activity relationship, the subcritical water extract with higher total phenol (2.7-fold) and flavonoid (5.3-fold) content was found to exhibit the most potent cholinesterase and tyrosinase inhibitory activities, where the activity results could be attributed to its rich phenolic composition. 70% ethanol extract was determined with a remarkable tyrosinase inhibitory activity

among the tested samples, while it was identified with the highest total anthocyanin content (3.71 mg Cya3GlueE/g) (Table 1). Identical results were also obtained for the microwave extracts. Elevated total anthocyanin content (Microwave II extract) could be related to a higher tyrosinase inhibition. Similar to previous studies, the strongest tyrosinase inhibition was observed with the anthocyanin rich extracts in microwave extracts of black mulberry fruits [9] and red cabbage leaves [7]. Although the subcritical water extract was not able to provide anthocyanin content, improved tyrosinase inhibitory activity could be attibuted to its dramatically increased total phenol and flavonoid amounts, which was also consistent with literature data [6,8].

General advantages of microwave extraction have been denoted as the protection of active compounds due to shortened process duration, while consuming less energy and extraction solvent [7]. With the microwave extraction of red meat radish, 3 times lowered solvent amounts and dramatically reduced extraction time (180 min. vs 4 min.) was used. However, compared to conventional extractions, no improvement was observed for the extraction of targeted bioactive content (Table 1). Microwave extraction was able to yield a total anthocyanin content of 1.54 mg Cya3GluE/g using a 50% ethanol solvent system. Both microwave and conventional solvent extracts were unable to possess remarkable acetylcholinesterase and butyrylcholinesterase inhibitory activities (<50% inhibition at 1.50 mg/ml), while tyrosinase inhibition was observed with 800 watt-4 min.-20 solvent:solid ratio (IC_{50}: 2.20 mg/ml) and 70% ethanol ultrasonic (IC_{50}: 1.88 mg/ml) extracts.

Subcritical water has emerged as a green extraction technique with the advantage of eliminating the use of organic solvents by allowing the polarity of the extraction solvent to be regulated in the subcritical phase of water. In addition to the highlighted advantage of being free of toxic and harmful residues to nature and humans, the ability to improve selectivity for bioactive compounds is important [6]. Without the need to use any organic solvent, subcritical water extraction was found to possess the highest total phenol (27.57 mg GAE/g) and flavonoid (4.80 mg QE/g) contents for the extraction of red meat radish (Table 1). Consistent with the bioactive composition, the highest enzyme inhibitory activity was determined with the subcritical water extract for all studied enzymes. However, the subcritical water system was found to be incapable of extracting the red meat radish anthocyanins with the studied extraction parameters.

The main drawbacks for both subcritical water and microwave extractions could be pointed out as; higher initial cost of setup for high-tech equipment, need for training and experience to operate and maintain the extraction systems, and limited applicability of the discovered optimum extraction parameters in literature directly to the investigation of new plant materials, as each plant material may require a novel optimization study to possess the highest potential for bioactive content and targeted activity.

This is the first study comparatively revealing cholinesterase and tyrosinase inhibitory potential of subcritical water and microwave assisted extracts of red meat radish. In the pursuit of sustainability goals, red meat radish can be considered as a potential source of phenolic compounds with enzyme inhibitory activity through the extraction of advanced techniques. Further studies are planned on the fractionation and isolation of the subcritical water extract of red meat radish.

ACKNOWLEDGEMENTS

This work was a part of a project supported by Izmir Katip Celebi University Scientific Research Projects Funds [2018-ÖNAP-ECZF-0009]. Access to the facilities of Novel Fluidic Technologies Laboratory and Pharmaceutical Sciences Research Center (FABAL) at Ege University, and Central Research Laboratories (MERLAB) at Izmir Katip Celebi University are highly appreciated.

AUTHOR CONTRIBUTIONS

Concept: H.K.; Design: H.K.; Control: H.K.; Sources: H.K.; Materials: H.K.; Data Collection and/or Processing: H.K.; Analysis and/or Interpretation: H.K.; Literature Review: H.K.; Manuscript Writing: H.K.; Critical Review: H.K.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1050-1057, 2024
Doi: 10.33483/jfpau.1512913

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IN VITRO EVALUATION OF THE ANTIBACTERIAL EFFECT OF CHITOSAN COATED TEICOPLANIN-LOADED LIPID NANOPARTICLES AGAINST *STAPHYLOCOCCUS AUREUS*

KİTOSAN KAPLI TEİKOPLANİN YÜKLÜ LİPİD NANOPARTİKÜLLERİN STAPHYLOCOCCUS AUREUS'A KARŞI ANTİBAKTERİYEL ETKİSİNİN İN VİTRO DEĞERLENDİRİLMESİ

Berrin KÜÇÜKTÜRKMEN[1]*, Merve Eylul KIYMACI[2]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06560, Ankara, Türkiye
[2]University of Health Sciences Turkey, Gülhane Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06018, Ankara, Türkiye

ABSTRACT

Objective: *The aim of this research is to formulate teicoplanin-loaded solid lipid nanoparticles (SLN) coated with chitosan to sustain teicoplanin release for effective antibacterial therapy.*
Material and Method: *"Double emulsion-solvent evaporation technique was used for the production of SLNs. The nanoparticles were characterised in terms of morphology, size, encapsulation efficacy, in-vitro drug release and antibacterial activity studies after optimization of process and formulation parameters.*
Result and Discussion: *Transmission electron microscopy images confirmed the formation of spherical SLNs. With chitosan coating, the size increased (from 80 nm to 106 nm) and the negative value of zeta potential (- 11.29) changed to positive (+34.41). The in-vitro release data showed prolonged release of teicoplanin from optimized SLN and chitosan-coated SLN (c-SLN) formulations over 1 week. The antibacterial activity study (S. aureus and Methicillin-resistant S. aureus) showed the activity of the teicoplanin loaded SLN formulations. In conclusion, this study demonstrated the potential of c-SLN for effective delivery of teicoplanin.*
Keywords: *Chitosan, coating, lipid nanoparticle, precirol ATO 5, teicoplanin*

ÖZ

Amaç: *Bu araştırmanın amacı, etkili antibakteriyel tedavi için teikoplanin salımını uzatmak üzere kitosanla kaplanmış teikoplanin yüklü katı lipit nanopartiküllerinin (SLN) formüle edilmesidir.*
Gereç ve Yöntem: *SLN'ler çift emülsifikasyon-solvent buharlaştırma tekniği ile hazırlanmıştır. Proses ve formülasyon parametrelerinin optimizasyonu sonrasında nanopartiküller morfoloji, boyut, enkapsülasyon etkinliği, in vitro etken madde salımı ve antibakteriyel aktivite çalışmaları açısından karakterize edilmiştir.*
Sonuç ve Tartışma: *Geçirimli elektron mikroskobu görüntüleri küresel SLN'lerin oluşumunu doğrulamıştır. Kitosan kaplama ile boyut artmış (80 nm'den 106 nm'ye) ve zeta potansiyelinin negatif değeri (-11.29) pozitife (+34.41) dönüşmüştür. İn vitro salım verileri, optimize edilmiş SLN ve kitosan kaplı SLN (c-SLN) formülasyonlarından teikoplaninin 1 hafta boyunca uzun süreli salınımını göstermiştir. Antibakteriyel aktivite çalışması (S. aureus ve Metisiline dirençli S. aureus),*

* **Corresponding Author / Sorumlu Yazar:** Berrin Küçüktürkmen
e-mail / e-posta: berrinkucukturkmen@gmail.com, **Phone / Tel.:** +903122033159

Submitted / Gönderilme : 09.07.2024
Accepted / Kabul : 23.07.2024
Published / Yayınlanma : 10.09.2024

teikoplanin yüklü SLN formülasyonlarının aktivitesini göstermiştir. Sonuç olarak, bu çalışma c-SLN'nin teikoplaninin etkin verilişi için potansiyelini ortaya koymuştur.
Anahtar Kelimeler: *Kaplama, kitosan, lipit nanopartikül, precirol ATO 5, teikoplanin*

INTRODUCTION

Antibiotics constitute the primary treatment step in pathogenic bacterial infections. However, in recent years, due to the uncontrolled or incorrect use, antibiotic-sensitive bacteria are constantly mutating, which triggers the emergence of bacterial drug resistance [1]. Against antibiotic resistance, the use of drug delivery systems that can deliver high doses of antibiotics that cannot be administered systemically or cause side effects directly to the site of infection has improved the therapeutic index while minimizing side effects [2]. However, antibiotics may be sensitive to production conditions such as temperature increase, sonication, use of organic solvents, etc., which are common in preparation methods of nanoparticles. For these reasons, conditions to which antibiotics are sensitive, such as temperature, should be optimized, especially during the preparation and encapsulation steps.

SLNs have great potential as nanocarriers for antibiotic encapsulation due to their industrial scale-up capability and minimal toxicity [3]. Their biodegradability, biosafety, long-term release behavior, and ability to hold both lipophilic and hydrophilic medicines make them appealing for improving drug targeting capabilities [4]. The most utilized methods for preparing SLNs are high-pressure/high shear homogenization [5] and microemulsion-based approaches [6]. When combined with the solvent evaporation approach, the double emulsion method (w/o/w), is more mild and prevents temperature or pressure stress on the trapped molecules [7].

The surface of SLNs can be modified for various purposes, such as preventing the removal of nanoparticles from the circulation by the reticuloendothelial system, increasing the half-life of the active molecule loaded into the particulate system, improving the stability of nanoparticles, reducing toxicity, increasing biocompatibility, and increasing mucosal penetration. Surface modification of nanoparticles with chitosan, a natural cationic polysaccharide derived from chitin, is a strategy used to increase the penetration of encapsulated molecules through mucosal surfaces. This biopolymer is preferred due to its antimicrobial and mucoadhesive properties, as well as being biocompatible, biodegradable, and low toxicity [8].

Teicoplanin is a glycopeptide antibiotic produced by Actinoplanes teichomiceticus that acts through inhibition of bacterial cell wall biosynthesis, is effective against Gram-positive bacteria and methicillin-resistant *Staphylococcus aureus* (MRSA). Compared to other glycopeptide antibiotics such as vancomycin, teicoplanin is significantly more surface active with multiple, unique, functional groups. Another advantage of teicoplanin is that it can be sterilized with γ-irradiation [9]. There are few studies in the literature on nano systems containing teicoplanin. In this paper, we investigated the synthesis and efficiency of teicoplanin-bearing SLNs as an innovative system for glycopeptide formulation.

The goal of this research was to produce teicoplanin-loaded SLN formulation by using double emulsion and solvent evaporation technique to achieve small particle size and high teicoplanin encapsulation. The surface of SLN formulations was coated with chitosan polymer, which has adhesive and antimicrobial properties. The developed SLN and chitosan-coated SLN formulations were characterized *in vitro*.

MATERIAL AND METHOD

Materials

Precirol ATO 5 was gifted from Gattefossé (France), teicoplanin, chitosan (low molecular weight), Pluronic F68 and dichloromethane were purchased from Sigma-Aldrich Co (USA). All other analytical grade reagents were used as received without further purification.

Preparation of Solid Lipid Nanoparticles and Coating with Chitosan

Double emulsion solvent evaporation method was used for the preparation of SLN [7,10]. First, Pluronic F68 (2%) was mixed with the cold water and kept in the refrigerator overnight for a clear

solution. 40 mg of Precirol ATO 5 was dissolved in 1 ml dichloromethane. Teicoplanin was dissolved in 0.5 ml of Pluronic F68 solution and added to the lipid phase and sonicated for 1 min (Bandelin Sonopuls HD2070 (Germany), 90% amplitude). The resulting emulsion was then immediately added to 10 ml of Pluronic F68 solution and sonicated 10 mins while cooling in an ice bath. Dichloromethane was evaporated by magnetic stirring and teicoplanin-loaded solid lipid nanoparticles were obtained with a Vivaspin® 20 centrifugal concentrator. To coat the solid lipid nanoparticles, chitosan (1%) was dissolved in acetic acid solution (1%). Lipid nanoparticles and chitosan solution (1:1, v/v) were magnetically stirred for 90 minutes, and chitosan-coated nanoparticles were obtained with a Vivaspin® 20 centrifugal concentrator.

Particle Size and Size Distribution

SLN aliquots were diluted tenfold with ultrapure water. The particle size and distribution of SLN formulations were measured at ambient conditions using Malvern Zetasizer Ultra (Malvern Instruments, UK) and each sample was analyzed in triplicate [11].

Encapsulation Efficiency (EE)

The EE of teicoplanin was determined by the indirect method [12]. In the end of the nanoparticle preparation process, nanoparticles were collected with the Vivaspin® 20 centrifugal concentrator. The amount of teicoplanin in the supernatant phase was analyzed by the microBCA analysis method and the EE was calculated by the following equation. All measurements were carried out in triplicate and results were expressed as mean values with a standard deviation (SD).

$$\text{EE } \% = \frac{\text{Total amount of Teicoplanin} - \text{Free Teicoplanin}}{\text{Total amount of Teicoplanin}} * 100$$

***In Vitro* Release**

Dialysis membrane method was used to determine the *in vitro* release of teicoplanin from the F8 and c-F8 SLN formulations [11]. SLN formulations were placed in the dialysis bag (12000-14000 Da MWCO), the dialysis bag was placed in pH 7.4 phosphate buffer (20 ml) at 37±0.5°C and shaken at 50 rpm in an incubated shaker. A volume of 0.5 ml was withdrawn at each specified time, replaced with the same amount of fresh pH 7.4 phosphate buffer and teicoplanin concentrations were measured by microBCA analysis. The cumulative drug release percentage was calculated, and *in vitro* release graphs were created. All experiments were performed in triplicate.

Transmission Electron Microscopy (TEM) Analysis

The morphology of the c-F8 formulation was determined by TEM (FEI Tecnai G2, the Netherlands). Briefly, a drop of the c-F8 SLN formulation was placed on 200-mesh copper grid, excess formulation was removed using filter paper, dried at room temperature and imaged by TEM (120 kV) [13].

DSC Analysis

The thermal properties of teicoplanin, Precirol ATO 5, plain SLN, teicoplanin loaded SLN and chitosan-coated teicoplanin-loaded SLN were analyzed with a Shimadzu DSC-60 device. Approximately 5 mg of samples were placed in an aluminum pan and compressed. Nitrogen gas (50 ml/min), heating rate of 10°C/min, temperature range 30-300°C were used in the analysis of the samples [11].

Antibacterial Activity

Antibacterial activity of the teicoplanin, F8 and c-F8 formulations were tested against *Staphylococcus aureus* (ATCC 29213) and methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC 43300). Antibacterial activity test was evaluated by broth microdilution method as a minimal inhibition concentration (MIC) according to European Committee on Antimicrobial Susceptibility Testing standards (ISO 20776-1:2019) [14]. In the broth microdilution test, 100 µl of cation-adjusted Mueller-Hinton broth (MHB) was added to each well of a sterile U-bottom microplate. The same volume of SLN

formulations (teicoplanin concentrations from 0.0625 μg μl^{-1} to 32 μg μl^{-1}) [15] and controls was added to the first well and diluted. Then, a 1:100 dilution of bacterial suspension made in 0.9% NaCl from fresh bacterial culture to a density of 0.5% McFarland turbidity was added to the wells. Following the incubation of microplates at 35 ± 1°C for 18 ± 2 hours, MIC was defined as the lowest concentration of formulations that inhibited the visible growth of bacteria.

RESULT AND DISCUSSION

Particle Size, PDI and Zeta Potential

Different drug/lipid ratios and sonication times were tested to optimize the particle size of SLN formulations (Table 1). All formulations were prepared with fixed concentration of drug (2 mg) and surfactant (2%), varying only lipid ratios (10-40 mg). When the sonication time was kept constant, the particle size clearly decreased as the drug/lipid ratio increased. In the opposite case, when the drug/lipid ratio was kept constant, the particle size clearly decreased as the sonication time increased. The formulation coded F8, where the drug/lipid ratio was 20:1 and the sonication time was 10 minutes, was found to have a significantly smaller particle size than the others. It was observed that PDI values increased significantly as the particle size decreased. Although the zeta potential value of the F8 formulation is lower than other formulations, it was decided to proceed to the next stage by choosing the F8 formulation since it is significantly smaller than the other formulations, considering that the chitosan coating planned to be applied afterwards will increase the stability and taking into account that the particle size will increase after coating. In the c-F8 formulation coated with chitosan, it was observed that the particle size increased slightly to 106.8 nm and the zeta potential turned positive as expected. The zeta potential of the c-F8 formulation was found to be +34.41 mV and was classified as highly stable according to the literature [13]. The coating mechanism of SLN is based on the electrostatic interaction of positively charged chitosan with the negatively charged SLN surface. The method was found to be advantageous as it preserves the structure and functionality of teicoplanin because it does not involve heat or chemical functionalization [8].

Table 1. Composition and characterization results of formulations (Data are expressed as mean ± SD of triplicate measurements)

	Lipid:Drug (w/w)	Sonication (min)	Particle size (nm)	PDI	Zeta potential (mV)
F1	10:1	2	512.8±3.67	0.284±0.014	-25.16±1.59
F2	20:1	5	545.5±41.8	0.454±0.092	-23.65±0.75
F3	5:1	5	309.8±11.3	0.421±0.026	-25.15±0.60
F4	10:1	10	273.1±90.4	0.357±0.026	-33.67±0.78
F5	10:1	5	445.1±22.4	0.402±0.044	-19.78±0.09
F6	5:1	2	375.2±7.66	0.315±0.080	-22.80±0.38
F7	5:1	10	249.4±19.1	0.324±0.022	-27.92±1.19
F8	20:1	10	80.69±1.06	0.594±0.033	-11.29±0.90
c-F8	20:1	10	106.8±4.41	0.681±0.048	+34.41±2.05

Encapsulation Efficiency (EE%)

Encapsulation efficiency of F8 and c-F8 formulations were found 58.23% ±1.18 and 54.47% ±7.94, respectively (Table 2). It was observed that the encapsulation efficiency decreased slightly in the c-F8 formulation coated with chitosan. This decrease may be due to a small loss of active ingredient on the surface of the nanoparticles during the 90 mins mixing time in the coating process.

Table 2. Encapsulation efficiency (%) results of F8 and c-F8 formulations (Data are expressed as mean ± SD of triplicate measurements)

	F8	c-F8
Encapsulation Efficiency (%)	58.23 ±1.18	54.47±7.94

In Vitro Release

The *in vitro* release of teicoplanin from F8 and c-F8 formulations were compared (Figure 1). It was observed that the *in vitro* release of free teicoplanin was completed in 8 hours. With F8 and c-F8 formulations, the *in vitro* release time of teicoplanin was extended to over 1 week. The *in vitro* release of teicoplanin from the c-F8 formulation is slower than that from the F8 formulation. This is an expected result since the c-F8 formulation is coated with chitosan. Similar results have been seen in the literature for nanoparticle systems coated with chitosan [16]. These data obtained as a result of *in vitro* release are preliminary indicators of advantageous features such as providing a long-term antibacterial effect in the in vivo environment, stable protection of the active substance in nanoparticle structure, and extending its half-life by not being eliminated immediately.

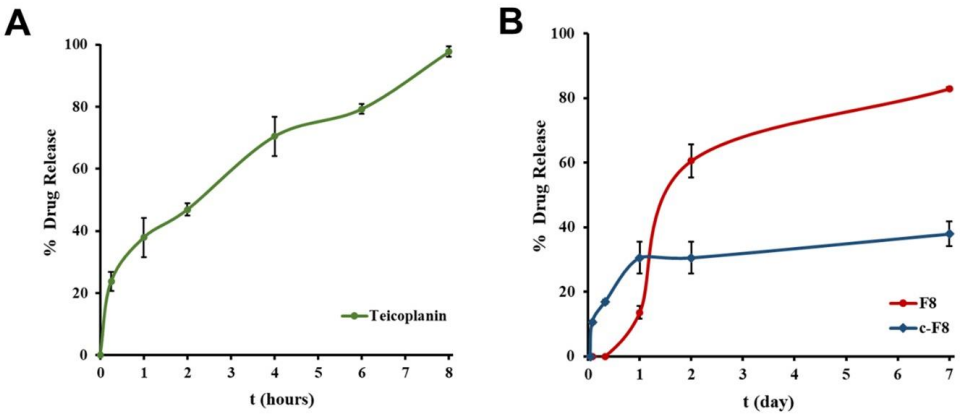

Figure 1. *In vitro* release of free teicoplanin (A) and *in vitro* release of teicoplanin from F8 and c-F8 formulations (B)

TEM Analysis

TEM analysis was performed to examine nanoparticle morphology. Figure 2 is the TEM image of the c-F8 formulation, and the particles observed to be smaller than 100 nm in spherical shape are consistent with the particle size analysis data.

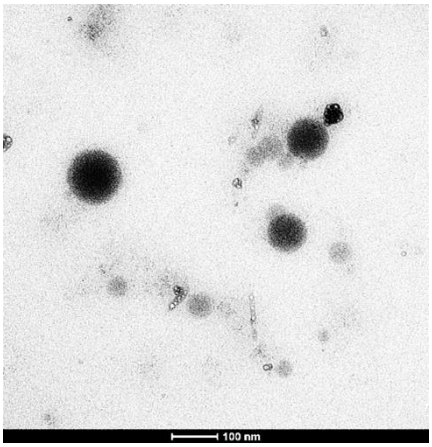

Figure 2. TEM image (scale: 100 nm) of c-F8 formulation

DSC Analysis

DSC analysis revealed an endothermic peak belonging to teicoplanin at 55.31°C, which is consistent with the literature [2]. Characteristic peak of Precirol ATO 5 observed at 55.18°C [17]. DSC

thermogram of drug-free SLN, F8 and c-F8 formulations exhibited similar peak around 47-48°C (Figure 3). It was observed that the peak of teicoplanin-loaded SLN formulations had similar pattern, location, and intensity as the blank formulation and this result confirmed incorporation of antibiotic in the SLNs.

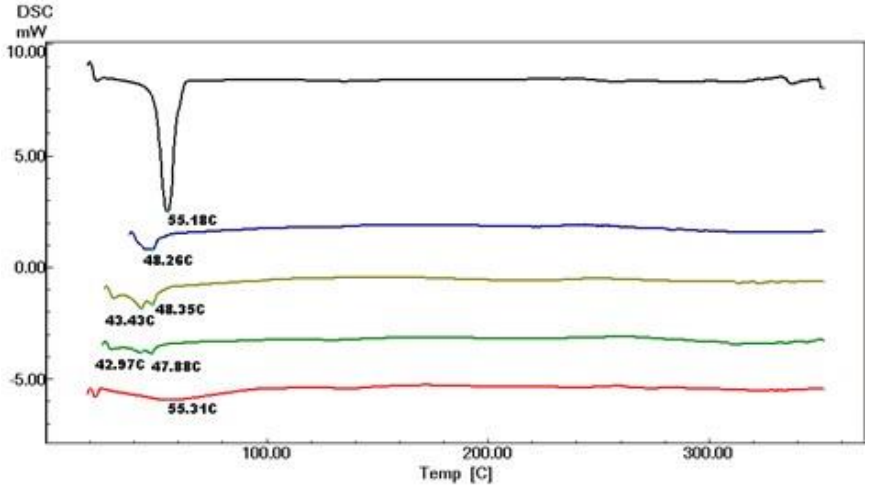

Figure 3. DSC thermograms of teicoplanin (red), F8 (green), drug-free SLN (khaki), c-F8 (blue), Precirol ATO 5 (black)

Antibacterial Activity

Antibacterial activity of teicoplanin loaded F8 and c-F8 formulations were investigated by broth microdilution method. MIC results for all formulations were listed at Table 3. Free teicoplanin application on both *S. aureus* and MRSA strains resulted in MIC of 0.061 µg ml^{-1}. An MIC value of 0.0625 µg ml^{-1} was obtained with the F8 and c-F8 formulations, similar to teicoplanin. This result may be due to the prolonged release of teicoplanin for up to 1 week, as seen in the *in vitro* release test. Therefore, the release rate may increase the inhibitory effect of teicoplanin over time. The MIC value of the c-F8 formulation was found to be 1 µg ml^{-1}. Chitosan's antimicrobial activity against a variety of bacteria is well known. Therefore, it was evaluated that this result was achieved due to the chitosan coating in the c-F8 formulation. Similar MIC values of different chitosan types against *S. aureus* strains are available in the literature [18].

Table 3. MIC values of the teicoplanin and formulations against *S. aureus* strains

Bacteria	Minimal inhibition concentration (µg/ml)				
	F8	c-F8	Drug-free F8	Drug-free c-F8	Teicoplanin
***Staphylococcus aureus* ATCC 29213**	0.0625	0.0625	0	1	0.061
Methicillin-resistant *Staphylococcus aureus* ATCC 43300	0.0625	0.0625	0	1	0.061

In this research, a teicoplanin-loaded chitosan-coated SLN formulation was developed and its potential for application as an antibacterial drug delivery system was evaluated. The double emulsion-solvent evaporation method was used to produce nanoparticles. It was concluded that the optimized teicoplanin-loaded c-SLN formulation consisted of spherical-shaped particles, the particle size was 106.8 nm, the encapsulation efficiency was 54.47%, and it was effective on both *S. aureus* and MRSA. These observations indicate that the developed nanoparticulate carrier system is promising for antibiotic delivery.

AUTHOR CONTRIBUTIONS

Concept: B.K.; Design: B.K.; Control: B.K., M.E.K.; Sources: B.K., M.E.K.; Materials: B.K., M.E.K.; Data Collection and/or Processing: B.K., M.E.K.; Analysis and/or Interpretation: B.K., M.E.K.; Literature Review: B.K., M.E.K.; Manuscript Writing: B.K., M.E.K.; Critical Review: B.K., M.E.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflicts of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethical committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1058-1067, 2024
Doi: 10.33483/jfpau.1522001

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ANTI-CANCER DRUG PALBOCICLIB FROM HUMAN BIOLOGICAL FLUIDS BY USING DIFFERENTIAL PULSE VOLTAMMETRIC METHOD AT BORON DOPED DIAMOND ELECTRODE

ANTİ-KANSER İLACI PALBOCICLIB'İN BOR KATKILI ELMAS ELEKTROTTA DİFERANSİYEL PULS VOLTAMETRİ YÖNTEMİ KULLANILARAK İNSAN BİYOLOJİK SIVILARINDAN TAYİNİ

Melike AKAN[1], Çiğdem KANBEŞ-DİNDAR[2]*, Nazife ASLAN[1]*, Bengi USLU[2]*

[1]Ankara Hacı Bayram Veli University, Polatlı Science and Arts Faculty, Chemistry Department, 06900, Ankara, Türkiye
[2]Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06560, Ankara, Türkiye

ABSTRACT

Objective: *A very efficient and sensitive electrochemical technique utilizing differential pulse voltammetry (DPV) at a boron-doped diamond electrode (BDDE) was devised to measure Palbociclib in this study.*
Material and Method: *All experiments employed typical three-electrode cell of 10 ml capacity in conjunction with boron-doped diamond electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode. During electrochemical measurements, DPV and cyclic voltammetry (CV) methods was utilized at BDDE.*
Result and Discussion: *Based on experimental findings from electrochemical characterization investigations, it was determined that oxidation behavior of Palbociclib in BDDE is irreversible and regulated by diffusion. Anodic peak current exhibited a linear relationship within concentration range of 0.01–1 µM, 0.02–0.8 µM, and 0.02–0.8 µM in pH 2.0 phosphate buffer solution (PBS) for reference substance solution, human serum, and urine samples, respectively. Limits of detection were found as 2.28 nM, 2.93 nM, and 1.31 nM for standard drug solution, human serum and urine samples, respectively. In order to validate the developed method, its repeatability, reproducibility, selectivity, precision and accuracy in all environments were investigated and calculated. This method was successfully applied for the analysis of Palbociclib in human serum and urine samples.*
Keywords: *Boron-doped diamond electrode, differential pulse voltammetry, human serum sample, palbociclib, urine sample*

* **Corresponding Author / Sorumlu Yazar:** Çiğdem Kanbeş-Dindar
e-mail / e-posta: ckanbes@gmail.com, **Phone / Tel.:** +905050491355

Corresponding Author / Sorumlu Yazar: Nazife Aslan
e-mail / e-posta: nazife.aslan@hbv.edu.tr, **Phone / Tel.:** +903125461356

Corresponding Author / Sorumlu Yazar: Bengi Uslu
e-mail / e-posta: buslu@pharmacy.ankara.edu.tr, **Phone / Tel.:** +903122033178

Submitted / Gönderilme : 24.07.2024
Accepted / Kabul : 03.08.2024
Published / Yayınlanma : 10.09.2024

ÖZ

Amaç: *Bu çalışmada Palbociclib'i doğru bir şekilde ölçmek için bor katkılı elmas elektrotta (BDDE) diferansiyel puls voltametrisi (DPV) kullanan çok verimli ve hassas bir elektrokimyasal teknik geliştirilmiştir.*

Gereç ve Yöntem: *Tüm deneylerde bor katkılı elmas elektrot, platin tel karşı elektrot ve Ag/AgCl referans elektrot ile birlikte 10 ml kapasiteli tipik üç elektrotlu hücre kullanılmıştır. Elektrokimyasal ölçümler sırasında BDDE'de DPV ve döngüsel voltametri (CV) yöntemleri kullanılmıştır.*

Sonuç ve Tartışma: *Elektrokimyasal karakterizasyon incelemelerinden elde edilen deneysel bulgulara dayanarak, Palbociclib'in BDDE'deki oksidasyon davranışının geri dönüşümsüz olduğu ve difüzyonla düzenlendiği belirlenmiştir. Anodik pik akımı, referans madde çözeltisi, insan serumu ve idrar numuneleri için DPV yöntemiyle pH 2.0 fosfat tampon çözeltisinde (PBS) sırasıyla 0.01-1 µM, 0.02-0.8µM ve 0.02-0.8 µM konsantrasyon aralığında doğrusal bir ilişki görülmüştür Tespit limitleri standart ilaç çözeltisi, insan serumu ve idrar numuneleri için sırasıyla 2.28 nM, 2.93 nM ve 1.31 nM olarak bulunmuştur. Geliştirilen yöntemin validasyonu için tekrarlanabilirliği, tekrar üretilebilirliği, seçiciliği, kesinliği ve doğruluğu araştırılmış ve hesaplanmıştır. Bu yöntem insan serum ve idrar örneklerinde Palbociclib analizi için başarıyla uygulanmıştır.*

Anahtar Kelimeler: *Bor katkılı elmas elektrot, diferansiyel puls voltammetri, idrar örneği, insan serum örneği, palbociclib*

INTRODUCTION

Breast cancer (BC) is a major global health problem for women, with more than 2.1 million new cases diagnosed each year [1]. It affects individuals of all age groups, races and cultural backgrounds [1]. Unfortunately, there is currently no commercially available drug or treatment that can completely eradicate BC [2]. Rapid and accurate diagnosis of BC can significantly improve patients' chances of survival. Precise identification of the disease and subsequent adjustment of the drug dosage is crucial in this context [3].

Palbociclib is a pharmaceutical compound designed by Pfizer specifically to treat breast cancer that is positive for hormone receptors (HR-positive) and negative for human epidermal growth factor receptor 2 (HER2-negative) [4]. Structure of palbociclib contains cyclin-dependent kinases, enabling it to selectively inhibit CDK4 and CDK6 (Figure 1) [5]. Furthermore, palbociclib was the initial cyclin-dependent kinase 4/6 (CDK4/6) inhibitor to get approval for its application in cancer therapy [6]. Administering palbociclib to postmenopausal women alongside letrozole as their first endocrine therapy led to a notable rise in the rates of progression-free survival and clinical benefit [6,7]. Moreover, the administration of palbociclib to women alongside fulvestrant led to a significant increase in the duration of progression-free survival and overall survival rates. These data have been gathered from randomized clinical research [7].

There are only a few analytical methods used for determining pablociclib such as LC-MS/MS [8,9], SPE-LC-MS[10], UV-VIS spectroscopy [11], electropheroses [12], MIP-electrochemical [13], and spectrofluorimetry [14], from pharmaceutical preparations. The sample and electrode preparation procedure of the proposed new method is simpler, practical and inexpensive. Although the previously described method has been used for the analysis of pharmacological forms, this study demonstrated that it can also be realized with human serum samples [15]. Compared to conventional carbon electrodes and other metallic electrodes, BDDE differs in its chemical stability and its ability to be used in extreme chemical environments such as strongly acidic environments [16,17]. Therefore, this study aims to propose the development of electrochemical method for the quantification of pablociclib in human serum samples using differential pulse voltammetric method on BDDE. The electrochemical behavior of the drug was also studied in terms of buffer solution and pH in BBDE using DPV and CV techniques. Also, the variation of electrochemical behavior depending on the scan rate was studied. The proposed method can be an environmentally friendly alternative to chromatographic techniques in therapeutic drug monitoring by eliminating time-consuming steps such as evaporation, adsorption, extraction and separation prior to drug analysis.

MATERIAL AND METHOD

Instruments

The parameters of step potential: 10 mV; modulation amplitude: 50 mV; modulation time: 50 ms; interval time: 500 ms were chosen for the measurement of Palbociclib with DPV. A three-electrode system was employed for all electrochemical measurements. This system consisted of an Ag/AgCl (BAS, 3 M NaCl) reference electrode, a BDDE (Windsor Scientific Ltd., 3 mm diameter) working electrode, and an auxiliary electrode made of platinum wire. The measurements were conducted using an AUTOLAB 204 PGSTAT electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands). Prior to every measurement, the electrode surface was prepared using alumina powder and a polishing cloth.

Reagents

Phosphate buffer solutions were prepared using K_2HPO_4 and KH_2PO_4 (PBS, 0.1 M, pH 2.0, 3.0, 5.0, 6.0, 7.0, 8.0). Sigma-Aldrich supplied synthetic human serum and sigmatrix urine diluent. Palbociclib was generously provided by Neutec Drug A.S., Istanbul. A stock solution of $1x10^{-3}$ M was prepared in double-distilled water. H_2SO_4 (0.1 M and 0.5 M), Britton-Robinson (BR) buffer solutions, phosphate buffer solutions, and acetate buffer solutions were prepared in distilled water.

Analysis of Human Serum Samples

The samples were stored frozen at -20°C until analysis [18]. A standard serum solution was prepared by adding 1.0 ml of a 1.0×10^{-3} M Palbociclib stock solution and 3.6 ml of the serum sample to 5.4 ml of acetonitrile to precipitate the proteins [19]. In order to eliminate any residual protein, the tubes were subjected to sonication for 15 minutes, followed by centrifugation at 5000 rpm for 20 minutes [20].The supernatant obtained was then used to perform the electrochemical experiments, where it was prepared in a selected buffer and added to the electrochemical cell.

Analysis of Spiked Urine Samples

An aliquot volume of urine sample was fortified with Palbociclib dissolved in bi-distilled water to achieve final concentration of 1×10^{-3} M and treated with 0.7 ml of acetonitrile as endogenous substance precipitating agent, and then the volume was completed to 2.0 ml with the same urine sample [21]. The tubes were vortexed for 10.0 min, and then centrifuged for 5 min at 5000 × g for getting rid of residues. The supernatant was taken carefully. These solutions were analyzed in the voltammetric cell containing PBS buffer at pH 2.0. The amount of Palbociclib in spiked human urine samples for the recovery studies was calculated from the related calibration equation [21].

RESULT AND DISCUSSION

Electrochemical Behavior of Palbociclib on the BDDE

To investigate the voltammetric behavior of Palbociclib using BDDE, repeated CV measurements were taken in different pH mediums. Figure 1 indicates repetitive cyclic voltammograms of 20 µM Palbociclib in 0.1 M H_2SO_4 solution (A), 0.5 M H_2SO_4 solution (B), pH 2.0 PBS (C), and pH 7.0 BRT (D) at a scan rate of 100 mV/s. Palbociclib showed one well-defined oxidation peak and a weak wave in pH 7.0 BRT solution while a well-defined oxidation peak and a wave in pH 2.0 PBS buffer (optimum pH value) and one well-defined oxidation peak in 0.5 M H_2SO_4 and 0.1 M H_2SO_4 buffer solutions. As can be seen from Figure 1, the intensity of the peaks was higher in the first scan compared to the second, third, fourth, and fifth scans for all different pH environments. The decrease in peak intensity can be explained by contamination on the electrode surface [22,23]. Also, for pH 7.0 BRT medium, in the cathodic direction, no peak was observed, which showed the irreversible electrode reaction (Figure 1D). Moreover, for acidic medium (0.1 M H_2SO_4, 0.5 M H_2SO_4, and pH 2.0 PBS), after the first CV scan, the oxidation product was formed, and the anodic peak of this product was obtained at 0.1 M H_2SO_4, 0.5 M H_2SO_4, and pH 2.0 PBS (Figure 1 A, B, and C).

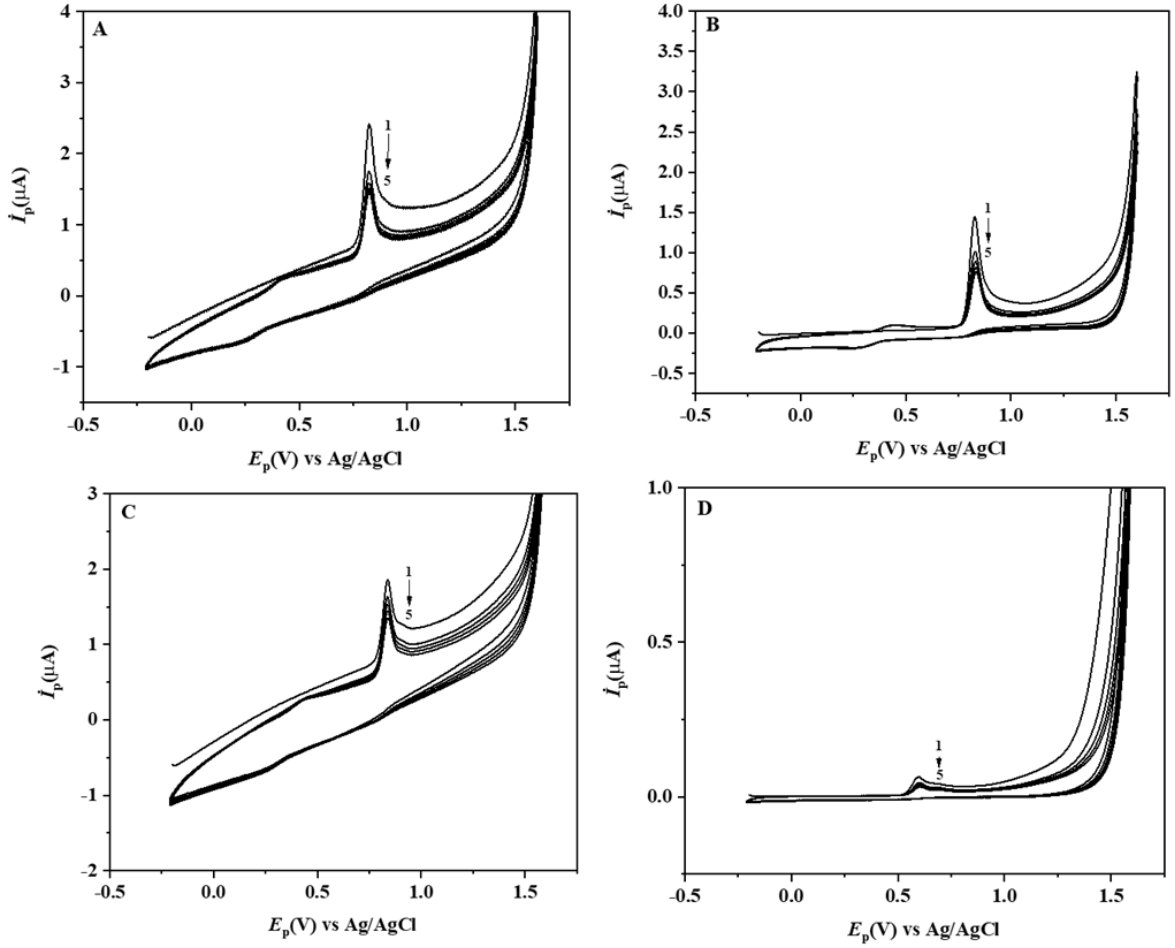

Figure 1. Repetitive cyclic voltammograms of 20 μM Palbociclib solutions in (a) 0.1 M H_2SO_4; (b) 0.5 M H_2SO_4 (c) pH 2.0 PBS (d) pH 7.0 BRT, as obtained at a scan rate of 100 mVs^{-1}

Due to the superior peak shape, maximum current, and most consistent measurement findings, the experiments including scan rate investigations and drug analysis quantification, were conducted using a pH 2.0 PBS solution. In order to determine whether the surface contact mechanism is influenced by diffusion, adsorption, or both, scan rate tests were conducted using CV. A solution of 20 μM palbociclib in pH 2.0 PBS was used, and the scan rate range was set between 0.005 and 1 Vs^{-1} (Figure 2).

When considering the change in scan rate values from 0.005 mV/s to 1 V/s, there is a shift of 63 mV towards positive potential values in relation to the irreversibility of the electrode reaction. For the proposed method, a linear response was found with the square root of the scan rate as follows:

$$I_p\,(\mu A) = 0.17\ v^{1/2}\,(mVs^{-1}) + 0.3009\ (r = 0.996) \quad (1)$$

The data analysis showed that when plotting the logarithm of the peak current against the logarithm of the scan rate, a straight line with a slope of 0.59 was obtained. The calculated slope closely approximates the theoretical value of 0.5, which is expected for a perfect reaction of solution species occurring as a diffusion-controlled process. The equation derived from the experimental data is as follows:

$$\log I_p\,(\mu A) = 0.59\ \log v\ (mV\ s^{-1}) - 1.05\ (r = 0.995) \quad (2)$$

Figures 3A and 3B show the potential values of the anodic oxidation peak and the graph of pH values versus observed current values, respectively, across the pH range of 2.0-9.0. The values obtained in pH 2.0 PBS, pH 2.0 BRT solutions were very close to each other. The peak obtained in pH 2.0 PBS was slightly higher but had more reproducible values. Thus, pH 2.0 PBS was utilized as working pH in further studies. Furthermore, the plot of the peak potential (E_p) vs. pH has one linear line (Figure 3B). The observed linear relationship between the values of Ep and pH ranging from 2.0 to 9.0 indicates that the electrochemical process involved an equal amount of protons and electrons, as indicated by the slope of the line.

$$E_p \text{ (mV)} = -47.2 \text{ pH} + 893;\ r = 0.9965 \text{ (pH 2.00 – 9.00 with DPV)} \qquad (3)$$

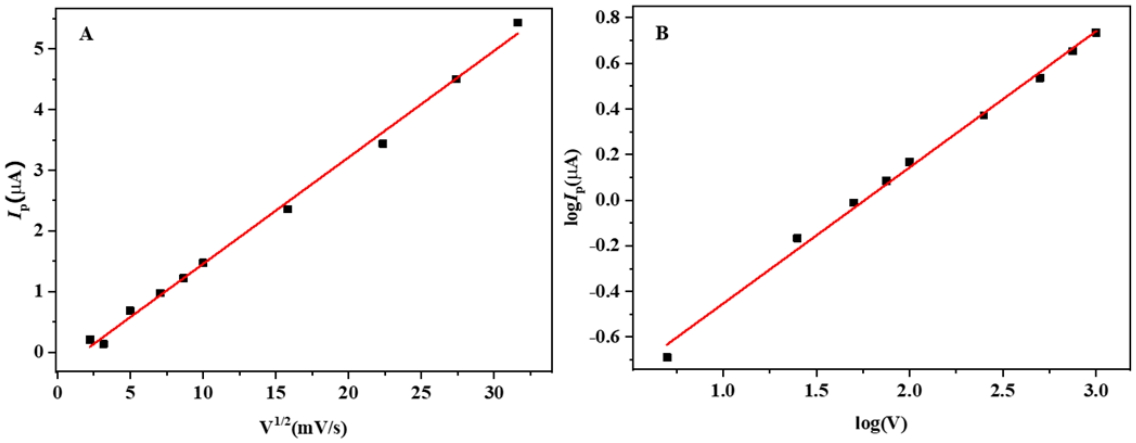

Figure 2. I_p vs $V^{1/2}$ (A) and log I_p vs log V (B) graphs of 1.0×10^{-5}M pablociclib in pH 2.0 PBS obtained in the range of 5-1000 mV/s

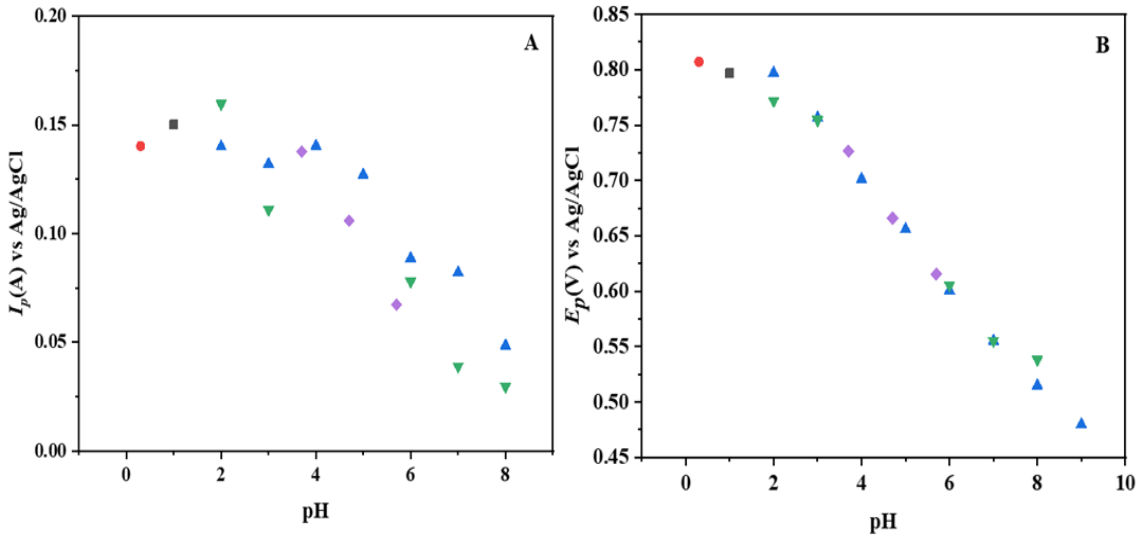

Figure 3. Effects of the pH on the pablocicilib peak potential (B) and peak current (A) in different supporting electrolytes, H_2SO_4 solutions (0.5M); (●)H_2SO_4 solutions (0.1 M); (■) Britton-Robinson buffer ; (▲) phospahate buffer; (▼) acetate buffer (♦) . These experiments were performed using DPV with the pablociclib concentration of 1×10^{-5}M

Electroanalytical Applications

An investigation was conducted to assess the analytical potential of pablociclib by examining the

correlation between pablociclib peak current and concentration. The experiments were carried out in pH 2.0 PBS, where the best peak symmetry and the highest peak current were obtained. The linearity was achieved in the concentration range of 10–1000 nM (Figure 4). The following was the related equation between peak current and concentration:

$$i_p\,(\mu A) = 0.311\ C\ (\mu M) - 0.0193 \text{ (n: 11, } r = 0.9907) \qquad (4)$$

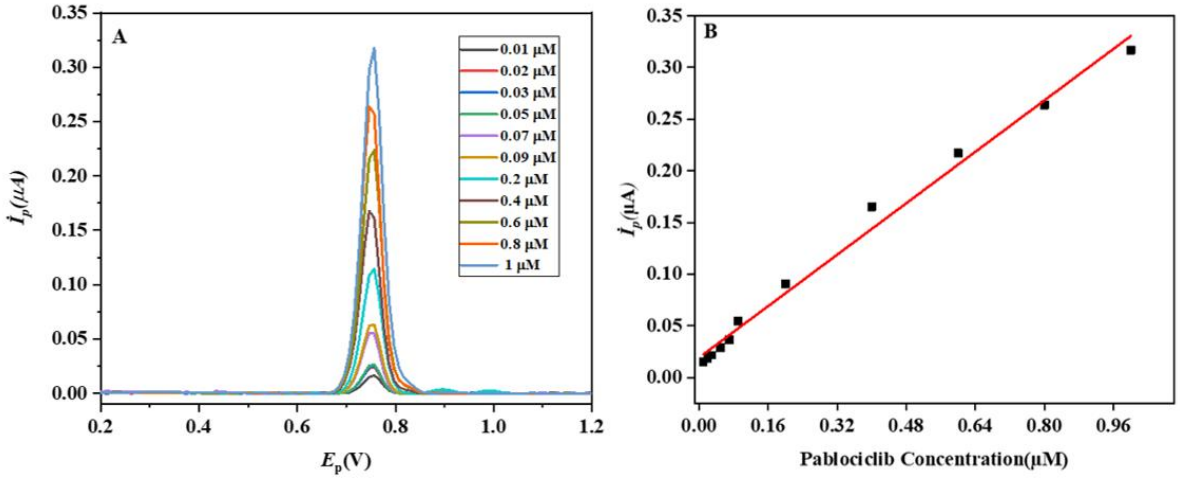

Figure 4. Calibration curve of DP voltammograms for standard solution of pablociclib in different concentration ranges from 0.01 µM to1 µM (A) and the calibration curve of standard pablocicilib (B) in pH 2 PBS

Determination of Pablocicilib in Spiked Biological Samples

The calibration equation was found for spiked biological samples to show that the proposed methods could be used on human serum samples (Figure 5) and urine (Figure 6).

$$I_p\,(\mu A) = 0.2408C\ (\mu M) - 0.0011 \text{ (n:9, } r = 0.9931) \qquad (5)$$

$$I_p\,(\mu A) = 0.3046C\ (\mu M) - 0.001 \text{ (n:9, } r = 0.9922) \qquad (6)$$

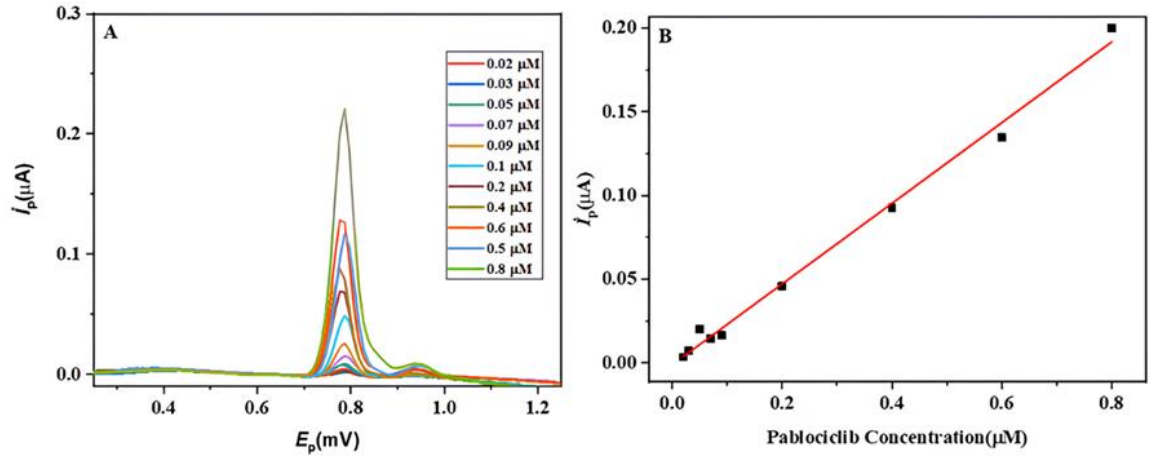

Figure 5. DP voltammograms of serum samples in the concentration range from 0.02 µM to 0.8 µM (A) and the calibration curve of serum samples (B) in pH 2 PBS

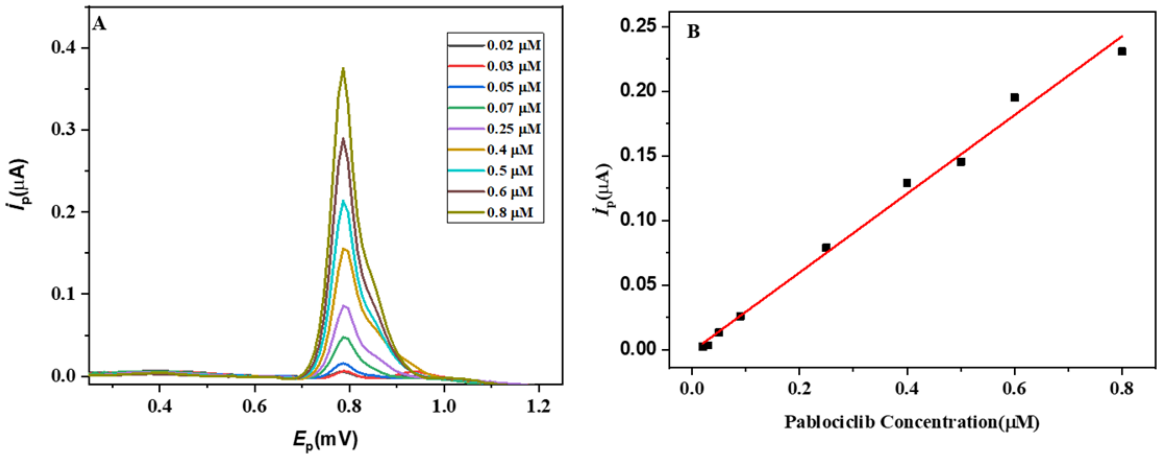

Figure 6. DP voltammograms of urine samples in different concentration ranges from 0.02 µM to 0.8 µM (A) and calibration curve of urine samples (B) in pH 2.0 PBS

Statistical data of the calibration are given in Table 1. Repeated measurements of pablocicilib peak potential and peak current within and between days demonstrate the precision of the developed method. The formulas 3 s/m and 10 s/m were used to determine the LOD and LOQ values, where "s" is the standard deviation of the response and "m" is the slope of the calibration curve. The LOD and LOQ values showed the sensitivity of the method (Table 1).

Table 1. Correlation data for pablociclib calibration generated using DPV in BDDE from standard solution, serum and urine samples

Parameters	**Standard**	**Serum**	**Urine**
Anodic potential (mV)	756	786	786
Linearity dynamic range (µM)	0.01-1	0.02-0.8	0.02-0.8
Slope (µA μM^{-1})	0.311	0.2408	0.3046
Intercept (µA)	0.02	-0.001	-0.001
Correlation coefficient (*r*)	0.9907	0.9931	0.9922
LOD (nM)	2.28	2.93	1.31
LOQ (nM)	7.60	9.77	4.89
Intra-day precision of peak current (RSD%)*	1.54	2.01	4.32
Inter-day precision of peak current (RSD%)*	1.87	1.48	1.14

*LOD and LOQ values were calculated based on the lowest value of the calibration range. For Serum and Urine, the intra-day precision of the peak current and the inter-day precision of the peak current values were calculated based on the midpoint of the calibration. Each value is the average of five experiments

The performance of the developed method was also compared with previous analytical methods (Table 2) and it was found that the developed method was the superior method in terms of sensitivity, while most of the other methods involved time-consuming preconcentration, high consumption of harmful and organic solvents and expensive equipment.

The proposed methods are simple to use and are based on human serum and urine obtained reproducible results that were sensitive enough to detect pablociclib in samples (Table 3).

Table 2. Comparison with other previously reported analytical methods to study the analytical performance of pablociclib determination

Analytical Method	Linearity range	LOD	Sample	Ref.
SPE-LC-MS	2-400 ng/ml	-	drug	[10]
Spectroflourimetry	1.0-20.0 µg/ml	0.021µg/ml	tablets	[14]
UV-Vis	2-10 mg/ml	0.0782	drug	[11]
LC-MS/MS	72.8-185.5 ng/ml	120 ng/ml	drug	[20]
LC-MS/MS	1-250 ng/ml	-	drug	[9]
LC-MS/MS	3.1-500 ng/ml	-	drug	[8]
Electrophoresis	10.0-100 µg/ml	0.11 µg/ml	tablet	[12]
MIP-voltammetry	2.5×10^{-11}-2.5×10^{-10}M	3.33×10-12M	Human serum	[13]
DPV	0.01–1 µM, 0.02–0.8 µM, 0.02–0.8 µM	2.28 nM 2.93 nM 1.31 nM	Standard serum urine	This Study

Table 3. Results of DPV recovery studies for BDDE from serum and urine samples

Parameters	Serum	Urine
Added concentration (µM)	0.2	0.2
Found concentration (µM)	0.195	0.1963
Average recorved %	98.75	99.75
Number of experiments	5	5
RSD % of recovery	2.012	4.32
Bias %	2.5	1.85

Conclusion

The voltammetric behavior of Palbociclib was examined by using a DPV approach to determine it from serum with a BDDE. The work stands out from the rest since no previous research has used voltammetry to measure Palbociclib with BDDE. Moreover, proposed electrochemical method was applied to human serum and urine samples.

AUTHOR CONTRIBUTIONS

Concept: C.K.D.; Design: C.K.D.; Control: N.A., B.U.; Sources: B.U.; Materials: C.K.D., B.U.; Data Collection and/or Processing: M.A., C.K.D.; Analysis and/or Interpretation: M.A., C.K.D.; Literature Review: M.A., C.K.D., N.A., B.U.; Manuscript Writing: M.A., C.K.D., N.A., B.U.; Critical Review: M.A., C.K.D., N.A., B.U.; Other: -

CONFLICT OF INTEREST

The authors declare that this article has no real, potential, or perceived conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1068-1077, 2024
Doi: 10.33483/jfpau.1484485

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ESSENTIAL OIL CONTENTS AND BIOLOGICAL ACTIVITIES OF *THYMUS CANOVIRIDIS* JALAS AND *THYMUS SIPYLEUS* BOISS.

THYMUS CANOVIRIDIS JALAS VE THYMUS SIPYLEUS BOISS. TÜRLERİNİN UÇUCU YAĞ İÇERİKLERİ VE BİYOLOJİK AKTİVİTELERİ

Serkan YİĞİTKAN[1]*, Mehmet FIRAT[2]

[1]Dicle University, Faculty of Pharmacy, Department of Pharmacognosy, 21280 Diyarbakir, Türkiye
[2]Van Yuzuncu Yil University, Faculty of Education, Department of Biology, 65080 Van, Türkiye

ABSTRACT

Objective: *Members of the Lamiaceae family are considered to be major sources of bioactive therapeutic agents. Many of them are important medicinal and aromatic plants used in traditional and modern medicine and in the food, cosmetic and pharmaceutical industries. The aim of this study was to investigate in detail the biological activities and chemical composition of the essential oils of Thymus canoviridis Jalas and Thymus sipyleus Boiss. species belonging to the genus Thymus, one of the most important genera of the Lamiaceae family.*
Material and Method: *The essential oil content of the species was determined by GC-MS. Antioxidant activities of the essential oils were determined using lipid peroxidation, DPPH free radical, ABTS cation radical and CUPRAC methods. In addition, cytotoxic activities against breast cancer (MCF-7) and colon cancer (HT-29) cell lines and anticholinesterase (against AChE and BChE enzymes), urease, tyrosinase, elastase, collagenase and angiotensin converting enzyme inhibition activities were determined.*
Result and Discussion: *When the essential oil composition of T. sipyleus was analyzed, the major compounds were 1,8-cineole (eucalyptol) (18.16%), camphor (15.08%) and endo-borneol (11.63%), while T. canoviridis was found to be rich in carvacrol (72.88%). T. canoviridis showed high antioxidant activity in lipid peroxidation (IC_{50}: 45.72±0.12 µg/ml), ABTS (IC_{50}: 6.12±0.03 µg/ml) and CUPRAC (IC_{50}: 5.31±0.01 µg/ml) methods. The selectivities of T. canoviridis and T. sipyleus species against MCF-7 cell line were 4.39 and 6.81, respectively. In the enzyme inhibition studies, both Thymus species showed moderate inhibition activity against BChE enzyme (Inhibition%: 57.88±1.14, 39.21±0.89, respectively). In addition, T. sipyleus showed moderate inhibition of elastase enzyme (Inhibition%: 25.33±0.79). When the results are evaluated in general, it can be said that T. canoviridis essential oil with its rich carvacrol content and high cytotoxic and antioxidant activity can be preferred as a safer and natural option instead of synthetic preservatives in food, pharmaceutical and cosmetic industries to extend shelf life and ensure food safety.*
Keywords: *Antioxidant activites, carvacrol, cytotoxic, GC-MS, T. canoviridis, T. sipyleus*

ÖZ

Amaç: *Lamiaceae ailesinin üyeleri, biyoaktif terapötik ajanların başlıca kaynakları olarak kabul edilir. Birçoğu geleneksel ve modern tıpta ve gıda, kozmetik ve ilaç endüstrilerinde kullanılan önemli tıbbi ve aromatik bitkilerdir. Bu çalışmada Lamiaceae familyasına mensup en önemli cinslerden biri olan Thymus cinsine ait Thymus canoviridis Jalas ve Thymus sipyleus Boiss.*

* **Corresponding Author / Sorumlu Yazar:** Serkan Yiğitkan
e-mail / e-posta: serkan.yigitkan@dicle.edu.tr, **Tel. / Phone:** +904122411001/7538

Submitted / Gönderilme : 15.05.2024
Accepted / Kabul : 07.08.2024
Published / Yayınlanma : 10.09.2024

türlerinin uçucu yağlarının biyolojik aktiviteleri ve kimyasal bileşiminin detaylı bir şekilde incelenmesi amaçlanmıştır.

Gereç ve Yöntem: *Türlerin uçucu yağ içeriği GC-MS cihazı ile belirlenmiştir. Ayrıca uçucu yağların lipit peroksidasyon, DPPH serbest radikal, ABTS katyon radikali ve CUPRAC metotları kullanılarak antioksidan aktiviteleri, meme kanseri (MCF-7) ve kolon kanseri (HT-29) hücre dizilerine karşı sitotoksik aktiviteleri ve antikolinesteraz (AChE ve BChE enzimlerine karşı), üreaz, tirozinaz, elastaz, kolajenaz ve anjiyotensin dönüştürücü enzim inhibisyon aktiviteleri belirlenmiştir.*

Sonuç ve Tartışma: *T. sipyleus uçucu yağ bileşimi incelendiğinde majör bileşikler 1,8-sineol ökaliptol (%18.16), camphor (%15.08) ve endo-borneol (%11.63) olarak tespit edilmiş, T. canoviridis ise karvakrol (%72.88) bakımından oldukça zengin bulunmuştur. T. canoviridis lipid peroksidasyon (IC_{50}: 45.72±0.12 µg/ml), ABTS (IC_{50}: 6.12±0.03 µg/ml) ve CUPRAC (IC_{50}: 5.31±0.01 µg/ml) yönteminde yüksek antioksidan aktivite göstermiştir. T. canoviridis ve T. sipyleus türlerinin MCF-7 hücre serisine karşı sağlıklı hücre serine göre selektivitelerinin sırasıyla 4.39 ve 6.81 olduğu belirlenmiştir. Yapılan enzim inhibisyon çalışmalarında Thymus türlerinin her ikisi de BChE enzimine karşı orta inhibisyon aktivitesi (% İnhibisyon: 57.88±1.14, 39.21±0.89, sırasıyla) göstermiştir. Ayrıca T. sipyleus'un, orta düzeyde elastaz enzim inhibisyonu (% İnhibisyon: 25.33±0.79) gösterdiği saptanmıştır. Sonuçlar genel olarak değerlendirildiğinde T. canoviridis uçucu yağının zengin karvakrol içeriği, yüksek sitotoksik ve antioksidan aktivitesi ile gıda, ilaç ve kozmetik endüstrisinde raf ömrünü uzatmak ve gıda güvenliğini sağlamak için sentetik koruyucular yerine daha güvenli ve doğal bir seçenek olarak tercih edilebileceği söylenebilir.*

Anahtar Kelimeler: *Antioksidan aktivite, GC-MS, karvakrol, sitotoksik, T. canoviridis, T. sipyleus*

INTRODUCTION

Among the 236 genera in the Lamiaceae family, the genus *Thymus* is among the eight most significant ones in terms of the total number of species it contains. Thyme is traditionally used both to identify the genus and to name *Thymus vulgaris* L., the most commercially used species [1]. The Lamiaceae family is represented by 45 genera, 546 species and 730 taxa in Türkiye, with an endemism rate of 44.2% [2]. Although their distribution in the world is mostly typical for the Mediterranean region, they are distributed especially in the Iberian Peninsula, Europe, Asia, North and West Africa, and North America. It has been observed that they are distributed in many places due to their ability to live in different habitats and grow at high altitudes [3]. The genus *Thymus* includes 40 species and 42 taxa in Türkiye, 16 of which are endemic [4].

Because of their attractive flavor, nutritional value, and medicinal properties, *Thymus* spp. are widely utilized in the food and pharmaceutical industries as spices, sauces, perfumes, and cosmetics [5]. In traditional medicine, infusion and decoction of the leaves and flowering parts have been used in the prevention and treatment of numerous complaints such as diabetes, colds, coughs, flu, indigestion, nausea and dysentery [6].

Numerous studies have been conducted on *Thymus* spp. to determine their chemical composition. The genus *Thymus* was found to be rich in essential oils characterized by considerable variability in chemical composition. The oil chemotypes include monoterpenes, sesquiterpenes and their oxygenated and hydrocarbon derivatives [7]. The main compounds in most *Thymus* essential oils are thymol and carvacrol, which are phenolic monoterpenes. This causes *Thymus* spp. to typically have a strong, spicy flavor. Other monoterpenes commonly detected in essential oils are linalool and, in lower percentages, borneol, camphor, limonene, *ß*-pinene, *α*-terpineol and terpinen-4-ol [1]. *Thymus* spp. are also rich in phenolic compounds, flavonoids, phenolic acids, terpenic compounds (di and triterpenes) responsible for their therapeutic effects such as antioxidant, antibacterial, cytotoxic, anticancer etc. [8]. A recent study revealed that *Thymus* spp. rich in thymol and carvacrol can be used in food preservation due to their antibacterial activity against many bacterial species [9].

Literature studies on *Thymus canoviridis* Jalas (TC-E) and *Thymus sipyleus* Boiss. (TS-E) belonging to the genus *Thymus*, which are widely used in the public and industry, were reviewed. In a study investigating the antioxidant and antimicrobial activities of essential oils of *Thymus* spp., thymol was found as the major compound in TC-E essential oil and it was determined that it showed good antimicrobial activity against *Proteus vulgaris* and *Staphylococcus aureus* bacteria. It has also been reported that TC-E and other *Thymus* spp. can be used as a natural preservative component in food and

medical industries [6]. Due to the popular use of *Thymus* spp., TC-E was examined for its effect on rhinosinusitis pathogens. The major component was found to be thymol, and the volatile components of the oil showed antibacterial and anti-inflammatory potential supporting the traditional use [10]. In this study, similar to previous studies, the chemical composition of the essential oils of *T. canoviridis* and *T. sipyleus* was evaluated by GC-MS. The present study, in contrast to others in the literature, aimed to comprehensively investigate the angiotensin converting enzyme activities, anticholinesterase, urease, tyrosinase, elastase, cholinase, and antioxidant activities of the species' essential oils.

MATERIAL AND METHOD

Plant Material

Thymus canoviridis Jalas (TC-E) was collected from Ezurum (19. km from Hınıs to Pasinler, steppe, 1921m, 39°32′35″ N, 41°43′43″ E) in August and *Thymus sipyleus* Boiss. (TS-E) was collected and identified by Dr. Mehmet Fırat from Erzincan (40. km from Kemaliye to Çemişkesek, dry steppe, 1500m, 39°11′34″ N, 38°38′14″ E) in July. Herbarium specimens were dried for storage in Van Yüzüncü Yıl University Faculty of Science herbarium. Herbarium numbers are M. Fırat 32626 and 32474 (VANF), respectively. The current name of the plant was written according to International Plant Names Index and The Plant List.

GC-FID and GC-MS Conditions for Essential Oils

The essential oils obtained by hydrodistillation method using Clevenger apparatus were determined by Agilent brand 7890A Model GC-FID gas chromatograph flame ionization detector combined with Agilent 5977B model mass spectrometry (MS) device in Dicle University Faculty of Pharmacy [11].

Antioxidant Activity

β-Carotene lipid peroxidation, ABTS cation radical, DPPH free radical and CUPRAC (Copper (II) ion reducing antioxidant capacity) methods were used to determine the antioxidant properties of the samples [12-14]. In these four antioxidant test methods, α-tocopherol and BHT (Butylated hydroxytoluene) compounds were used as standard reference. IC_{50} calculations were performed using samples with concentrations of 100, 50, 25, 10 and 5 µg/ml [36].

Toxic-Cytotoxic Activity

In order to determine toxic and cytotoxic effects of the essential oils, the method developed by Mojarraba et al. (2013) was used with minor modifications [15,16].

Anti-cholinesterase Inhibitory Activity

The spectrophotometric method based on AChE and BChE inhibition developed by Ellman et al. (1961) was used to determine anticholinesterase enzyme activity. In this test method, galantamine was used as a standard reference substance [17].

Anti-urease Inhibitory Activity

The method developed by Hina et al. (2015) was used to determine the urease inhibition activity of the samples. Thiourea was used as a standard reference for the anti-urease activity test method [18].

Anti-Aging Inhibitory Activity

Tyrosinase, elastase and collagenase inhibitory activity methods were used to determine the anti-aging potential of the samples. For tyrosinase inhibitory activity, the method developed by Hearing and Jimenez (1987) was applied with minor modifications. Elastase inhibition activity was performed using the method developed by Kraunsoe et al. (1996) with minor modifications. Collagenase activity was performed using the protocol developed by Thring et al. (2009) with minor modifications [19-21].

$$\text{Enzim inhibition (\%)} = 100\text{-(OD test well /OD control)} \times 100$$

Antihypertensive Activity (Angiotensin I-Converting Enzyme, ACE, Inhibition)

The method developed by Kwon et al. (2006) was used with minor modifications. Lisinopril was used as a standard. ACE inhibition was calculated using peak areas with the following equation [22].

Inhibition% = [Areacontrol – (Areasample – Areasample blank)]/(Areacontrol – Areablank) x 100

Statistical Analysis

The results of the activity assays were shown as means ± standard error meaning. The results were evaluated using an unpaired and one-way analysis of variance ANOVA. The differences were regarded as statistically significant at $p < 0.05$.

RESULT AND DISCUSSION

Essential Oil Contents

The essential oil content of the species was determined by GC-FID and GC-MS (Table 1 and Figure 1). When the results were evaluated, 97.31% and 32 compounds of TS-E and 96.78% and 30 compounds of TC-E were determined. Oxygenated monoterpenes (69.14%) and oxygenated sesquiterpenes (14.13%) constituted the major part of TS-E composition. When the composition of TS-E was analyzed, the major compounds were oxygenated monoterpenes 1,8-cineole (eucalyptol) (18.16%), camphor (15.08%) and *endo*-borneol (11.63%). The composition of TC-E was also found to contain mostly oxygenated monoterpenes (80.1%) and was rich in carvacrol (72.88%), an oxygenated monoterpene. In a study conducted with TS-E, the main compounds were carvacrol (18.2%) and 1,8-cineole (11.6%), in addition to significant amounts of *p*-cymene (9.2%), camphor (8.3%), camphene (7.2%), 3-octanol (5.8%), *β*-caryophyllene (5.0%), borneol (4.9%) [23]. In another study, the main components of the species were identified as borneol (11.2%), *α*-muurolol (9.2%), *β*-caryophyllene (7.6%), geranial (7.3%) and neral (5.4%) [24]. In a study conducted with TC-E, it was found that the essential oil of the species contained mainly thymol (60.44-64.79%) and *β*-caryophyllene (8.49-6.58%) [6]. Partially concurrently with this investigation, Baser et al. (1998) found that the principal constituents of the species' essential oil were carvacrol (29.51%), geraniol (13.25%), and thymol (9.9%). It can be generally stated that the chemical composition of the essential oils of *Thymus* spp. and subspecies of various origins varies greatly and is highly diverse because of several factors such as soil and climate, the vegetative cycle, seasonal change, etc [25-27].

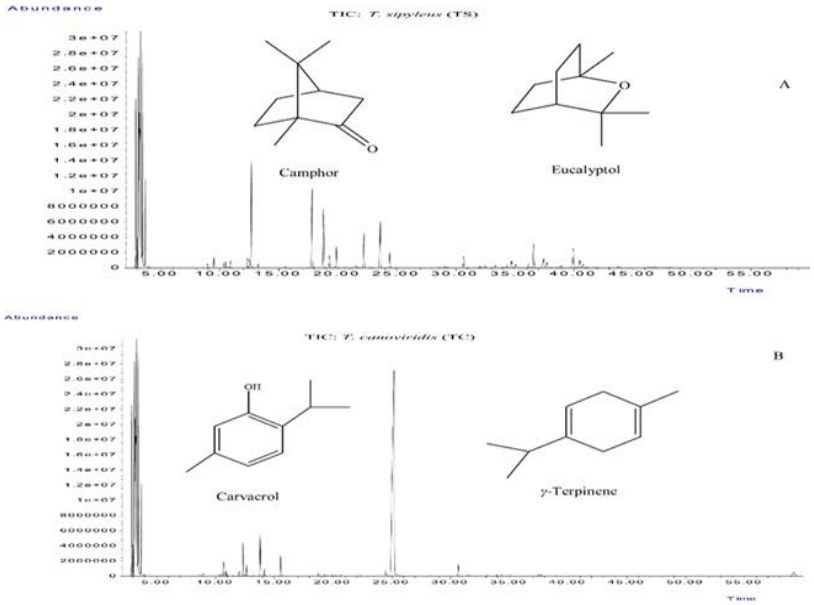

Figure 1. GC-MS chromatograms, **A**: GC-MS chromatogram of essential oil of *T. sipyleus*, **B**: GC-MS chromatogram of essential oil of *T. canoviridi*

Table 1. Chemical composition of the essential oil of *Thymus* species

No	RI[a]	Constituents[b]	TS-E	TC-E	Identification Methods
1	928	3-Thujene	tr	0.20	Co-GC, MS, RI
2	935	*α*-Pinene	0.56	0.30	Co-GC, MS, RI
3	951	Camphene	1.41	tr	Co-GC, MS, RI
4	976	Sabinene	0.72	tr	Co-GC, MS, RI
5	980	*β*-Pinene	0.81	tr	Co-GC, MS, RI
6	985	3-Octanone	tr	1.51	Co-GC, MS, RI
7	991	*β*-Myrcene	0.80	0.60	Co-GC, MS, RI
8	995	3-Octanol	tr	0.11	Co-GC, MS, RI
9	1019	*α*-Terpinene	tr	0.55	Co-GC, MS, RI
10	1027	*o*-Cymene	1.40	4.06	Co-GC, MS, RI
11	1031	Limonene	1.23	0.40	Co-GC, MS, RI
12	1034	1,8-Cineole (Eucalyptol)	18.16	1.41	Co-GC, MS, RI
13	1047	*cis*-*β*-Ocimene	0.60	0.13	Co-GC, MS, RI
14	1060	*γ*-Terpinene	tr	5.00	Co-GC, MS, RI
15	1069	Sabinene hydrate	tr	0.90	Co-GC, MS, RI
16	1101	Linalool	0.30	2.77	Co-GC, MS, RI
17	1149	Camphor	15.08	tr	Co-GC, MS, RI
18	1170	*endo*-Borneol	11.63	0.51	Co-GC, MS, RI
19	1181	4-Terpineol	2.06	0.18	Co-GC, MS, RI
20	1195	*β*-Fenchyl alcohol	4.00	0.19	Co-GC, MS, RI
21	1243	*cis*-Citral	6.00	tr	Co-GC, MS, RI
22	1246	Carvacrol methyl ether	tr	0.10	Co-GC, MS, RI
23	1255	*trans*-Geraniol	0.11	tr	Co-GC, MS, RI
24	1273	*trans*-Citral	8.56	tr	Co-GC, MS, RI
25	1290	Bornyl acetate	3.24	tr	Co-GC, MS, RI
26	1297	Thymol	tr	1.16	Co-GC, MS, RI
27	1303	Carvacrol	tr	72.88	Co-GC, MS, RI
28	1393	*β*-Bourbonene	0.36	0.07	Co-GC, MS, RI
29	1429	Caryophyllene	2.10	1.89	Co-GC, MS, RI
30	1448	Aromandendrene	tr	0.26	Co-GC, MS, RI
31	1463	*α*-Humulene	0.27	0.08	Co-GC, MS, RI
32	1470	Alloaromadendrene	0.46	tr	Co-GC, MS, RI
33	1484	*γ*-Muurolene	1.60	0.14	Co-GC, MS, RI
34	1490	Germacrene D	0.55	tr	Co-GC, MS, RI
35	1504	Varidiflorene	tr	0.30	Co-GC, MS, RI
36	1514	*β*-Bisabolene	0.61	0.22	Co-GC, MS, RI
37	1523	*γ*-Cadinene	tr	0.10	Co-GC, MS, RI
38	1531	*δ*-Cadinene	0.56	0.17	Co-GC, MS, RI
39	1567	Nerolidol	3.95	tr	Co-GC, MS, RI
40	1588	Spathulenol	2.04	0.29	Co-GC, MS, RI
41	1595	Caryophyllene oxide	1.03	0.30	Co-GC, MS, RI
42	1625	Epicubenol	0.50	tr	Co-GC, MS, RI
43	1650	*T*-Cadinol	5.00	tr	Co-GC, MS, RI
44	1664	*α*-Cadinol	1.61	tr	Co-GC, MS, RI
Hydrocarbons			-	1.62	
Monoterpenes hydrocarbons			7.53	11.24	
Oxygenated monoterpenes			69.14	80.1	
Sesquiterpenes hydrocarbons			6.51	3.23	
Oxygenated sesquiterpenes			14.13	0.59	
	Total identified (%)		97.31	96.78	

[a]Kovats index on HP–5MS fused silica column, [b]A nonpolar Agilent HP-5MS fused silica column, [c]Percentage concentration, [Co-GC]Co-injection with authentic compounds, [RI]Retention Index literature comparison, [tr] trace: %<0.05

Results of Biological Activities

Antioxidant and Toxic-Cytotoxic Activities

The antioxidant activity of the essential oils of the species was determined by lipid peroxidation, DPPH free radical, ABTS cation radical and CUPRAC methods (Table 2). In lipid peroxidation and DPPH methods, TC-E showed good antioxidant activity (IC_{50}: 45.72±0.12 and 60.60±1.68 μg/ml, respectively) and TS-E showed low antioxidant activity (IC_{50}: 650.12±2.41 and 1000 μg/ml, respectively). High antioxidant activity was observed for TC-E in ABTS and CUPRAC methods. In ABTS method, TC-E showed higher antioxidant activity (IC_{50}: 6.12±0.03 μg/ml) than BHT and α-TOC used as reference (IC_{50}: 13.67±0.33 μg/ml and IC_{50}: 10.43±0.44 μg/ml, respectively). In the CUPRAC method, it was determined that TC-E showed higher antioxidant activity (IC_{50}: 5.31±0.01 μg/ml) compared to the references (IC_{50}: 8.24±0.05 μg/ml and IC_{50}: 18.57±0.27 μg/ml, respectively), supporting the ABTS results. In a study conducted with 4 different *Thymus* spp., including *T. canoviridis* species, antioxidant activity was examined using only DPPH method and it was found to show good antioxidant activity. In addition, it was determined that thymol and carvacrol detected in the species were the active components of essential oils [6]. It is available in the literature that oxygenated monoterpenes, especially thymol and carvacrol, two well-known phenolic compounds, are mainly responsible for the antioxidant potential of essential oils containing them [28,29]. In this context, it can be said that the antioxidant capacity of *T. canoviridis* essential oil is related to carvacrol (72.88%), which is the major compound in this study. In another study in which the antioxidant activity of TS-E extract was compared with a different *Thymus* spp., the antioxidant activity of TS-E was found to be low similar to this study. The reason for the low activity was attributed to the low amounts of both monoterpene hydrocarbons and oxygenated monoterpenes [24].

The toxic effects of the samples were determined by MTT method on healthy cell line (PDF) and cytotoxic effects on cancerous MCF-7 (breast cancer) and HT-29 (colon cancer) (Table 2). In general, the essential oil of the two species showed low toxicity on healthy human cell line (PDF). TC-E and TS-E samples showed moderate cytotoxicity (43.12±0.99 and 65.92±1.32 % viability against HT-29 cells, 200 μg/ml) on colon cancer. The two species showed very high cytotoxic activity (16.84±0.06 and 10.72±0.03 % viability, 200 μg/ml, respectively) against MCF-7 cells in breast cancer.

There are no studies in the literature on the cytotoxic activity of essential oils of *T. sipyleus* and *T. canoviridis*. In a study comparing the cytotoxic activity of methanol extracts of *T. sipyleus* and *Thymus leucostomus* Hausskn. et Velen, the cytotoxic effects of the extracts of these species on cervical (HeLa) and ovarian cancer (Skov-3) cells were investigated using colorimetric assay. The results showed that the methanol extract of *T. sipyleus* was moderately active against Skov-3 cell line (IC_{50}: 60 μg/ml) compared to *T. leucostomus* (IC_{50}: 360 μg/ml). Against HeLa cell line, *T. sipyleus* showed no effect after 72 hours (IC_{50}: 907 mu g/ml) [30]. In another study investigating the anticancer effects of *Thymus* spp., the effect of *Thymus* vulgaris on breast cancer was examined. Thymol (55.88%), linalool (13.71%), carvacrol (8.36%) and p-cymene (6.00%) were found as the main components in the essential oil and the anticancer activity results revealed that treatment of MCF-7 cells with 100 μg/ml of essential *T. vulgaris* oil promoted apoptosis and induced DNA damage on the cells [31]. In a study investigating the selective cytotoxic and antiproliferative properties of *Thymus* caramanicus Jalas extract, MCF-7 human breast cancer cells were used and cytotoxicity was determined using MTT and neutral red assays. Vincristine was used as an anticancer control drug in the extract combination treatment. As a result, it was reported that *Thymus* extract has a potential antiproliferative property against human breast cancer cells and its combination with the chemotherapeutic agent vincristine can effectively induce cell death and that the most active components of *T. caramanicus*, carvacrol (51.0%) and thymol (20.84%) may be responsible for the anticancer effect observed in this study [32].

Enzyme Inhibitory Activities

Anticholinesterase (acetylcholinesterase and butyrylcholinesterase), urease, tyrosinase, elastase, collagenase, and angiotensin converting enzyme (ACE) inhibition activities of essential oils of TC-E and TS-E species were determined *in vitro* (Table 2). When the results were evaluated, it was determined that the two studied species showed moderate inhibition activity against BChE enzyme (Inhibition%:

57.88±1.14, 39.21±0.89, respectively). In addition, TS-E showed moderate inhibition of elastase enzyme (Inhibition%: 25.33±0.79) while TC-E showed no inhibition of elastase enzyme. In the AChE, urease, tyrosinase, tyrosinase, collagenase, ACE inhibition activities of both *Thymus* spp., the essential oils showed low or no activity. In another study conducted with *T. canoviridis*, *Thymus pubescens* Boiss. et Kotschy ex Celak. var. *pubescens* and *Thymus leucotrichus* Hal. var. *leucotrichus*, the biological activities of the essential oils of the species were investigated. IC_{50} values were calculated as 34.99 µg/ml, 44.15 µg/ml and 36.75 µg/ml for anticholinesterase enzyme, respectively. *T. canoviridis*, which is very rich in carvacrol (52.87%), was found to have higher antioxidant, antimicrobial and anticholinesterase activity than other essential oils [33]. In addition, studies have reported that carvacrol has antioxidant, antimicrobial, antidiabetes, antiparkinsonian, antialzheimer and antiglaucoma effects [34].

Table 2. Biological activities of the *Thymus* species[1]

	Antioxidant activity				**Toxic-cytotoxic activity**		
	IC_{50} (µg/ml)			**$A_{0.5}$ (µg/ml)**	**(%viability 200 µg/ml)**		
Samples	**Lipid peroxidation**	**DPPH**	**ABTS**	**CUPRAC**	**PDF**	**HT-29**	**MCF-7**
TS-E	650.12±2.41^a	>1000^a	312.45±3.21^a	217.50±5.12^a	73.00±1.23^a	65.92±1.32^a	10.72±0.03^a
TC-E	45.72±0.12^b	60.60±1.68^b	6.12±0.03^b	5.31±0.01^b	74.06±1.02^a	43.12±0.99^b	16.84±0.06^b
Butyl hydroxytoluene[2]	10.38±0.11^c	55.12±1.03^c	13.67±0.33^b	8.24±0.05^c	-	-	-
α- Tocopherol[2]	16.23±0.12^d	16.43±0.34^d	10.43±0.44^d	18.57±0.27^d	-	-	-
Doxorubicin (20 µg/ml)	-	-	-	-	-	23.26±0.86^c	-
Cisplatin (20 µg/ml)	-	-	-	-	-	-	48.12±0.99^c
Enzyme activity (inhibition %, at 100 µg/ml)							
Samples	**AChE**	**BChE**	**Urease**	**Tyrosinase**	**Elastase**	**Collagenase**	**ACE**
TS-E	NA	39.21±0.89^a	3.75±0.02^a	7.16±0.08^a	25.33±0.79^a	12.19±0.12^a	15.10±0.23^a
TC-E	4.19±0.01^a	57.88±1.14^b	11.94±0.11^b	NA	NA	3.17±0.03^b	11.19±0.18^b
Galantamine[2]	88.16±1.43^b	80.13±1.02^c	-	-	-	-	-
Thiourea[2]	-	-	95.89±1.36^c	-	-	-	-
Kojic acid[2]	-	-	-	92.07±1.21^b	-	-	-
Oleanolic acid[2]	-	-	-	-	43.75±1.38^b	-	-
Epicatechin gallate[2]	-	-	-	-	-	86.84±1.68^c	-
Lisinopril[2]							98.8±1.23^c

[1]Values expressed are means ± S.D. of three parallel measurements and values were calculated according to negative control. Values with different letters in the same column were significantly different ($p < 0.05$). [2]Standard compound. NA: Not active

In a study investigating the antioxidant and anticholinesterase activities of *Cyclotrichium origanifolium* (Labill.) Manden & Scheng (CO) and *T. sipyleus* (TS), the AChE and BChE activities of CO and TS were determined at a concentration of 200 µg/ml using galantamine as a standard compound. The best inhibition values against AChE and BChE enzymes were determined for CO (Inhibition%: 58.40 and 60.73 respectively) and TS (Inhibition%: 56.65 and 48.76 respectively) compared to galantamine [35]. The BChE enzyme inhibition activity of *T. sipyleus* was found to be moderate, similar to our study.

The chemical composition of the essential oils of *T. canoviridis* and *T. sipyleus* species were analyzed by GC-MS. There are no studies in the literature on the toxic-cytotoxic activities of the essential oils of the species and this study is the first in this sense. In addition, the enzyme inhibition (anticholinesterase, urease, tyrosinase, elastase, cholinase and angiotensin converting enzyme activities) of both species were examined in detail in this study. Especially *T. canoviridis* species showed high antioxidant activity. Both species showed high cytotoxic effect on breast cancer cells. When the results

are evaluated, it can be said that *T. canoviridis* essential oil with its rich carvacrol content and high cytotoxic and antioxidant activity can be preferred as a safer and natural option instead of synthetic preservatives in the food, pharmaceutical and cosmetic industries to extend shelf life and ensure food safety.

ACKNOWLEDGEMENTS

The author would like to thank Abdulselam Ertaş, İsmail Yener and Mehmet Akdeniz for GC-MS analysis.

AUTHOR CONTRIBUTIONS

Concept: S.Y.; Design: S.Y.; Control: S.Y., M.F.; Sources: S.Y.; Materials: M.F.; Data Collection and/or Processing: S.Y.; Analysis and/or Interpretation: S.Y.; Literature Review: S.Y.; Manuscript Writing: S.Y., M.F.; Critical Review: S.Y., M.F.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1078-1088, 2024
Doi: 10.33483/jfpau.1519926

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT OF A GREEN HPLC METHOD USING ETHANOL IN THE MOBILE PHASE COMPOSITION FOR THE DETERMINATION OF SODIUM BENZOATE AND POTASSIUM SORBATE IN BEVERAGES

İÇECEKLERDE SODYUM BENZOAT VE POTASYUM SORBAT TAYİNİ İÇİN HAREKETLİ FAZ BİLEŞİMİNDE ETANOL KULLANILAN YEŞİL HPLC YÖNTEMİNİN GELİŞTİRİLMESİ

Sercan YILDIRIM[1]*

[1]Karadeniz Technical University, Faculty of Pharmacy, Department of Analytical Chemistry, 61080, Trabzon, Türkiye

ABSTRACT

Objective: *This work aims to develop a novel and green high-performance liquid chromatography (HPLC) method for determining sodium benzoate (Na-BZT) and potassium sorbate (K-SBT) in beverages using ethanol as an environmentally friendly solvent in the mobile phase.*
Material and Method: *The chromatographic parameters were optimized using the Box-Behnken design. Validation studies were carried out in accordance with international guidelines.*
Result and Discussion: *The developed method displayed high accuracy (98.54-106.3%), precision (RSD≤5%), and specificity, with a total run time of 7 minutes. The limit of detection values for Na-BZT and K-SBT were 0.06 and 0.14 µg/ml, respectively. The use of ethanol, a less toxic solvent, minimized environmental impact compared to traditional solvents. The method's applicability was confirmed by analyzing ten different beverage samples. The results demonstrate the potential for broader application of ethanol-based HPLC methods in the beverage industry.*
Keywords: *Design of experiments, food analysis, HPLC, potassium sorbate, sodium benzoate*

ÖZ

Amaç: *Bu çalışma, hareketli fazda çevre dostu bir çözücü olarak etanol kullanarak içeceklerde sodyum benzoat (Na-BZT) ve potasyum sorbat (K-SBT) tayini için yeni ve çevreci bir yüksek performanslı sıvı kromatografi (HPLC) yöntemi geliştirmeyi amaçlamaktadır.*
Gereç ve Yöntem: *Kromatografik parametreler Box-Behnken tasarımı kullanılarak optimize edilmiştir. Validasyon çalışmaları uluslararası kılavuzlara uygun olarak gerçekleştirilmiştir.*
Sonuç ve Tartışma: *Geliştirilen yöntem, 7 dakikalık çalışma süresiyle yüksek doğruluk (%98.54-106.3), kesinlik (RSD≤%5) ve spesifiklik sergilemiştir. Na-BZT ve K-SBT için tespit limiti değerleri sırasıyla 0.06 ve 0.14 µg/ml idi. Daha az toksik bir çözücü olan etanol kullanımı, geleneksel çözücülere kıyasla çevresel etkiyi en aza indirmiştir. Yöntemin uygulanabilirliği on farklı içecek örneğinin analiz edilmesiyle doğrulanmıştır. Sonuçlar, içecek endüstrisinde etanol bazlı HPLC yöntemlerinin daha geniş uygulama potansiyelini göstermektedir.*
Anahtar Kelimeler: *Deneysel tasarım, gıda analizi, HPLC, potasyum sorbat, sodyum benzoat*

* **Corresponding Author / Sorumlu Yazar:** Sercan Yıldırım
e-mail / e-posta: sercanyildirim@ktu.edu.tr, **Phone / Tel.:** +904623778812

Submitted / Gönderilme : 21.07.2024
Accepted / Kabul : 07.08.2024
Published / Yayınlanma : 10.09.2024

INTRODUCTION

Na-BZT and K-SBT are commonly preferred preservatives in the food and beverage industry. The growth of bacteria, yeast, and fungi can be inhibited by Na-BZT in acidic settings. K-SBT is also capable of preventing the growth of molds and yeasts in food [1]. These preservatives are regulated by various health authorities, including the United States Food and Drug Administration and the European Food Safety Authority, which set maximum allowable concentrations to ensure consumer safety [2]. In Türkiye, the regulation and monitoring of these compounds are overseen by the Ministry of Agriculture and Forestry. According to the Turkish Food Codex Regulation on Food Additives, the maximum concentrations of Na-BZT and K-SBT can vary depending on the type of beverage, generally ranging from 150 to 200 mg/l for Na-BZT and from 250 to 2000 mg/l for K-SBT [3].

Although K-SBT and Na-BZT are considered safe, excessive use of these preservatives may lead to genotoxicity, causing DNA damage and chromosomal aberrations in various cell types. Studies also suggest that Na-BZT can lead to general genomic injuries, particularly in pregnant and their fetuses, emphasizing the potential health risks associated with the consumption of these additives. In this manner, accurate quantification of Na-BZT and K-SBT in beverages is necessary not only to ensure compliance with regulations but also to prevent potential adverse health effects [4].

A number of analytical methods based on spectrophotometry [5], gas chromatography [6], capillary electrophoresis [7], and liquid chromatography [1,2,8-12] have been developed for the determination of Na-BZT and K-SBT in food and beverages. Among these, HPLC is the most commonly utilized owing to its improved sensitivity, accuracy, and ability to handle complex matrices [10,11].
Lately, there has been a growing interest in developing "green" HPLC methods that minimize environmental impact. The idea of green chemistry (GrC) was introduced in the 1990s, which involves creating chemical products and procedures that minimize or eradicate the production and utilization of harmful substances. [13]. A roadmap for attaining sustainability in chemical procedures is presented through the twelve principles of GrC. Some of the fundamental principles involve the utilization of more secure substances and processes, boosting energy effectiveness, and the design of products for easy degradation. The importance of GrC lies in its potential to protect human health and the environment, reduce costs, and improve safety in chemical manufacturing [14].

Green analytical chemistry (GAC) extends the principles of GrC to chemical analysis. It aims to develop analytical methods that are environmentally benign, consume fewer resources, and generate less waste [15]. This shift towards greener practices is particularly relevant in chromatography, a widely used technique in analytical chemistry. Green chromatography focuses on minimizing the environmental impact of chromatographic methods at all stages, from sample collection and pretreatment to final instrumental analysis. It emphasizes the use of solventless extraction techniques, reducing solvent consumption and waste generation, and replacing toxic solvents with more environmentally benign alternatives [16]. Traditional HPLC methods often use acetonitrile or methanol as solvents, which are toxic and generate significant hazardous waste. In contrast, ethanol, a less toxic and more environmentally friendly solvent, offers a safer alternative and provides comparable performance in terms of solubility and elution strength for various analytes. The implementation of ethanol in the mobile phase aligns with the principles of GrC, aiming to moderate the utilization of harmful substances and generate less waste [17-20].

The optimization of HPLC methods using chemometric tools such as experimental design can significantly enhance the efficiency and robustness of the analytical process. Traditional one-factor-at-a-time (OFAT) approaches to method optimization can be time-consuming and labor-intensive, as they involve varying one parameter while keeping others constant. Furthermore, the OFAT approach often fails to identify optimal conditions due to the interaction effects between variables [21,22]. Chemometric tools, such as experimental design, address these limitations by allowing simultaneous variation of multiple parameters. This approach saves time and effort and provides a more comprehensive understanding of the effects and interactions of different factors on the analytical response. A design frequently utilized in chromatographic method optimization is the Box-Behnken design (BBD), which is advantageous as it requires fewer experiments than full factorial designs while still providing sufficient information to model quadratic response surfaces. It is particularly effective for identifying

optimal conditions and understanding the interactions between variables in HPLC method development [23,24].

In this study, a novel HPLC method was introduced using eco-friendly ethanol in the mobile phase to analyze Na-BZT and K-SBT in beverages. The optimization approach was based on experimental design, ensuring optimal separation and detection of the preservatives. This approach addresses the need for safer and more sustainable analytical practices and maintains the analytical performance required for accurate food preservative determination. To the best of our knowledge, the use of ethanol in the mobile phase for the separation of Na-BZT and K-SBT was reported for the first time in this work.

MATERIAL AND METHOD

Chemicals and Materials

Ethanol was purchased from ISOLAB Laborgeräte GmbH (Eschau, Germany). Sodium acetate trihydrate was obtained from Tekkim (İstanbul, Türkiye). Na-BZT, K-SBT, hydrochloric acid (HCl), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), and methanol were sourced from Sigma Aldrich (St. Louis, USA). Purified water from a Sartorius Stedim Arium pro UV instrument (Göttingen, Germany) was used throughout the study.

Instrumentation

A Prominence-20 HPLC system From Shimadzu (Kyoto, Japan) equipped with an SPDM20A diode array detector (DAD) was used. The operation of the system was overseen by Lcsolutions 1.25 software. An MX-S vortex from Isolab Laborgerte (Wertheim, Germany), an NF 615 centrifuge from Nüve (Ankara, Türkiye), An HI 2211 pH meter from Hanna Instruments (Woonsocket, United States), and an ultrasonic bath from Thermomac (İstanbul, Türkiye) were utilized for sample and standard preparation.

Preparation of Standard Solutions

Stock solutions (1000 µg/ml) of Na-BZT and K-SBT were prepared in methanol. Stock solutions were diluted with deionized water to prepare calibration and quality control (QC) solutions. All solutions were kept in the dark and at +4ºC until use.

Chromatographic Conditions

The chromatographic analyses were carried out using a Chromolith HighResolution RP18e monolithic column (100 × 4.6 mm). The mobile phase consisted of ethanol and pH 4.3, 20 mM acetate buffer in a ratio of 17.5:82.5 (v/v), delivered isocratically at a flow rate of 1.1 ml/min. The column temperature was adjusted to 25ºC. The DAD detector was set at 235 nm. A 10 µL of sample or standard solution was injected into the system.

Sample Preparation

Beverage samples, including two kinds of orange-flavored carbonated drinks, blackberry-, pineapple-, ginger-flavored drinks, lemon-flavored mineral water, tonic water, fizzy drink, energy drink, and coke, were purchased from a local market in Trabzon, Türkiye. Before HPLC analysis, the liquid sample was transferred to a beaker and sonicated for 5 min to remove the dissolved CO_2. Then, the tube was centrifuged at 5000 rpm for 3 min. The supernatant obtained was filtered through a syringe filter (0.45 µm). The final filtrate was diluted 10-fold with water before being injected into the system.

Method Validation

For the system suitability test (SST), a 25 µg/ml standard solution of Na-BZT and K-SBT was analyzed six times. Linearity was assessed by analyzing standard solutions at six different concentrations (0.5, 1, 2.5, 5, 10, 25, and 50 µg/ml) in triplicate. To evaluate the intra- and inter-day accuracy and repeatability of the method, quality control (QC) solutions at three concentrations (1, 25, and 40 µg/ml) were analyzed. Intra-day experiments involved three analyses at each level within the

same day, while inter-day experiments were performed over three consecutive days with seven analyses. Relative standard deviation (RSD) and % accuracy were used to represent repeatability and accuracy, respectively. The recovery of analytes following the dilute-and-shoot approach was also examined. For this, pre-analyzed beverages (blackberry flavored drink, coke, orange-flavored carbonated drink) were spiked with Na-BZT and K-SBT at 20 µg/ml level. Theoretical and experimental results were compared to estimate recovery. The limits of detection (LOD) and quantification (LOQ) were statistically estimated as previously described [25]. The method's selectivity was evaluated by checking the peak purity index values obtained for analytes during sample analysis.

Experimental Design

The influence of three factors, including ethanol ratio (%) in the mobile phase, pH, and flow rate, on the capacity factor (*k*) of the first peak and resolution of (*Rs*) Na-BZT and K-SBT was examined by BBD. The desirability approach was used to optimize several responses simultaneously [26]. Design Expert 11.1.2 was utilized to construct the BBD matrix and evaluate the model. The accuracy of the results was ensured by duplicating the measurements for each run.

RESULT AND DISCUSSION

Optimization of Chromatographic Conditions

Traditionally, chromatographic method optimization is based on the OFAT approach, which is time-consuming, involves many experiments, and cannot identify interactions among factors. On the other hand, by examining the interaction between critical factors and their combined influence on the response, experimental design facilitates the efficient manipulation of various variables. Therefore, optimization of chromatographic conditions was performed based on an experimental design aiming to obtain acceptable retention for the first-eluting peak and a satisfactory *Rs* between K-SBT and Na-BZT.

Chromatographic optimization is generally performed by response surface designs such as BBD and central composite design (CCD) [27]. Compared to CCD, BBD is a more straightforward approach since it involves conducting fewer experiments and eliminates the need for simultaneous experimental runs with all factors at extreme levels [28]. Consequently, BDD was utilized to optimize the chromatographic conditions for separating K-SBT and Na-BZT.

Since the initial screening runs demonstrated that analytes could be separated by isocratic elution, the first parameter was chosen as the ethanol ratio (%). The other two parameters were determined to be the pH and flow rate. The *k* of the first peak and *Rs* between K-SBT and Na-BZT were selected as responses to be optimized. Parameter ranges for ethanol ratio (%), pH, and flow rate were determined as 15-25%, 2.5-7, and 0.8-1.2 ml/min according to initial screening experiments. The design matrix and experimental results are given in Table 1.

Following the implementation of multiple regression analysis, the second-order polynomial equations were employed to represent the effects of factors on responses. All variables were subjected to a log10 transformation to enhance the models' capacity to interpret the data. Below are the mathematical expressions for the two responses:

$$\text{Log}_{10}(Rs) = 0.695774 - 0.151219A - 0.134066B - 0.0189029C - 0.0340106AB + 0.00785617AC + 0.00120551BC - 0.578631A^2 - 0.0186688B^2 + 0.0152894C^2$$

$$\text{Log}_{10}(k \text{ of first peak}) = - 0.0328285 - 0.55373A - 0.125051B - 0.00851598C + 0.0645791AB + 0.00637101AC - 0.0110173BC + 0.0481244A^2 - 0.011943B^2 + 0.0148512C^2$$

Where A, B, and C are pH, ethanol ratio (%), and flow rate, respectively. ANOVA was used to evaluate the models statistically, with the findings shown in Tables S1 and S2. The statistical significance of both models was confirmed by ANOVA ($p<0.0001$). The models effectively captured the relationships between factors and responses as indicated by the determination coefficient (R^2), adjusted R^2, and predicted R^2 values exceeding 0.96.

Table 1. The BBD matrix and experimental results for two responses

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	**Run**	**A: pH**	**B: Ethanol Ratio**	**C: Flow rate**	**Resolution**	***k* of first peak**
			%	**ml/min**		
4	1	7	25	1	0.642	0.242
5	2	2.5	20	0.8	2.09	3.78
8	3	7	20	1.2	0.909	0.313
17	4	4.75	20	1	5.00	0.922
11	5	4.75	15	1.2	6.70	1.28
9	6	4.75	15	0.8	7.30	1.28
15	7	4.75	20	1	4.96	0.928
10	8	4.75	25	0.8	3.60	0.715
12	9	4.75	25	1.2	3.34	0.645
1	10	2.5	15	1	2.09	5.65
13	11	4.75	20	1	4.92	0.929
7	12	2.5	20	1.2	1.84	3.57
3	13	2.5	25	1	1.44	2.50
6	14	7	20	0.8	0.963	0.312
16	15	4.75	20	1	4.95	0.930
14	16	4.75	20	1	4.98	0.927
2	17	7	15	1	1.28	0.301

In experimental design, lack-of-fit (LOF) serves as a statistical tool to evaluate the adequacy of a model in representing the collected data. LOF can be utilized to assess if there is a notable difference between the model predictions and the actual data. The LOF F-value is calculated by comparing the difference between the actual and predicted values from the model with the variation in repeated measurements. Therefore, a statistically significant LOF may occur due to the high precision at the central points of the design and the presence of error at axial points [21]. RSD values for two responses at the central point were all below 0.62% for five repeated measurements. In this context, a significant LOF was attributed to the limited variation at the central point.

Response surfaces obtained by the selected regression models are presented in Figure 1. pH was found to be the most significant factor for the *k* of the first peak. Retention of the analytes increased with the decrease of pH as both are found in neutral form under acidic conditions (Figure 1A). It should be noted that the elution order of the analytes changed with the increase in pH. K-SBT eluted earlier than Na-BZT at pH 2.5, while Na-BZT was the first-eluting analyte for pH 4.75 and 7. Retention of analytes increased with the decrease in ethanol ratio due to the decrease in elution strength of the mobile phase with a low organic solvent ratio. As expected, the flow rate did not change the *k* of analytes significantly. pH was also found to be the most significant parameter for the *Rs* of Na-BZT and K-SBT. The *Rs* of Na-BZT and K-SBT initially increased with pH from 2.5 to 4-5, then decreased with a further increase in pH to 7 (Figure 1B). The ethanol ratio had a negative effect on *Rs* due to decreased retention of analytes. A decrease in *Rs* was observed with the increase in flow rate, which can be attributed to the decline in column performance at elevated flow rates.

The optimum chromatographic condition was determined by Design-Expert software based on the desirability function [26]. The studies aimed to achieve a *k* value exceeding 1 for the first-eluting peak, i.e., Na-BZT, and an *Rs* better than 2.5. The optimal pH, ethanol ratio (%), and flow rate values were estimated as 4.3, 17.5%, and 1.1 ml/min. The chromatogram recorded under these conditions is given in Figure 2. The selected models proved reliable as the variations between predicted and actual values were under 4%. Na-BZT and K-SBT were eluted from the column at 3.82 and 5.05 min with a run-to-run analysis time of 7 min.

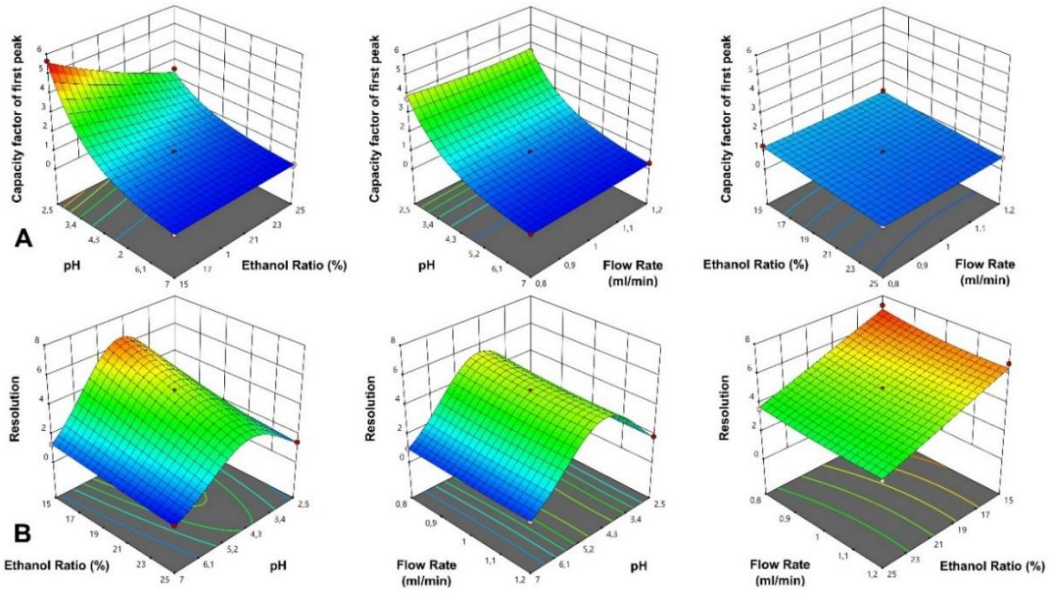

Figure 1. Response surfaces for (A) *k* of the first peak and (B) *Rs* of K-SBT and Na-BZT

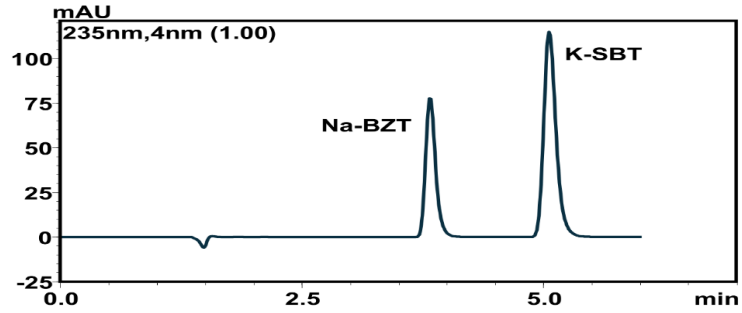

Figure 2. Chromatogram obtained at 235 nm under optimized conditions for a standard mixture (25µg/ml) of Na-BZT and K-SBT

Method Validation

Validation studies followed ICH guidelines to evaluate the method's reliability [29]. Before validation experiments, an SST was performed to ensure the chromatographic system's performance [2,20]. The results of the SST are presented in Table 2. The tailing factors were 1.29 for Na-BZT and 1.27 for K-SBT, both well within the acceptable limit of less than 2. The *k* values were 1.45 for Na-BZT and 2.24 for K-SBT, demonstrating the adequate retention of both analytes. The *Rs* between the two peaks was 5.46. Theoretical plates were higher than the minimum requirement of 2000. The selectivity factor was 1.54. The RSDs for retention time and peak area were less than 0.2% for both analytes.

Calibration graphs were plotted for Na-BZT and K-SBT within the 0.5-50 µg/ml concentration range. (Table 3). A correlation coefficient (*r*) of 0.9999 was observed for both analytes, confirming the linearity of the method. The LOD was determined to be 0.06 µg/ml for Na-BZT and 0.14 µg/ml for K-SBT. LOQ values were 0.18 µg/ml and 0.43 µg/ml for Na-BZT and K-SBT, respectively (Table 3).

The accuracy and precision of the method were assessed by evaluating the intra-day (n=3) and inter-day (n=7) variations at three concentration levels (1, 25, and 40 µg/ml) for both Na-BZT and K-SBT (Table 4). The method showed an intra-day accuracy range of 98.74%-106.3%, with RSD values ranging from 0.02% to 4.2%. Inter-day accuracies ranged from 98.54% to 105.3%, with RSD values ranging from 0.10% to 3.3%. Results demonstrate that the developed method displays acceptable accuracy and precision for determining Na-BZT and K-SBT. Furthermore, excellent mean recoveries in the ranges of 100.3-101.1% and 99.65-101.8% were obtained for Na-BZT and K-SBT, respectively,

which can be attributed to the direct injection of samples following the simple dilution with water (Table 5).

Table 2. Results of SST experiments (n=6)

	Na-BZT	K-SBT	Recommended value
Retention time (min)	3.82	5.05	-
Tailing factor (T)	1.29	1.27	<2
Capacity factor (*k*)	1.45	2.24	>1
Resolution (Rs)	-	5.46	>1.5
Theoretical plates (N)	5524	6685	>2000
Selectivity factor (*a*)	-	1.54	>1.05
RSD% of retention time	0.12	0.086	<1
RSD% of peak area	0.15	0.13	<1

Table 3. Results of validation experiments related to linearity and sensitivity

	Na-BZT	K-SBT
Linear range (µg/ml)	0.5-50	0.5-50
Slope	22557	41876
Intercept	948.2	-1990.4
SE of slope	18.96	84.23
SE of intercept	409	1817
Correlation coefficient (*r*)	0.9999	0.9999
LOD (µg/ml)	0.06	0.14
LOQ (µg/ml)	0.18	0.43

Table 4. Results of accuracy and precision experiments conducted at three quality control levels (1, 25, and 40 µg/ml)

		Intra-day*		Inter-day*	
Analyte	**Concentration level (µg/ml)**	**Accuracy (%)**	**RSD (%)**	**Accuracy (%)**	**RSD (%)**
Na-BZT	1	100.3	4.2	98.54	3.3
	25	99.89	0.019	99.88	0.11
	40	100.5	0.058	100.5	0.11
K-SBT	1	106.3	2.2	105.3	1.8
	25	98.74	0.029	98.66	0.10
	40	100.8	0.072	100.7	0.14

*The number of experiments is 3 and 7 for intra- and inter-day experiments, respectively.

Table 5. Results of recovery experiment performed on three different beverage samples (n=3)

Analyte	Sample	Added (µg/ml)	Found ±SD (µg/ml)	Recovery (%)
Na-BZT	Blackberry-flavored drink	20	20.08±0.02	100.4
	Coke	20	20.07±0.02	100.3
	Orange-flavored carbonated drink	20	20.22±0.02	101.1
K-SBT	Blackberry-flavored drink	20	19.93±0.03	99.65
	Coke	20	20.10±0.04	100.5
	Orange-flavored carbonated drink	20	20.36±0.05	101.8

Analysis of Food Samples

The feasibility of the proposed technique was proved by analyzing ten different beverages.

Representative chromatograms of 6 selected samples are given in Figure 3.

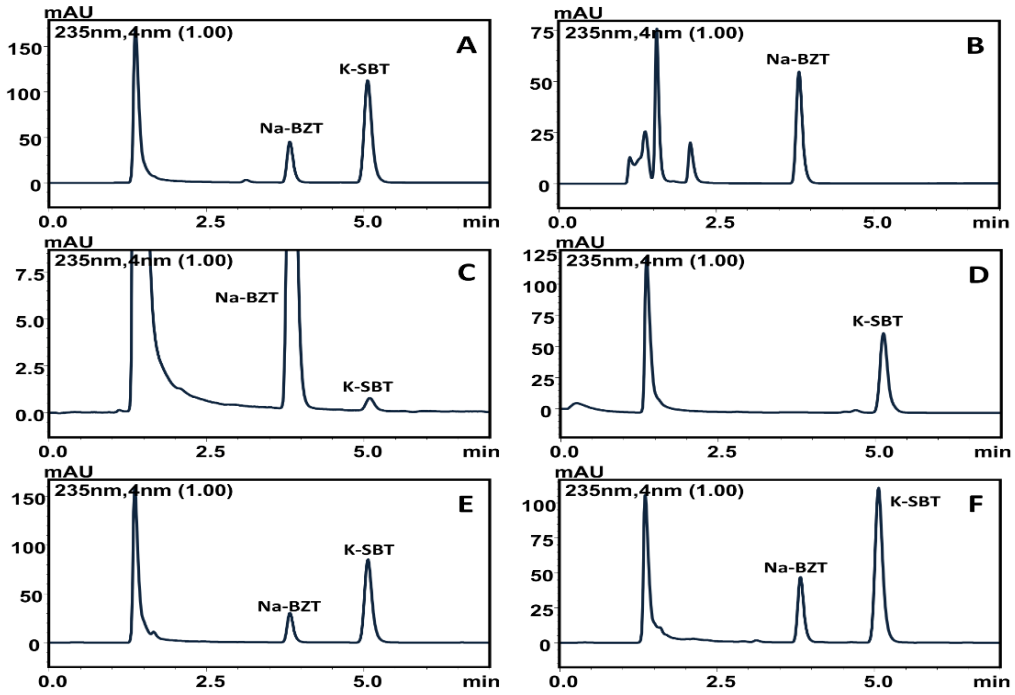

Figure 3. Representative HPLC chromatograms of beverages. (A) Pineapple-flavored drink, (B) Coke, (C) Lemon-flavored mineral water, (D) Tonic water, (E) Orange-flavored carbonated drink, and (F) Blackberry-flavored drink

The peaks were identified by comparing the retention times and absorption spectra of unknown peaks with Na-BZT and K-SBT standards. As can be seen, no interference from sample matrices was noticeable. This observation was further confirmed by the obtained peak purity index values higher than 0.9999 for all samples, indicating the selectivity of the proposed method. The amounts of Na-BZT and K-SBT in beverages are given in Table 6. Concentrations of preservatives ranged from 1.83 to 171.2 for Na-BZT and from 2.03 to 236.7 for K-SBT. Notably, the concentrations of Na-BZT and K-SBT in all samples were within the maximum limits set by the Turkish Food Codex Regulation. On the other hand, despite the absence of any mention on the label, orange-flavored carbonated drink-2 and lemon-flavored mineral water showed the presence of Na-BZT and K-SBT traces.

Table 6. Contents (mean ± SD) of Na-BZT and K-SBT in beverage samples (n = 3)

Sample	Na-BZT (µg/ml)	K-SBT (µg/ml)	Na-BZT declared	K-SBT declared
Blackberry-flavored drink	142.0±0.19	229.6±0.27	Yes	Yes
Coke	171.2±0.16	-	Yes	No
Orange-flavored carbonated drink	93.0±0.15	176.9±0.54	Yes	Yes
Orange-flavored carbonated drink-2	1.83±0.08	230.5±0.18	No	Yes
Pineapple-flavored drink	140.0±0.20	236.7±0.16	Yes	Yes
Ginger-flavored drink	141.2±0.32	223.4±0.21	Yes	Yes
Tonic water	-	135.6±0.13	No	Yes
Fizzy drink	166.8±0.01	-	Yes	No
Energy drink	-	215.9±0.20	No	Yes
Lemon-flavored mineral water	142.01±0.24	2.03±0.02	Yes	No

Evaluation of Method's Greenness

AGREE (Analytical GREEnness Metric Approach) is a comprehensive tool designed to evaluate the environmental greenness of analytical methodologies [30]. It is based on the 12 principles of GAC [31] and provides a unified 0-1 scale to assess various criteria, including sample preparation, reagent toxicity, waste generation, energy consumption, and operator safety. The AGREE tool generates a pictogram with a score in the center, where a score closer to 1 indicates a greener method. Each segment around the perimeter of the pictogram represents one of the GAC principles, using colors (green, yellow, red) to indicate the method's environmental performance in each area visually.

The method's greenness is assessed using the AGREE software, achieving a score of 0.77 (Figure 4), which indicates significant adherence to GrC principles. The sample preparation process involves minimal steps, including sonication, centrifugation, and filtration, which reduces chemical and energy usage. The method employs a small sample amount (e.g., 0.1 ml sample was enough), minimizing resource consumption and waste generation. Although the device positioning is marked in red due to offline sample analysis, this aspect often depends on laboratory setups and may not be easily modified. The method avoids derivatization, reducing the need for additional chemicals and associated waste. Waste management is enhanced by using ethanol, a less toxic and more environmentally friendly solvent compared to traditional solvents, such as acetonitrile and methanol. The method demonstrates high throughput with a total run time of 7 min. Energy consumption is generally optimized in HPLC systems, but further improvements could involve using more energy-efficient equipment such as UPLC. The reagents used, particularly ethanol, are sourced to minimize environmental impact. Overall, the developed method aligns well with GrC principles, offering a sustainable solution for analyzing Na-BZT and K-SBT.

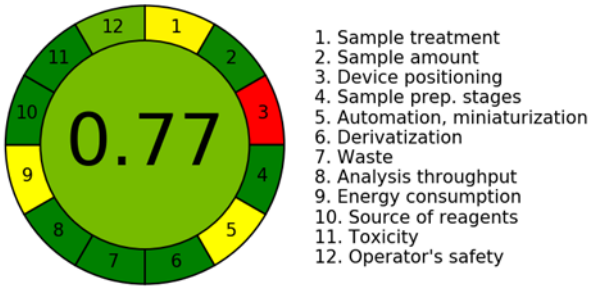

Figure 4. Greenness assessment of the HPLC method using the AGREE metric

Conclusion

A green HPLC method was presented to determine Na-BZT and K-SBT in beverages, utilizing ethanol in the mobile phase as an eco-friendly alternative. Chromatographic conditions were optimized via a chemometric approach using BBD to ensure optimum separation and detection of these preservatives. The proposed method aligns with the principles of GrC by minimizing environmental impact and maintains high analytical performance. Using ethanol to separate Na-BZT and K-SBT proved to be an effective and environmentally benign alternative to traditional solvents such as acetonitrile and methanol. The proposed method demonstrated high accuracy, precision, and specificity. The technique's feasibility was realized by analyzing ten beverages with excellent recoveries. Overall, this study highlights the feasibility of incorporating GrC principles into routine analytical methods, offering a sustainable solution for food preservative analysis. The findings emphasize the potential for broader application of ethanol-based HPLC methods in food safety monitoring and regulatory compliance.

AUTHOR CONTRIBUTIONS

Concept: S.Y.; Design: S.Y.; Control: S.Y.; Sources: S.Y.; Materials: S.Y.; Data Collection

and/or Processing: S.Y.; Analysis and/or Interpretation: S.Y.; Literature Review: S.Y.; Manuscript Writing: S.Y.; Critical Review: S.Y.; Other: -

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1089-1097, 2024
Doi: 10.33483/jfpau.1522582

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ANTIVIRAL-DRUG FAVIPIRAVIR FROM BIOLOGICAL SAMPLES BY USING MOLECULAR IMPRINTED POLYMER-BASED ELECTROCHEMICAL SENSOR

BİYOLOJİK ÖRNEKLERDEN MOLEKÜLER BASKILI POLİMER TABANLI ELEKTROKİMYASAL SENSÖR KULLANILARAK ANTİVİRAL-İLAÇ FAVİPİRAVİRİN TAYİNİ

Çiğdem KANBEŞ-DİNDAR[1]*, Bengi USLU[1]*

[1]Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06560, Ankara, Türkiye

ABSTRACT

Objective: *The molecularly imprinted polymer (MIP) technique was applied in this study for selective, fast, and sensitive electrochemical determination of antiviral drug favipiravir (FAVI).*
Material and Method: *By electropolymerizing the functional monomer o-phenylenediamine (o-PD) on a glassy carbon electrode (GCE) in the presence of a template molecule FAVI, the new MIP-based sensor (MIP@o-PD/GCE) was constructed using cyclic voltammetry (CV). For the removal and rebinding procedures, as well as the optimization of conditions and performance measurement of MIP@o-PD/GCE, differential pulse voltammetry (DPV) was used. The ferrocyanide/ferricyanide redox marker was used to monitor each step of the experimental procedure using DPV.*
Result and Discussion: *MIP@o-PD/GCE has a linear response to FAVI in the range from 10 pM to 90 pM under optimal experimental conditions for human serum samples. The detection limit of MIP@o-PD/GCE was obtained to be 1.80 pM, whereas the quantification limit was found to be 6.23 pM. The designed sensor was successfully applied to a synthetic human serum sample to verify its applicability and validity. Electrochemical sensor selectivity was evaluated by comparing the binding of paracetamol and tenofovir, which are similar to favipiravir, and also oseltamivir and famciclovir, which are other drugs used in the treatment of COVID-19.*
Keywords: *Antiviral drug, electroanalysis, electrochemical sensor, favipiravir, molecularly imprinted polymer*

ÖZ

Amaç: *Bu çalışmada moleküler baskılanmış polimer (MIP) tekniği, antiviral ilaç favipiravirin (FAVI) seçici, hızlı ve hassas elektrokimyasal tayini için uygulanmıştır.*
Gereç ve Yöntem: *Döngüsel voltammetri (CV) kullanılarak, fonksiyonel monomer o-fenilendiaminin (o-PD) bir şablon molekül FAVI varlığında camsı karbon elektrot (GCE) üzerinde elektropolimerize edilmesiyle, yeni MIP tabanlı sensör (MIP@o-PD/GCE) oluşturulmuştur. MIP@o-PD/GCE'nin uzaklaştırma ve yeniden bağlama prosedürlerinin yanı sıra etki edebilecek koşulların optimizasyonu ve performans ölçümü için diferansiyel puls voltametrisi (DPV) kullanılmıştır. Ferrosiyanid/ferrisiyanid redoks işaretleyicisi, DPV kullanılarak deneysel*

* **Corresponding Author / Sorumlu Yazar:** Çiğdem Kanbeş-Dindar
e-mail / e-posta: ckanbes@gmail.com, **Phone / Tel.:** +905050491355

Corresponding Author / Sorumlu Yazar: Bengi Uslu
e-mail / e-posta: buslu@pharmacy.ankara.edu.tr, **Phone / Tel.:** +903122033178

Submitted / Gönderilme : 25.07.2024
Accepted / Kabul : 07.08.2024
Published / Yayınlanma : 10.09.2024

prosedürün her adımını izlemek için kullanılmıştır.
Sonuç ve Tartışma: *İnsan serum numune örnekleri için MIP@o-PD/GCE, optimum deneysel koşullar altında 10 pM ila 90 pM aralığında FAVI'e doğrusal bir yanıt vermektedir. MIP@o-PD/GCE'nin tespit limiti 1.80 pM olarak elde edilirken, miktar belirleme limiti 6.23 pM olarak bulunmuştur. Tasarlanan sensör, uygulanabilirliğini ve geçerliliğini doğrulamak için sentetik bir insan serumu örneğine başarıyla uygulanmıştır. Elektrokimyasal sensör seçiciliği, FAVI ile benzerlik gösteren parasetamol ve tenofovir ile Covid-19 tedavisinde kullanılan diğer ilaçlar olan oseltamivir ve famsiklovirin bağlanması karşılaştırılarak değerlendirilmiştir.*
Anahtar Kelimeler: *Antiviral ilaç, elektroanaliz, elektrokimyasal sensör, favipiravir, moleküler baskılanmış polimer*

INTRODUCTION

FAVI also known as 6-fluoro-3-hydroxy-2-pyrazinecarboxamide (Figure 1), is a pro-drug that Fujifilm Toyama Chemical Company in Japan developed for treating influenza [1]. It was invented in 2002 and received approval in 2014 [2]. Following its arrival at the target cell, it undergoes a metabolic pathway that creates its active form, FAVI-ribofuranosyl-5'-triphosphate, within the cell [3]. This molecule is then recognized as a substrate by the RNA-dependent RNA polymerase, which is crucial for viral transcription, and that enzyme is selectively blocked [4].

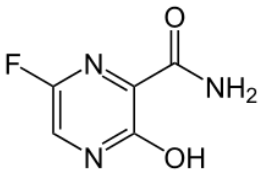

Figure 1. Chemical structure of FAVI

Various research focuses on determining the FAVI (Free Active Pharmaceutical Ingredient) in drugs or biological liquids. The previously conducted research employed several analytical methods, including capillary electrophoresis [5], spectroflourometry [6], liquid chromatography with UV detection [7], liquid chromatography with mass spectroscopy [8,9] spectrophotometry, and voltammetry [10,11]. These studies showed that FAVI could be analysed selectively when chromatographic systems were applied. However, these methods are time-consuming and too many organic solvents were used. Consequently, researchers have created electrochemical approaches as more feasible and eco-friendly alternatives in several investigations. Nevertheless, there is little empirical evidence demonstrating the resolution of the selectivity issue in these strategies. Furthermore, they lack the necessary sensitivity for analyzing biological materials. Hence, it is imperative to establish a more refined and discerning analytical technique. Within this particular framework, the integration of electrochemistry with molecular imprinting polymer technology presents a very efficient and practical resolution.

Biomimetic recognition elements, or MIP are polymer matrices with specific recognition sites for target molecules, or templates. Specific spots that identify the target molecule are created during the polymerization process. Subsequently, using the suitable solvent, these areas are permitted to create the suitable hollow spaces for identifying purposes. MIP technology is often used with electrochemical analysis techniques to selectively determine drug compounds.

The aim of this study was to develop a selective MIP surface using an electrochemical polymerisation technique for the stable and selective determination of FAVI from biological media. The stable, selective, and sensitive surface was optimized by adjusting essential parameters such as polymerization cycle, monomer: drug ratio, removal duration, and rebinding time. Subsequently, the linear operating range of the selective sensor surface was determined. Finally, we examined the sensor's linear working range in serum medium to demonstrate the applicability of the sensor for biological matrices.

MATERIAL AND METHOD

Instruments

A traditional three-electrode cell powered by AUTOLAB (Netherlands) was used to conduct the electrochemical experiments, namely CV and DPV. Our system was executed on a PC with the help of the NOVA 2.1.4 software. A reference electrode made of saturated Ag/AgCl (3 M KCl), a Pt plate counter electrode, and a GCE (3 mm diameter, BASi) were used in a standard three-electrode setup. The precision balance was calibrated by Ohaus Instruments (Shanghai, China) to measure the acquired quantities. A pH meter with a precision of ±0.05 was used to test the solutions' pH levels (Mettler-Toledo pH/ion S220, Switzerland). The removal and rebinding operations were carried out using a Thermo-Shaker (Biosan TS 100).

Reagents

The FAVI standard solution was made using distilled water and thereafter kept in a refrigerator. Methanol (99.8%), sodium hydroxide (>97%), acetic acid (AA) o-PD (≥98%), and sodium acetate trihydrate (>99%) were obtained from Sigma-Aldrich. For electrochemical measurements, the acetate buffer solution (pH 5.2) was prepared by using double-distilled water. All prepared solutions were stored at 4◦C in a refrigerator. The human serum stock solution (derived from male AB plasma) was purchased from Sigma-Aldrich in St. Louis, MO, USA.

Preparation of the MIP and Non-Imprinting Polymer (NIP) Based Electrochemical Sensors

The GCE was subjected to ultrasound for twenty minutes in a mixture of one-to-one methanol and two-part distilled water before the electrochemical polymerization (EP) procedure. The next step was to use a polishing pad to clean the electrode surface with alumina slurry. The surface was then rinsed with double-distilled water and let to dry at room temperature. To prepare the MIP@o-PD/GCE, the GCE was immersed in the polymeric film solution (5 mM o-PD, 2 mM FAVI in 0.1 M pH 5.2 acetate buffer) and electropolymerized scanning between -0.2 and 0.8 V for 20 cycles with 50 mV/s scan rate(Figure 2A). The polymeric film was prepared in the same protocol without FAVI to prepare the NIP-based sensor.

After the EP of the functional monomer o-PD and the template FAVI on the GCE surface, the imprinted electrode was rinsed with double-distilled water. Removal of template molecule FAVI was performed by immersing the imprinted GCE into a solution containing methanol and 1 M AA (2:3, v/v) for 5 min using a Thermo-Shaker at 550 rpm and room temperature. Afterward, the rebinding process was carried out by incubating the MIP-based electrode in different concentrations of FAVI for 5 min using a Thermo-Shaker at 550 rpm and room temperature. Before incubation of each different concentration, the electrode was washed with distilled water for 30 s.

In order to compare the analytical performance of the MIP-based electrochemical sensor, the NIP-based sensor was employed. Using the identical experimental procedure and circumstances as described above, but without the addition of FAVI during the EP stage, NIP-based GCE was synthesized. A GCE in NIP and MIP-based electrochemical sensors were used to conduct electrochemical measurements in a solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl (1:1). The methods used were CV and DPV.

RESULT AND DISCUSSION

Polymerization of MIP and NIP

Firstly, in order to perform the electrochemically selective determination of FAVI, the most widely used monomer in the literature, o-PD, was used and a MIP layer was formed on the surface of the electrode by electropolymerisation method as shown in Figure 2A. At this stage, the optimal parameters often seen in current studies, namely a monomer-to-template ratio of 10:1 and a polymerization cycle of 20, were selected[15]. Subsequently, analysis was conducted using the DPV method at each stage in the presence of a redox agent, and the change on the electrochemical surface was found to be shown in Figure 2B, where the highest current value is first obtained from the electrode surface. However, when the polymer coating increases, the surface becomes closed due to the insulating

properties of the polymer, making electron transport more difficult, and resulting in a significant decrease in current value. Later, the target molecule, namely FAVI, was removed in the presence of a suitable solvent to create selective cavity voids on the surface, resulting in the formation of voids that allow electron transfer. As a result, the current value of the redox marker has increased. Later, to demonstrate the determination of FAVI from the desired environment, cavity voids were created on the surface of the electrode. The surface was then incubated in a solution containing FAVI for 15 minutes. As seen in the image, when FAVI selectively settled into the cavity voids, there was a decrease in the current signal of the redox marker due to a decrease in electron transfer from the surface.

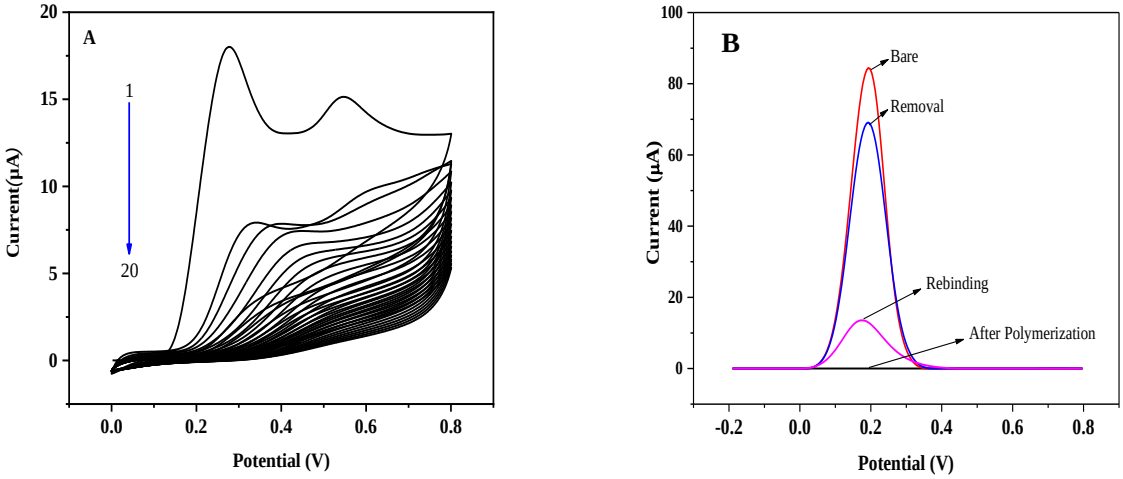

Figure 2. Cyclic voltammograms during electropolymerization of 5 mM o-PD in the presence of 2 mM FAVI in 0.5 M acetate buffer, pH 5.2 (Scan rate: 50 mV/s, 20 cycles) (A); Differential pulse voltammograms (B) Bare GCE: red. FAVI-MIP/GCE after electropolymerization (black), template removal (blue), rebinding in buffer (pink)

Optimization of Important Sensor Parameters

Following the successful demonstration of the feasibility of creating a selective surface using o-PD, several parameters such as the number of polymerization cycles, monomer: template ratio, removal time, and rebinding times were fine-tuned to establish a durable and consistent selective surface. Only the optimum parameters were modified in each phase, while the remaining values were kept fixed. The polymerization cycle was analyzed to determine the total number of cycles when the surface was fully coated with the polymer. According to Figure 3A, the highest current value after removal was obtained when the electrode surface was coated by MIP solution for 20 cycles. For this purpose, the monomer-drug ratio was four different monomer: drug ratios (10:1; 5:1; 1:1; and 2.5:1) were tested, and the peak current as a 68 µA was obtained at 2.5:1 (Figure 3B). Different removal solutions including: 0.1 M AA, ethanol, methanol, and methanol: AA (2:3) were tried to remove FAVI from the cavities formed during polymerization. The results obtained are close to each other for each solution media. However, it did not rebind after ethanol use and the methanol: AA (2:3) show the highest reproducibility. By using methanol:AA mixture as removal solution, removal time optimization was performed to remove FAVI in the most appropriate time without damaging the polymer surface and the most appropriate removal time was found to be 5 minutes (Figure 3C). The optimization of the incubation period of FAVI is a crucial component in the MIP sensor. A 100 µM solution of FAVI was used at this step, with an incubation duration of 5 minutes determined as the most suitable (Figure 3D).

Selectivity of FAVI -MIP Electrode Surface

The selectivity of the electrochemical sensor was assessed by comparing the affinity of paracetamol and tenofovir, which are comparable to FAVI, as well as oseltamivir and famciclovir, which are additional medications used in the management of Covid-19. A one-to-one ratio was used for all

drugs. It was found that the incubation in these solutions did not result in any notable alteration in the sensor (Figure 4). Recovery values of paracetamol, oseltamivir, tenofovir, and famciclovir were 97.25, 104.85, 98.35, and 102.14, respectively. The 97.25-102.14% recovery indicated that the interfering agents did not significantly affect the FAVI/MIP/GCE analytical performance.

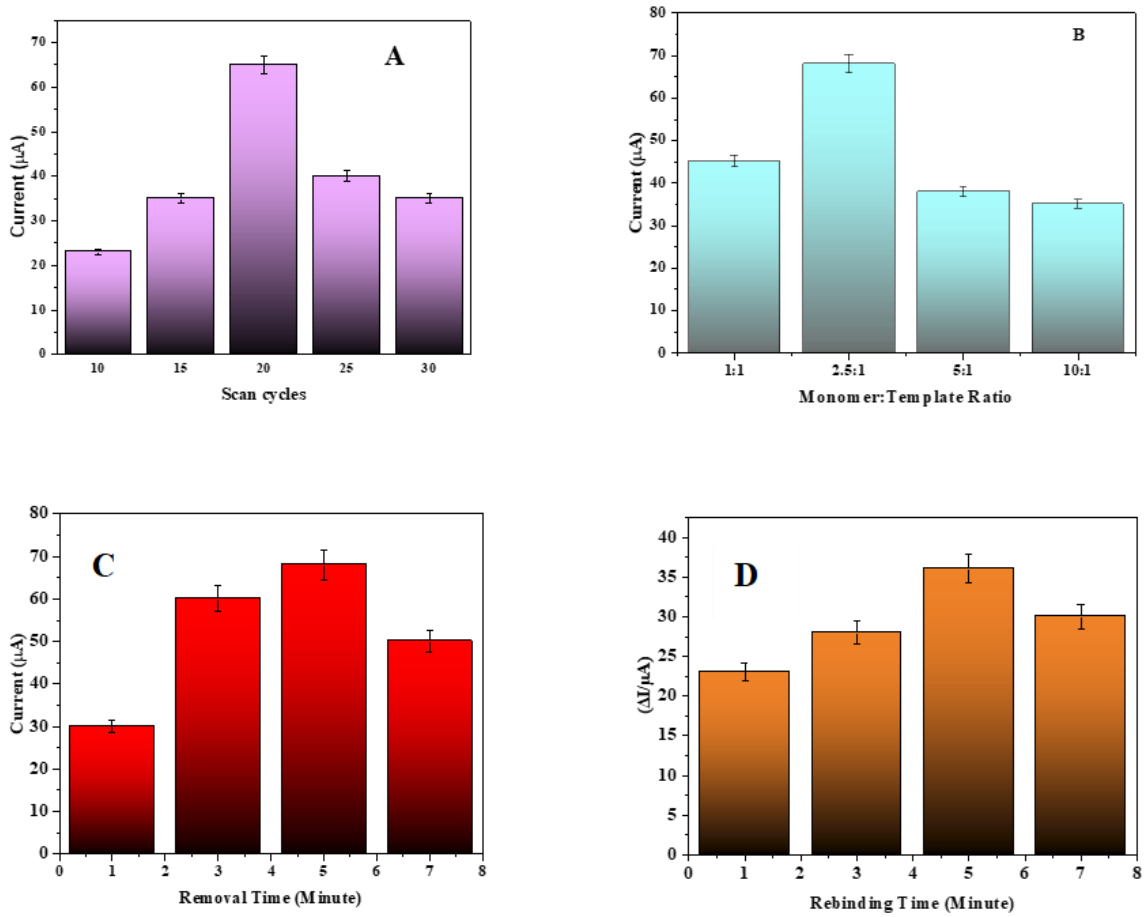

Figure 3. The optimization studies of electropolymerization scyle (A), monomer: template ratio(B), removal time (C), rebinding time (D), for development of MIP(FAVI)/GCE

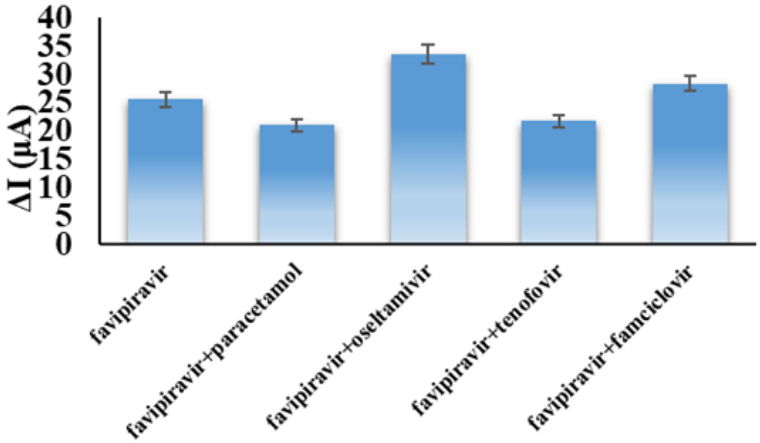

Figure 4. The structure of FAVI, FAVI:paracetamol (1:1), FAVI:oseltamivir (1:1), FAVI:tenofovir (1:1), FAVI:famciclovir (1:1)

Electroanalytical Applications

To investigate the analytical performance of MIP-modified GCE was incubated in different standard FAVI concentrations and the voltammograms obtained are shown in Figure 5A. Also, the analytical performance of MIP-modified GCE was evaluated by incubating it in various serum FAVI concentrations. The resulting voltammograms can be displayed in Figure 5C. The calibration curve was generated by plotting the ΔI current value against the variable FAVI standard concentration (Figure 5B) and the varying FAVI serum concentration (Figure 5D). Figure 5B and Figure 5D also shows the signals obtained after incubation with NIP-modified GCE at different FAVI concentrations to compare the selectivity of the sensor. The linearity was achieved for standard FAVI solutions and serum samples in the concentration range of 20–100 pM and 10-90 pM, respectively (Figure 5). While by using standard FAVI concentration, the related equation between peak current and concentration was founded as ΔI (μA) = 0.4562 *C* (pM) – 23.85 (*r* = 0.9924), by utilizing spike human serum FAVI solution, the related equation between peak current and concentration was described as ΔI (μA) = 0.5213 *C* (pM) – 21.63 (*r* = 0.9948).

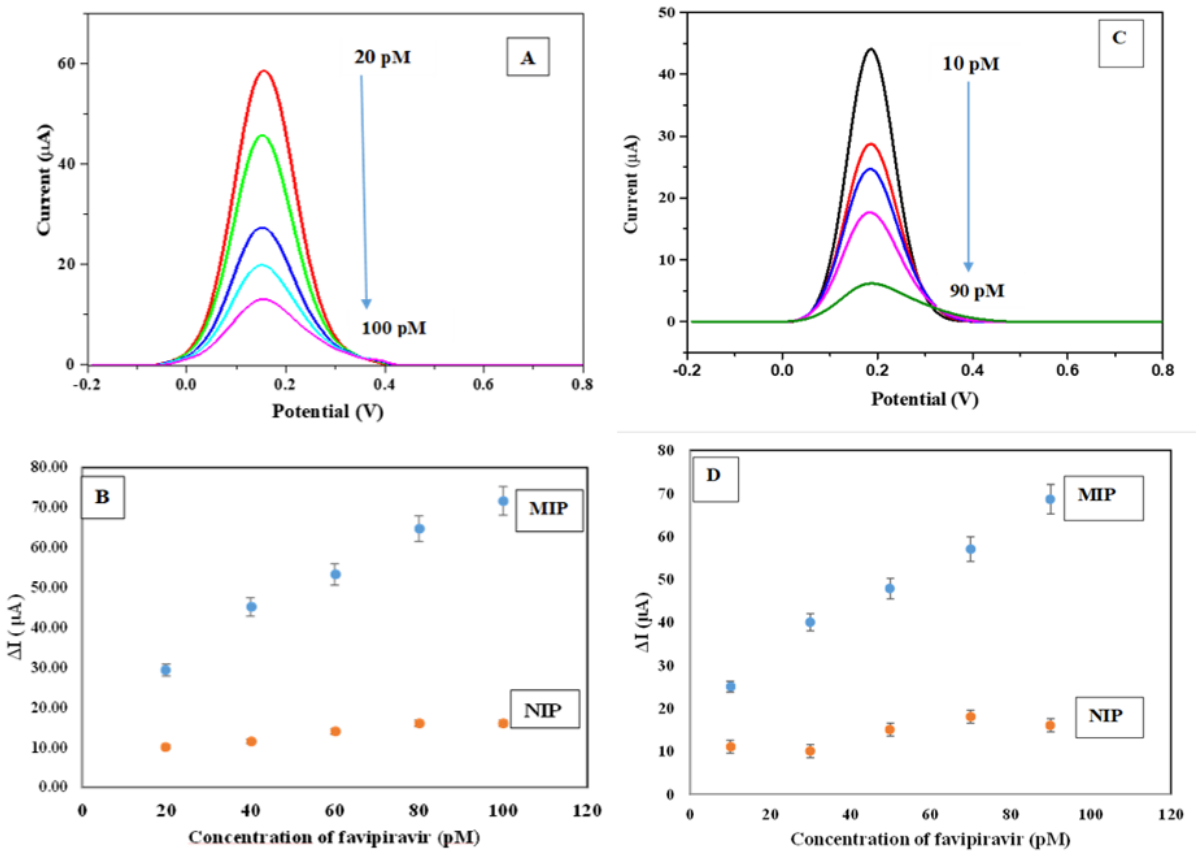

Figure 5. DP voltammograms of MIP-FAVI/GCE with different FAVI concentrations in (A) buffer solution, and (C) commercial serum solution. The calibration curve of FAVI with MIP-FAVI/GCE and NIP/GCE in (B) standard solution, and (D) commercial serum solution

Statistical data of the calibration are given in Table 1. Repeated measurements of FAVI peak potential and peak current within and between days demonstrate the sensitivity of the developed method. The formulas 3 s/m and 10 s/m were used to determine the LOD and LOQ values, where "s" is the standard deviation of the response and "m" is the slope of the calibration curve. The LOD and LOQ values (Table 1) showed the sensitivity of the method. The proposed methods are simple to use and are based on human serum obtained reproducible results that were sensitive enough to detect FAVI in samples (Table 1).

The performance of the developed method was also compared with previous analytical methods

(Table 2) and it was found that the developed method was the superior in terms of sensitivity, while most of the other methods involved time-consuming preconcentration, high consumption of harmful and organic solvents and expensive equipment.

Furthermore, the recovery studies were performed with commercial serum samples (Table 3). Recovery and RSD % results have proven the accuracy and precision of MIP@FAVI/GCE.

Table 1. Correlation data for FAVI calibration generated using DPV in from standard solution and serum

Parameters	**Standard**	**Serum**
Linearity dynamic range (pM)	20-100	10-90
Slope ($\mu A\ pM^{-1}$)	0.4562	0.5213
Intercept (μA)	23.85	21.62
Correlation coefficient (*r*)	0.9924	0.9948
LOD (pM)	2.22	1.80
LOQ (pM)	7.53	6.23
Intra-day precision of peak current (Relative Standard Deviation, RSD%)*	1.78	3.56
Inter-day precision of peak current (RSD%)*	2.43	4.70

*LOD and LOQ values were calculated based on the lowest value of the calibration range. For serum the intra-day precision of the peak current and the inter-day precision of the peak current values were calculated based on the midpoint of the calibration. Each value is the average of five experiments

Table 2. Comparison with other previously reported analytical methods to study the analytical performance of FAVI determination

Method	**LOD**	**Linearity range**	**Applications**	**References**
Spectrofluorometry	0.06 μM	0.25-1.78 μM	Tablets and human plasma	[6]
LC-MS/MS	37.5 μM and 28.6 μM for positive and negative modes	30-318 μM and 39-318 μM for positive and negative modes	Human plasma	[9]
Capillary Electrophoresis	1.59 μM	1.59-19.09 μM	Plasma and urine	[5]
AdSSWV (BDDE)	18 nM	64-6400 nM	Tablets and human urine	[16]
SWV (MnO_2-rGO/SPE)	9.0 nM	10-55000 nM	Tablets and plasma	[17]
DPV (MIP)	**1.80 pM**	10-90 pM	**Human serum**	***Proposed method***

* MnO_2-rGO: Manganese oxide-reduced graphene oxide; SPE: screen printed electrode;BDDE: boron doped diamond electrode

Table 3. Results of DPV recovery studies by using MIP sensor from serum samples

Parameters	**Serum**
Added concentration (pM)	10.00
Found concentration (pM)	10.14
Average recovered %	101.4
Number of experiments	5
RSD % recovery	5.08

Conclusion

This study attempts to design a molecularly imprinted polymer using the advantages of easy preparation of an electrochemical sensitive sensor. FAVI was used to template molecule. Optimized electrochemical MIP sensors were used to analyze FAVI from human serum samples. Results showed that developed electrochemical MIP sensors provide simple, low cost and sensitive determination of FAVI. The effect of some interfering agents onto the FAVI signal was also evaluated and and a change of less than 5% in the FAVI response was observed. In order to analyze serum samples with easier sample preparation and cheaper apparatus, this approach seems to be promising.

AUTHOR CONTRIBUTIONS

Concept: C.K.D., B.U.; Design: C.K.D., B.U.; Control: C.K.D., B.U.; Sources: C.K.D., B.U.; Materials: C.K.D., B.U.; Data Collection and/or Processing: C.K.D., B.U.; Analysis and/or Interpretation: C.K.D., B.U.; Literature Review: C.K.D., B.U.; Manuscript Writing: C.K.D., B.U.; Critical Review: C.K.D., B.U.; Other: -

CONFLICT OF INTEREST

The authors declare that this article has no real, potential, or perceived conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that this study does not require the ethics committee's approval.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1098-1109, 2024
Doi: 10.33483/jfpau.1505078

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE FABRICATION OF MIP BASED ELECTROCHEMICAL SENSOR FOR THE DETERMINATION OF DOBUTAMINE

DOBUTAMİN TAYİNİ İÇİN MIP ESASLI ELEKTROKİMYASAL SENSÖRÜN GELİŞTİRİLMESİ

Göksu ÖZÇELİKAY-AKYILDIZ[1]*, Sariye İrem KAYA[2]

[1]Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06560, Ankara, Türkiye
[2]University of Health Science, Gülhane Faculty of Pharmacy, Department of Analytical Chemistry, 06018, Ankara, Türkiye

ABSTRACT

Objective: *Dobutamine (DBT), one of the most common synthetic catecholamines, is important in the renal, cardiovascular, hormonal, and central nervous systems. In our study, it is proposed to produce the first MIP-based electrochemical sensor for DBT analysis. MIP(DBT)/GCE was developed by electropolymerization of 4-aminobenzoic acid (4-ABA) in the presence of DBT. Analytical performance and validation evaluation were performed on both standard solution and commercial serum samples.*
Material and Method: *DBT was supplied by the Vem pharmaceutical company. For the preparation of 5 mM $[Fe(CN)_6]^{3-/4-}$ solution, known amounts of potassium ferricyanide ($[K_3Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6].3H_2O$ mixed in 0.1 M KCl solution. The 4-ABA was used to create the polymeric film in the presence of DBT. Electrochemical measurements were actualized in IVIUM compactStat.h potentiostat (Eindhoven, The Netherlands) using a three-electrode system consisting of Pt wire, Ag/AgCl reference electrode, and glassy carbon electrode (GCE).*
Result and Discussion: *The MIP(DBT)/GCE sensor was applied to standard solution and commercial serum samples. When DBT concentrations were plotted against ΔI values, a linear response between 1×10^{-13} and 1×10^{-12} M was obtained in both environments. LOD results were found to be 0.087×10^{-13} M and 0.033×10^{-13} M in standard solution and commercial human serum, respectively. Recovery% and RSD% were found to be 99.68-100.32% and 1.38-1.66%, respectively.*
Keywords: *Commercial samples of human serum, determination, dobutamine, electrochemical MIP sensor, validation*

ÖZ

Amaç: *En yaygın sentetik katekolaminlerden biri olan dobutamin (DBT), renal, kardiyovasküler, hormonal ve merkezi sinir sistemlerinde önemlidir. Çalışmamızda DBT analizi için ilk MIP tabanlı elektrokimyasal sensörün üretilmesi önerilmiştir. MIP(DBT)/GCE, 4-aminobenzoik asidin (4-ABA) DBT varlığında elektropolimerleştirilmesiyle geliştirildi. Analitik performans ve doğrulama değerlendirmesi hem standart çözelti hem de ticari serum örneklerinde gerçekleştirildi.*
Gereç ve Yöntem: *DBT, Vem ilaç firmasından temin edilmiştir. 5 mM $[Fe(CN)_6]^{3-/4-}$ çözeltisinin hazırlanması için, potasyum ferrisiyanür ($[K_3Fe(CN)_6]$), potasyum ferrosiyanür ($K_4[Fe(CN))_6].3H_2O$, 0.1 M KCl'de karıştırıldı. DBT varlığında polimerik filmi oluşturmak için 4-ABA kullanıldı.*

* **Corresponding Author / Sorumlu Yazar:** Göksu Özçelikay-Akyıldız
e-mail / e-posta: goksu.ozcelikay91@gmail.com, **Phone / Tel.:** +905349160728

Submitted / Gönderilme : 25.06.2024
Accepted / Kabul : 08.08.2024
Published / Yayınlanma : 10.09.2024

Elektrokimyasal ölçümler, IVIUM kompaktStat.h potansiyostatta (Eindhoven, Hollanda) Pt teli, Ag/AgCl referans elektrotu ve camsı karbon elektrottan (GCE) oluşan üç elektrotlu bir sistem kullanılarak gerçekleştirildi.

Sonuç ve Tartışma: *MIP(DBT)/GCE sensörü, DBT'yi belirlemek için standart çözeltide ve insan serumunun ticari numunelerinde test edildi. DBT konsantrasyonları ΔI değerlerine göre çizildiğinde her iki ortamda da 1×10^{-13} ile 1×10^{-12} M arasında doğrusal bir yanıt elde edildi. TS sonuçları ise sırasıyla standart çözelti ve ticari insan serumda 0.087×10^{-13} ve 0.033×10^{-13} olarak bulundu. %Geri kazanım ve %BSS sırasıyla %99.68-100.32 ve %1.38-1.66 olarak belirlendi.*

Anahtar Kelimeler: *Dobutamin, elektrokimyasal MIP sensör, insan serumunun ticari örnekleri, tayin, validasyon*

INTRODUCTION

Organic compounds that play a physiological regulatory role and are found in the structure of disease markers and pharmaceutical substances are called catecholamines. Dobutamin (DBT) (Figure 1), one of the most common synthetic catecholamines, is widely used in clinical practice as a cardiostimulator, antimutagen, antioxidant, and anticarcinogen. DBT's catechol, secondary amine, and phenol functional groups have a high potential to participate in redox reactions, and therefore, it is an electroactive compound.

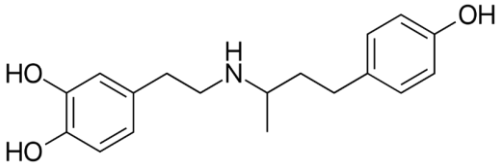

Figure 1. Chemical structure of DBT

Many analytical methods were performed to determine DBT. Especially the chromatographic techniques such as ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS) [1], two-dimensional liquid chromatography-mass spectrometry (2D-LC-MS) [2], high-performance liquid chromatography-mass spectrometry (HPLC–MS/MS) [3], reverse phase-high performance liquid chromatography (RP-HPLC) [4], high-performance liquid chromatography (HPLC) [5,6], were used to analyze DBT. However, scientists have been looking for new analytical methods recently because of the high cost of chromatographic techniques and the long analysis time. The electrochemical methods are strong alternatives to chromatographic techniques [7]. The voltammetric sensors record the current as a function of potential. In the measurement, the potential is variously applied either step by step or continuously to obtain a voltammogram. Several electrochemical sensors based on the voltammetry technique include cyclic, differential pulse, square wave, linear sweep, stripping, and hydrodynamic [8]. Electrochemical sensor has several advantages such as high sensitivity and selectivity to analyze the drugs, rapid response time, small sampling volume, and cost-effective protocols. There is no need for expensive equipment for signal transduction since the electron-transfer process directly generates an electronic signal [9].

Due to all these advantages of electrochemical methods, it would be attractive to develop electrochemical methods to detect DBT. Shvedene et al. (2002) performed electrochemical DBT determination using a screen-printed electrode modified with 3-(4-tolylazo)phenyl-boronic acid and ionic liquid graphite powder compositions [10]. Zhang (2004) determined DBT by the adsorptive stripping voltammetry method using electrodes modified with poly(acridine orange) film [11]. Chernyshov et al. (2008) reported that carbon paste electrodes were prepared and applied for the electrochemical determination of DBT. This electrode consists of graphite powder and ionic liquids [12]. Rastogi et al. (2012) used electrodes modified with sulfonic acid and acrylamide-derived copolymer to determine neurotransmitter substances, including DBT, by ion exchange voltammetry [13]. Ling et al. (2013) developed a nanosensor for the electrochemical oxidation of DBT [14]. The microflower-structured magnesium oxide-nafion composite layer-modified glassy carbon electrode

(GCE) was used. Asadian et al. (2014) produced graphene nanoribbon/polyaniline modified GCE (GNS/PAN/GCE) by in situ electropolymerization process. It showed that this modification agent enhanced the performances of electrodes at 10-fold [15]. Ekram et al. (2018) developed a Sr_2PdO_3 nano perovskite mixed with carbon nanotubes (CNT) modified new electrochemical sensor for the DBT analysis. With the modification, sensitivity, repeatability, and stability performance of nanosensors were improved [16]. Atta et al. (2019) determined the DBT by nanosensor. The electrode surface was modified by layer-by-layer technique. The multi-walled carbon nanotubes (MWCNT), ionic liquid (ILC), graphene (RGO), and 18-crown-6 (CW) were dropped to the electrode surface, respectively. This observed increase in current is associated with the ILC filling the gaps between the fMWCNT and G layers [17]. Ibrahim and Temerk (2020) fabricated the new nanosensor modified with nanocomposite (In_2O_3/ fMWCNT) for the determination of DBT. This modification agent provided the synergistic effect of In_2O_3 and fMWCNTs. It was enhanced the selectivity and sensitivity of the electrochemical sensor [18].

With the introduction of the key-lock theory by German chemist Hermann Emil Fischer, he opened a new page in the studies on the concept of molecular interaction from the past to the present [19]. Among molecular interaction applications, the molecular imprinting technique stands out. The molecular imprinting technique is a technique used to develop polymers with selective molecule recognition sites [20]. Polymer preparation components in the molecular imprinting technique; It consist of a functional monomer, target molecule, cross-linker, and initiator. The first step of polymer synthesis is the interaction of the target molecule (imprint molecule) and the functional monomer to form a target molecule complex. While this complex is being formed, covalent, non-covalent, semi-covalent, or metal-coordinated interactions occur between the target molecule and the functional monomer. This process is called the polymerization process [21].

With this study, the first MIP-based electrochemical sensor was fabricated for DBT analysis. For the formation of polymerization of 4-ABA, the electropolymerization technique was used in the presence of DBT. The developed MIP(DBT)/GCE was applied to a commercial serum sample for DBT analysis.

MATERIAL AND METHOD

Reagents and Chemicals

DBT was supplied from the Vem pharmaceutical drug company (İstanbul, Türkiye). The commercial serum sample (product no: H4522), uric acid, ascorbic acid, paracetamol, Na_2SO_4, $MgCl_2$, $[K_3Fe(CN)_6]$, $K_4[Fe(CN)_6].3H_2O$ and KCl were supplied from Sigma-Aldrich (St. Louis, Missouri, USA). For the preparation of 5 mM $[Fe(CN)_6]^{3-/4-}$ solution, known amounts of potassium ferricyanide ($[K_3Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6].3H_2O$ mixed in 0.1 M KCl solution. The 4-ABA was used to create the polymeric film. Moreover, the preparation of buffer, phosphate buffer $NaH_2PO_4.2H_2O$, H_3PO_4, acetic acid sodium salt trihydrate, acetic acid, and Na_2HPO_4 were purchased from Merck (Darmstadt, Germany) to realize the removal process and create the cavities in a polymeric matrix.

Apparatus

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed for electrochemical measurements. Electrochemical measurements were actualized in IVIUM compactStat.h potentiostat (Eindhoven, The Netherlands) using a three-electrode system consisting of Pt wire as counter electrode, Ag/AgCl as reference electrode, and glassy carbon (GC) as working electrode. The three electrodes were supplied to BASi company (USA). All chemical compounds (drugs, monomers, and so on) were weighted with balance (Ohaus company, China). The prepared solutions were kept in the ultrasonic bath for the sonication of the solution. The pH of the buffer was adjusted with a pH meter. A centrifuge (3500 rpm, 30 min) (NF200, Nuve Company, Türkiye) was used for the separation of precipitation from the supernatant. The supernatant taken was used to apply commercial serum samples. The thermo-shaker (650rpm, 25◦C) (TS-100, Biosan, Riga, Latvia) was used for the rebinding process. The surface characterization of MIP(DBT)/GCE was performed by a scanning

electron microscope (SEM) with an energy-dispersive X-ray spectrometer (EDX)(SEM_EDX, TESCAN GAIA 3, Czech Republic) and electrochemical impedance spectroscopy (EIS) (Metrohm, Autolab, Utrecht, Netherland).

Fabrication of MIP(DBT)/GCE and NIP-Based Electrochemical Sensors

Alumina was applied to the GCE surface on the polishing pad to clean the electrode surface thoroughly. Then, the GCE surface was washed with distilled water (dwater) and methanol, respectively. To prepare the polymeric film solution, 0.2 mM DBT, and 0.6 mM 4-ABA were mixed as a 1:3 ratio (v/v) in the pH 7.0 phosphate buffer. The GCE was electropolymerized in a polymeric film solution by scanning the potential with CV between −0.2 and 1.5 V. To obtain the cavity in the polymeric matrix, the GCE was immersed in the removal solution (pH 10.5 phosphate buffer), and the CV was performed by scanning the potential between −0.2 and 1.5 V. To control the MIP sensor, the NIP sensor was formed using the same fabrication protocol without the analyte.

Preparation of Commercial Human Serum Sample

MIP(DBT)/GCE practicability was examined with commercial serum samples. The commercial serum sample in the presence of DBT as the target molecule was prepared. 1 mM DBT (1 ml), commercial serum (3.6 ml), and 5.4 ml ACN (to precipitate the protein residues) were mixed in the centrifuge tube. DBT was not added to the commercial serum sample for the blank serum solution. Two centrifuge tubes were settled in a centrifuge as an opposite and centrifuged to separate the supernatant from the precipitate. The gathered supernatant was used to evaluate the accuracy of MIP(DBT)/GCE. Moreover, the calibration plot was obtained, and recovery studies were done in commercial serum samples.

RESULT AND DISCUSSION

Surface Characterization of the Molecularly Imprinted Polymeric Film and Non-Imprinting Polymeric Film

Characterization of the polymeric film formed on the GCE surface is carried out in terms of the surface's morphological properties and electrochemical properties. When examining surface morphology, the main goals are to contrast the surface characteristics of MIP with those of NIP and to pinpoint the distinct characteristics of MIP. Images obtained as a result of SEM measurements for MIP and NIP are shown in Figure 2. As can be clearly observed in the MIP image (Figure 2A), a very porous structure and, therefore, a rough appearance have been formed. The layered view of the polymeric structure is also visible in the figure. Observation of the features of the polymeric structure in this image supports the formation of MIP on the GCE surface. On the other hand, uniformity and smoothness are present in the NIP's SEM image (Figure 2B). This reveals that NIP is not suitable for binding to the target molecule. EDX spectra showing the presence of C, N, and O atoms expected to be in the MIP structure are shown in Figure 2C.

Electrochemical Characterization of the MIP-Based Electrochemical Sensors

The main methods preferred for evaluating electrochemical properties are CV and EIS. In both methods, the characteristic behavior of the redox probe is examined. As mentioned before, the redox probe in this study is a $5x10^{-3}$ M $[Fe(CN)_6]^{3-/4-}$ solution. Changes in the redox probe's anodic/cathodic peak currents were examined using the CV method (Figure 3A). When the GCE surface is unmodified before polymerization, the reversible peaks of the redox probe are observed as quite clear and high as a result of oxidoreduction, which occurs easily. As a result of coating the GCE surface with polymer after electropolymerization, a non-conductive surface is formed that prevents electron transfer. This manifests in the absence of redox probe peaks observed in the CV voltammogram. Thanks to the removal step, the template molecule DBT is removed from the polymer network of MIP, and the resulting cavities create spaces that re-enable electron transfer. This step is displayed as a re-increase of the reversible peaks of the redox probe in the CV voltammogram. Binding of DBT molecules to imprinted cavities through the rebinding step results in blocking electron transfer and decreasing oxidoreduction peaks.

Nyquist plots obtained with EIS measurements of the redox probe are displayed in Figure 3B. Here, the results regarding the charge transfer resistance (R_{ct}) value at different stages of MIP can be observed. Low R_{ct} values express fast electron transfer and easy oxidoreduction. The values recorded at different stages of MIP are included in Table 1 and confirm this.

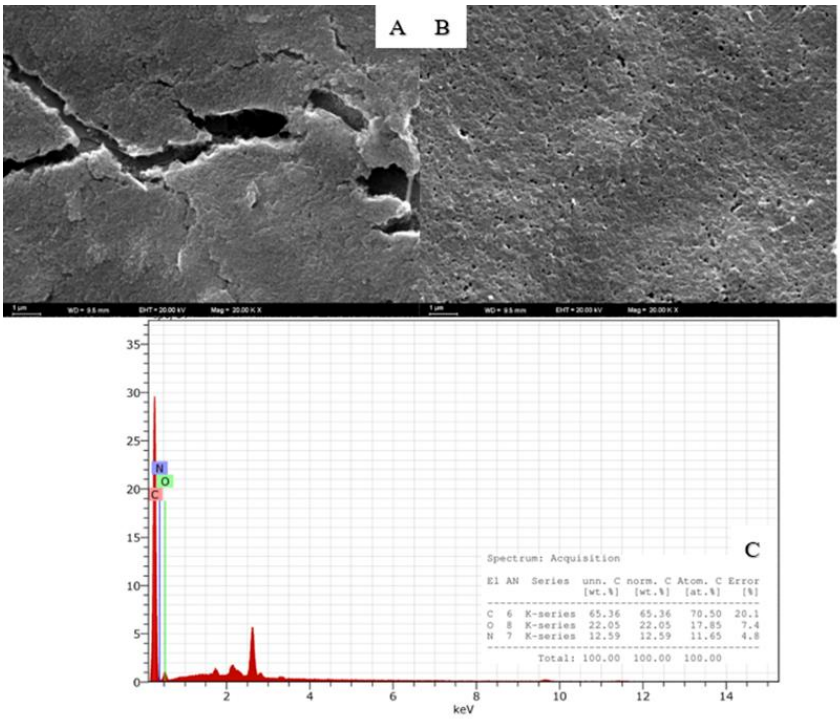

Figure 2. SEM images of A) MIP(DBT)/GCE and B) NIP(DBT)/GCE, EDX spectra of C) MIP(DBT)/GCE

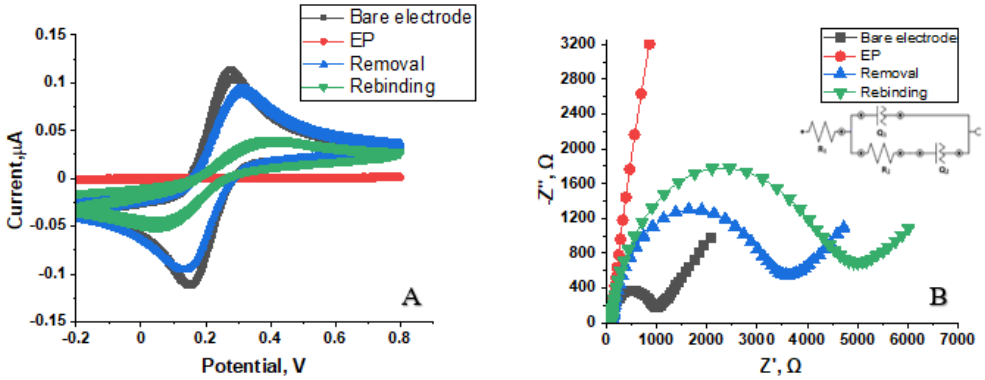

Figure 3. A) CV voltammograms B) Nyquist plots measured at different stages of MIP(DBT)/ GCE (Inset: R(Q[RQ]) equivalent circuit)

Table 1. R_{ct} values measured at different stages of MIP(DBT)/GCE

	R_{ct} (Ω)
Bare electrode	804.5
EP	24019
Removal	2813
Rebinding	3616.6

Optimization of Important Parameters

Critical parameters of the MIP process (number of cycles for EP, removal process, rebinding time, etc.) were optimized carefully and step by step for the determination of DBT. The DBT:4-ABA monomer ratio, number of scans, removal solution, removal cycles, and rebinding time were optimized. The optimization results are given in Figure 4.

The DBT:4-ABA ratio

The polymeric film formation is the most important parameter in the molecular imprinting process. According to the target molecule structure, the 4-ABA was selected. While this complex is being formed, non-covalent interactions occur between the 4-ABA and the DBT. While the results are evaluated, the difference of peak currents (Δ*Ip)* which are obtained after the polymerization process and removal process were calculated. Different proportions of 4-ABA and DBT were prepared and polymerized onto the electrode surface by an electrochemical process. When the 4-ABA ratio was 1 and 2 while keeping the DBT constant, it was not sufficient for the formation of the polymeric film. For this reason, the DBT:4-ABA ratio was selected as 1:3 (Figure 4A).

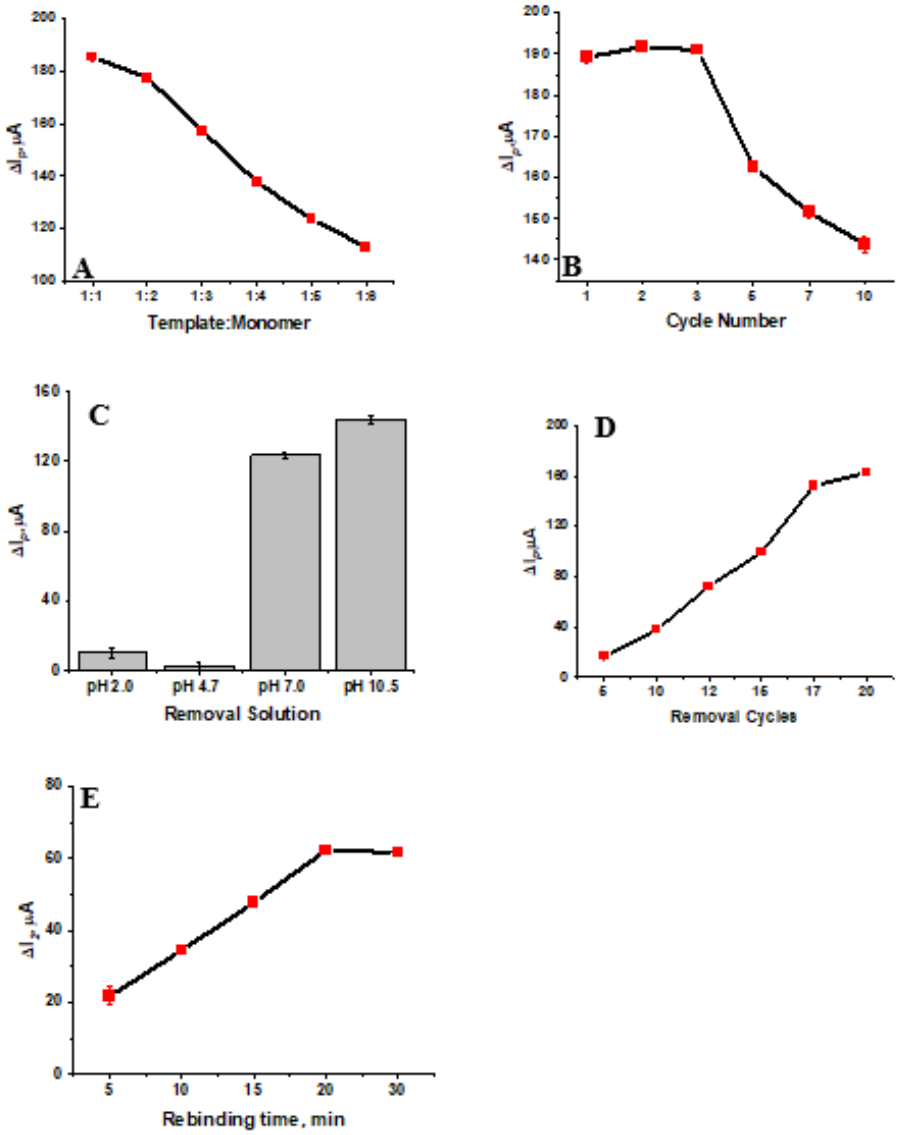

Figure 4. The optimization parameters of DBT:4-ABA monomer ratio(A), cycle number(B), removal solution(C), removal cycles(D), rebinding time(E) for development of MIP(DBT)/GCE

Cycle Number for the Electropolymerization

The electropolymerzation process arranges the polymer thickness. Another important parameter is to be determined by the cycle number. This step provides the sensitivity, repeatability, and stability of polymeric structures. For the electropolymerization process on the GCE surface, the repeated CVs were taken between −0.2 and 1.5 V. The number of cycles for electropolymerization was optimized from 1 to 10 cycles. The ΔI_p values were obtained similarly while numbers of 1, 2, 3. The cycle numbers were not sufficient for the formation of the polymeric film up to 5 cycles. For this reason, 5 cycles were selected for electropolymerization in the next stages (Figure 4B).

Selection of Removal Solution and Removal Cycles

The formation of a polymeric matrix in the presence of DBT provides the cavities that are suitable to shape, structure, and functional groups of DBT when the removal of the DBT from the polymeric matrix. Non-covalent bonds between DBT and 4-ABA are broken by exposure to various solutions and buffers of different pH environments. Some removal solutions (15 M acetic acid, 5 M NaOH, acetone, acetonitrile, ethanol) were applied by treatment of shaking in the thermal-shaker (650 rpm, 25◦C). This removal process didn't affect the cavities in the polymeric matrix. For this reason, buffers in different pH environments (pH 2.0, 7.0, and 10.5 phosphate buffer, pH 4.7 acetate buffer) were tried for electrochemical removal. The basic medium was given a good response for the removal process (Figure 4C). Therefore, pH 10.5 phosphate buffer was selected as the best removal solution. For the removal process, the CV was performed by scanning the potential between −0.2 and 1.5 V. To obtain the suitable removal cycles, the cycles from 5 to 20 min were optimized. According to Figure 4D, the removal cycle was chosen as 17.

Rebinding Time

The cavities formed during the removal process were immersed in DBT solution and exposed to a thermal shaker for a certain period to bind the DBT. The 10^{-3} M DBT, the target molecule, was diluted to different concentrations. While the results are evaluated, the difference of peak currents (ΔI_2) which are obtained after the removal process and rebinding process were calculated. According to Figure 4E, the rebinding time was found as 20 min for MIP(DBT)/GCE.

Analytical Performances of MIP(DBT)/GCE and NIP-Based Electrochemical Sensors

Analytical applicability and performance evaluation of the MIP(DBT)/GCE sensor was conducted using the indirect method. Accordingly, the DBT concentration is not measured directly; instead, the correlated decreases in the current of the redox probe as a result of binding DBT solutions to MIP(DBT)/GCE at increasing concentrations are measured. While the calibration curve is obtained, the differences between the current values obtained after removal and after rebinding (ΔI) are plotted against increasing DBT concentrations (Figure 5A). The key point to note here is that, unlike the linear response obtained with MIP(DBT)/GCE, there is no logical relationship between concentration and ΔI values due to the absence of specific cavities in NIP. The regression equation of the resulting calibration curve between 1×10^{-13} and 1×10^{-12} M is as follows: $\Delta I\ (\mu A) = 7.72 \times 10^{13}\ (\mu A/M) \times C\ (M) + 20.76$ ($r = 0.992$). An overlay of the differential pulse voltammograms of the redox probe obtained after rebinding increasing DBT concentrations is shown in Figure 5B.

We used the following ICH-recommended formulae to establish the limit of detection (LOD) and the limit of quantification (LOQ) values: LOD = 3 × standard deviation/slope and LOQ = 10 × standard deviation/slope [22]. LOD and LOQ values, along with other relevant regression data, are reported in Table 2.

Analytical Application of MIP(DBT)/GCE and NIP-Based Electrochemical Sensors in Commercial Human Serum Sample

Another stage of analytical performance evaluation and validation is analysis in a biological environment. For this purpose, the MIP(DBT)/GCE sensor was tested in commercial samples of human serum to determine DBT. When DBT concentrations were plotted against ΔI values, as in the standard

solution, a linear response was obtained between 1×10^{-13} and 1×10^{-12} M (Figure 5C). Associated differential pulse voltammograms are included in Figure 5D. The equation for the calibration curve is ΔI (µA) = 5.79×10^{13} (µA/M) × C (M) + 18.81 (r = 0.995). The LOD and LOQ values were calculated as 0.033×10^{-13} M and 0.111×10^{-13} M, respectively. Other regression data are included in Table 2.

A recovery study was conducted to test the feasibility and accuracy of MIP(DBT)/GCE in the serum sample. For this purpose, two known concentrations of standard DBT solution were spiked into the serum sample. The recovery% and RSD% values found as a result of the relevant applications and calculations are in Table 3 and demonstrate the accuracy and applicability of the MIP(DBT)/GCE sensor.

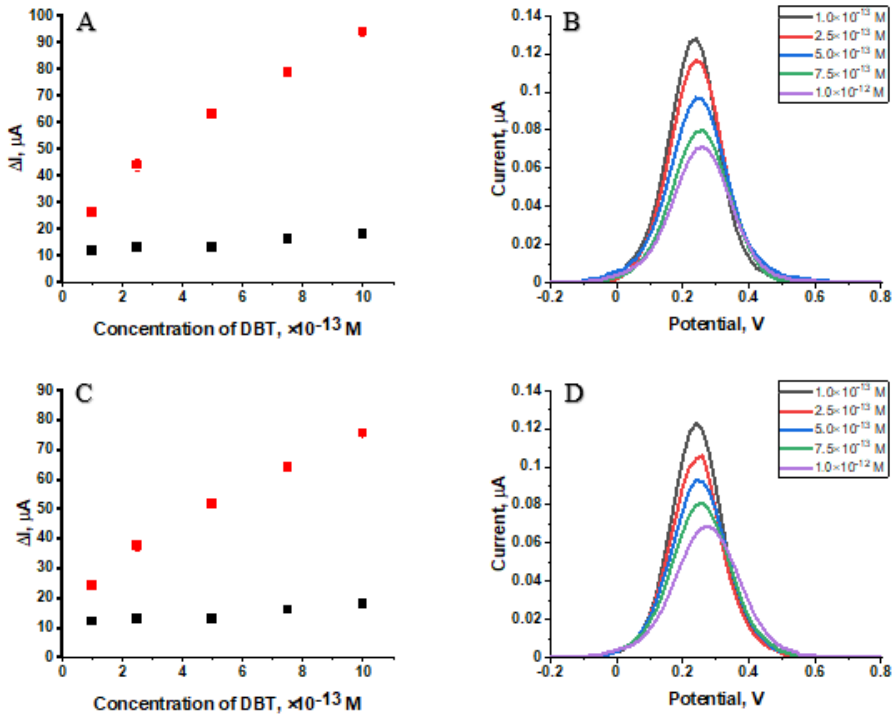

Figure 5. Calibration dependence on the MIP(DBT)/GCE for DBT in A) standard solution and C) commercial serum sample (red dots: MIP; black dots: NIP); differential pulse voltammograms obtained after rebinding increasing DBT concentrations in B) standard solution and D) commercial serum sample

Table 2. Analytical validation parameters for DBT determination on MIP(DBT)/GCE in standard solution and commercial human serum sample

	Standard solution	**Commercial human serum sample**
Linear range of DBT (M)	1×10^{-13}-1×10^{-12}	1×10^{-13}-1×10^{-12}
Slope (µA /10^{13} M)	7.717	5.791
S.E. of slope	0.397	0.235
Intercept (µA)	20.760	18.806
SE of intercept	2.742	1.273
Correlation coefficient (r)	0.992	0.995
LOD (M)	0.087×10^{-13}	0.033×10^{-13}
LOQ (M)	0.288×10^{-13}	0.111×10^{-13}
Repeatability of peak current (RSD%)*	0.756	0.270
Reproducibility of peak current (RSD%)*	1.544	1.650

* Each value is the mean of three experiments

Table 3. Recovery results of commercial human serum samples for DBT determination on MIP(DBT)/GCE

	Serum Samples#1	**Serum Samples#2**
Sample Concentration(M)	1.0×10^{-13}	2.5×10^{-13}
Spiked amount (M)	4.0×10^{-13}	2.5×10^{-13}
Found amount (M)*	4.98×10^{-13}	5.02×10^{-13}
Average recovery (%)*	99.68	100.32
RSD%	1.66	1.38
Bias%	-0.32	+0.32

* Each value is the mean of five experiments

Selectivity of MIP(DBT)/GCE and NIP-Based Electrochemical Sensors

Selectivity studies were performed with imprinting factor(IF) experiments and calculations. Since the cavities in the polymeric structures can recognize DBT in terms of shape, structure, and functional groups, it must be shown that molecules with a similar structure to the target molecule do not bind to the MIP(DBT)/GCE sensor. For this reason, catecholamine-derived compounds (carbidopa (CAR), levodopa (LEV), and dopamine (DOP)) shown in Figure 6 were chosen.

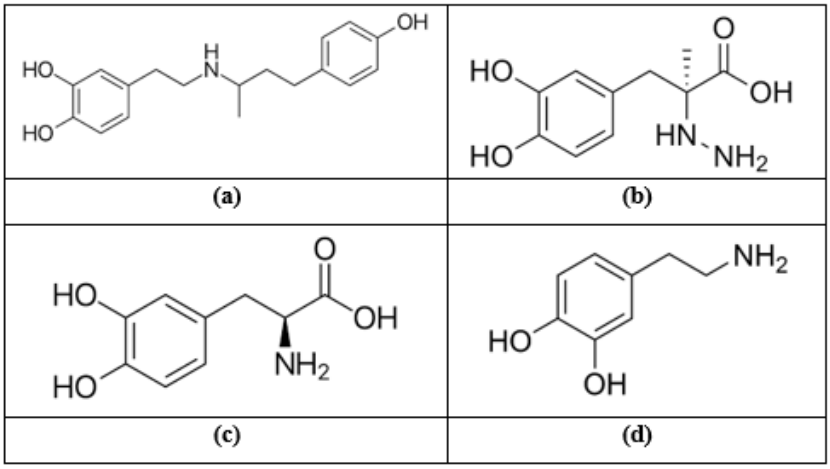

Figure 6. The molecular structures of the DBT(a), CAR(b), LEV(c) and DOP(d)

To calculate IF and IF' with the MIP(DBT)/GCE sensor using the ΔI_2 value, the equations below were employed. The results demonstrating the specificity of the sensor are presented in Table 4. It was emphasized that the sensor can determine DBT without being affected even in the presence of molecules that may affect selectivity at similar molecular structures.

$$IF_{(MIP)} = \frac{\Delta I_2(MIP)\ for\ DBT}{\Delta I_2(MIP)\ for\ other\ drug}$$

$$\mathrm{IF}_{(NIP)} = \frac{\Delta I_2(NIP)\ for\ DBT}{\Delta I_2(NIP)\ for\ other\ drug}$$

$$\mathrm{IF}' = \frac{IF_{(MIP)}}{IF_{(NIP)}}$$

Table 4. IF study results for MIP(DBT)/GCE sensor on DBT determination

Molecules	MIP/GCE		NIP/GCE		IF'$_{(MIP/NIP)}$
	$\Delta I_2/\mu A$	IF$_{(MIP)}$	$\Delta I_2/\mu A$	IF$_{(NIP)}$	
DBT	68.4	-	14.5	-	-
DOP	23.4	2.9	8.2	1.8	1.70
LEV	22.7	3.0	9.1	1.53	1.96
CAR	32.7	2.1	12.4	1.2	1.80

Interference Study of MIP Based Electrochemical Sensors

To evaluate the sensor's selectivity, studies are being carried out to analyze the DBT signal of the target molecule in the presence of potentially interfering substances (uric acid, ascorbic acid, paracetamol, Na_2SO_4, $MgCl_2$). DBT values were evaluated in the presence of interfering substances at different rates (1; 10; 100). The obtained recovery% and RSD% values for the sensor varied between 95.605– 103.431% and 0.57-1.98%, respectively (Table 5). These results demonstrate that the MIP(DBT)/GCE sensor can successfully detect DBT with interfering molecules and ions.

Table 5. Effect of interferents on the determination of DBT

Interferening Molecule/Ion	DBT: Interferent agents	Recovery of DBT (%)	RSD (%)*
Na^+	1:1	95.605	1.35
	1:10	98.716	1.03
	1:100	102.153	0.98
Cl^-	1:1	102.234	0.86
	1:10	96.182	0.57
	1:100	103.431	1.02
Mg^{2+}	1:1	102.234	0.86
	1:10	96.182	0.57
	1:100	103.431	1.02
SO_4^{2-}	1:1	95.605	1.35
	1:10	98.716	1.03
	1:100	102.153	0.98
Ascorbic acid	1:1	98.716	0.98
	1:10	98.364	1.57
	1:100	97.449	1.04
Paracetamol	1:1	101.531	0.79
	1:10	98.526	0.99
	1:100	101.305	1.79
Uric acid	1:1	102.153	1.65
	1:10	98.716	1.78
	1:100	96.935	1.98

*Each value is the mean of three experiments

Conclusion

This study explains the first MIP-based sensor for the electrochemical determination of DBT, a synthetic catecholamine. The contribution of the MIP approach used in this study and its advantage over existing studies is superior selectivity. EP was performed with the 4-ABA monomer selected in the MIP(DBT)/GCE sensor design. Critical parameters of the MIP process (number of cycles for EP, removal process, rebinding time, etc.) were optimized carefully and step by step. Morphological and electrochemical characterizations of the MIP(DBT)/GCE sensor, all stages of which were optimized, were performed with SEM, EDX, CV, and EIS methods, and the characteristic features of MIP were revealed. Analytical performance and validation assessment performed in both standard solution and commercial serum samples showed very good performance of the MIP(DBT)/GCE sensor, considering parameters such as sensitivity, accuracy, precision, and repeatability. In addition to all these, the effects of dopamine, levodopa, and carbidopa, molecules that are similar to DBT in terms of chemical structure and functional groups, were examined to reveal the selectivity, which is the most vital feature of the MIP-based approach. The high affinity of the MIP(DBT)/GCE sensor for DBT compared to these molecules is emphasized. Moreover, the MIP(DBT)/GCE sensor can selectively determine DBT without being affected by the possible interference effect of some molecules and ions commonly found in the body. In conclusion, this newly developed sensor is a very advantageous option for routine analysis of DBT with its remarkable selectivity.

AUTHOR CONTRIBUTIONS

Concept: G.Ö.A., S.İ.K.; Design: G.Ö.A., S.İ.K.; Control: G.Ö.A., S.İ.K.; Sources: G.Ö.A., S.İ.K.; Materials: G.Ö.A., S.İ.K.; Data Collection and/or Processing: G.Ö.A., S.İ.K.; Analysis and/or Interpretation: G.Ö.A., S.İ.K.; Literature Review: G.Ö.A., S.İ.K.; Manuscript Writing: G.Ö.A., S.İ.K.; Critical Review: G.Ö.A., S.İ.K.; Other:-

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1110-1117, 2024
Doi: 10.33483/jfpau.1474376

ORIGINAL ARTICLE / ÖZGÜN MAKALE

VALIDATED HPLC METHOD TO ANALYZE PHYTOCHEMICAL STRUCTURE OF *SCORZONERA* SPECIES GROWN IN TÜRKİYE

TÜRKİYE'DE YETİŞEN SCORZONERA TÜRLERİNİN FİTOKİMYASAL ANALİZİ İÇİN VALİDE EDİLMİŞ YPSK YÖNTEMİ

Seda ERCAN[1], Ekin KURTUL[2]*, Özge YILMAZ[1], Özlem BAHADIR ACIKARA[1]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06560, Ankara, Türkiye
[2]Zonguldak Bülent Ecevit University, Faculty of Pharmacy, Department of Pharmacognosy, 67600, Zonguldak, Türkiye

ABSTRACT

Objective: *The current study evaluated Scorzonera L. (Asteraceae) species, which are used as vegetables and medicinal plants in different countries where they grow naturally, such as Türkiye, Europe, Mongolia, and China, for their phenolic composition.*
Material and Method: *The twenty-five members of the Scorzonera genus, collected from different parts of Turkey, were investigated using a newly developed and validated High-Performance Liquid Chromatography (HPLC) method using some standard compounds, including chlorogenic acid, hyperoside, isoorientin, orientin, 7-O-methyl-isoorientin, isoquercetin, luteolin-7-O-β-glycoside, rutin, swertisin, and vitexin. The limit of detection and quantification levels were determined for each standard compound.*
Result and Discussion: *This study has revealed that the aerial parts are rich in phenolic compounds, with significantly higher amounts than the roots. Chlorogenic acid was detected in aerial parts and roots of all tested species and Scorzonera kotschyi aerial parts contained the highest amount (1787.26±32.88 µg/g). Most of the tested species contained varying amounts of hyperoside, isoorientin, isoquercetin, and orientin. Scorzonera aucheriana (572.93±0.04 µg/g), Scorzonera laciniata ssp. laciniata (524.07±5.06 µg/g), Scorzonera tomentosa (892.00±4.58 µg/g) and Scorzonera cana var. jacquiniana (309.23±1.69 µg/g) aerial parts contain these compounds respectively in higher amount. In contrast, vitexin, rutin and luteolin-7-O-β-glycoside were detected in a relatively small number of the tested species.*
Keywords: *Asteraceae, flavonoid, phytochemical analysis, Scorzonera, validation*

ÖZ

Amaç: *Bu çalışmada, Türkiye, Avrupa, Moğolistan ve Çin gibi yetiştiği ülkelerde sebze ve tıbbi bitki olarak kullanılan Scorzonera L. (Asteraceae) türleri fenolik bileşikleri açısından değerlendirilmiştir.*
Gereç ve Yöntem: *Türkiye'nin farklı bölgelerinden toplanan Scorzonera cinsine ait yirmi-beş örnek, klorojenik asit, hiperozit, izoorientin, orientin, 7-metil izoorientin, izokersetin, luteolin-7-O-β-glikozit, rutin, swertisin, viteksin gibi bazı standart bileşikler kullanılarak yeni geliştirilen ve valide edilen bir Yüksek Performanslı Sıvı Kromatografisi (YPSK) yöntemi ile incelendi. Her standart bileşik için tespit limiti ve tayin limiti hesaplandı.*
Sonuç ve Tartışma: *Toprak üstü kısımların köklere kıyasla fenolik bileşikler açısından çok daha*

* **Corresponding Author / Sorumlu Yazar:** Ekin Kurtul
e-mail / e-posta: e.kurtul@beun.edu.tr, **Phone / Tel.:** +905448871390

Submitted / Gönderilme : 11.07.2024
Accepted / Kabul : 08.08.2024
Published / Yayınlanma : 10.09.2024

zengin olduğu ortaya konmuştur. Test edilen tüm türlerin toprak üstü kısımları ve köklerinde klorojenik asit tespit edilirken en yüksek miktarda ise Scorzonera kotschyi'nin toprak üstü kısımlarında (1787.26±32.88 µg/g) bulunmuştur. Test edilen türlerin çoğunda değişen miktarlarda hiperozit, izoorientin, izokersetin ve orientin saptanmıştır. Sırasıyla, Scorzonera aucheriana (572.93±0.04 µg/g), Scorzonera laciniata ssp. laciniata (524.07±5.06 µg/g), Scorzonera tomentosa (892.00±4.58 µg/g) ve Scorzonera cana var. jacquiniana (309.23±1.69 µg/g)'nın toprak üstü kısımları bu bileşikleri yüksek miktarlarda içerirken, viteksin, rutin and luteolin-7-O-β-glikozit test edilen türlerde daha düşük oranda tespit edilmiştir.

Anahtar Kelimeler: *Asteraceae, fitokimyasal analiz, flavonoit, Scorzonera, validasyon*

INTRODUCTION

Scorzonera L. is a genus of plants that belongs to the Asteraceae family, which is widely distributed across diverse regions of Eurasia and Northern Africa. With around 160 species, *Scorzonera* is a diverse and widely distributed genus. This genus is particularly interesting in Türkiye due to its remarkable 52 species, 31 of which are endemic to the region [1]. *S. hispanica* L. is a European plant grown as a vegetable in some selected European countries. In Türkiye, various species such as *S. cana* (C.A. Mey.) Hoffm., *S. latifolia* (Fisch. and Mey.) DC., *S. mollis* Bieb., and *S. suberosa* C. Koch are also used as vegetables at the beginning of spring as their fresh twigs and roots [2,3]. Various species of *Scorzonera* have been used for medicinal purposes in traditional medicines worldwide. In European traditional medicine, various species of *Scorzonera* have been utilized for their therapeutic properties in treating respiratory diseases, colds, wounds, and other ailments. In addition to these medicinal benefits, *Scorzonera* is also renowned for its stomachic, diuretic, galactagogue, antipyretic, and appetizing effects. Similarly, traditional Mongolian medicine has been used to treat diarrhoea, lung oedema, parasitic diseases, and fever caused by bacterial and viral infections. The members of *Scorzonera* species have also been used in Libyan, Chinese, Tibetan, and Turkish traditional medicine to relieve hepatic pains and treat breast inflammation, abscesses, rheumatism, arteriosclerosis, hypertension, kidney diseases, and diabetes due to their anti-inflammatory and antipyretic properties [2,4]. Several in vitro and preclinical studies have demonstrated a diverse range of biological activities associated with the *Scorzonera* genus. Among these activities are wound healing effects, anti-inflammatory, antioxidant, and analgesic properties. Various phytochemicals such as dihydroisocoumarins [5-9], bibenzyl derivatives [10-12], flavonoids [6,13-16], lignans [12,17,18], stilbene derivatives [9,19,20], quinic and caffeic acid derivatives [6,13,16,19] sesquiterpene and sesquiterpene lactones [16,18,21-23] and triterpenes [13,24-27] have been isolated from the *Scorzonera* species. In the current study, twenty-five species of *Scorzonera* collected from different parts of Türkiye [28] were investigated for their phenolic content, particularly for their flavonoid composition by newly developed and validated HPLC method using some standard compounds, including chlorogenic acid, hyperoside, isoorientin, orientin, 7-methylisoorientin, isoquercetin, luteolin-7-glycoside, swertisin, rutin and vitexin. Limit of detection (LOD) and limit of quantification (LOQ) levels were determined for each standard compound.

MATERIAL AND METHOD

Preparation of Standard Solutions and Calibration

Ten different stock solutions were prepared, each with a concentration of 1 mg/ml, for the following compounds: chlorogenic acid, hyperoside, isoorientin, orientin, 7-methylisoorientin, isoquercetin, luteolin-7-glycoside, swertisin, rutin, and vitexin. Each stock solution was diluted to obtain six different concentration levels (0.01 mg/ml, 0.02 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, and 0.5 mg/ml). To establish the calibration curves, a total of three 10 µl injections were carried out for each standard solution. The resulting curves were constructed by plotting the peak area of each solution against its corresponding concentration. The HPLC chromatogram of the standard compounds was given in Figure 1.

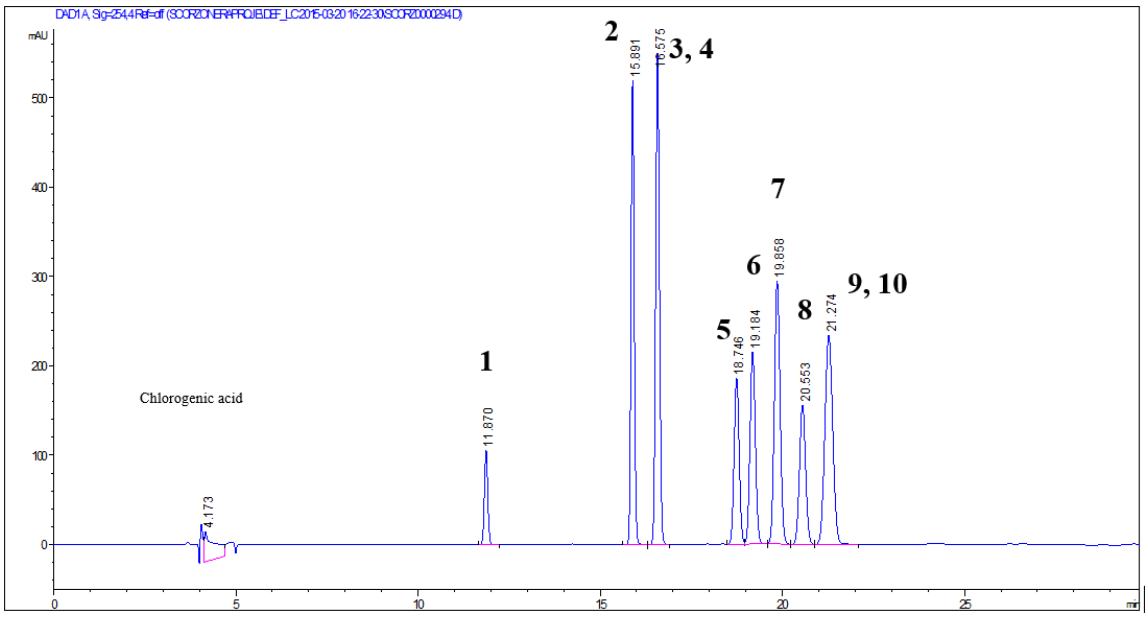

Figure 1. HPLC chromatogram of standard compounds. **1:** Chlorogenic acid; **2:** Isoorientin; **3:** Orientin; **4:** 7-*O*-methylisoorientin; **5:** Isoquercetin; **6:** Rutin; **7:** Hyperoside; **8:** Vitexin; **9:** Luteolin-7-*O*-glucoside; **10:** Swertisin. When standard substances were given as a mixture, the presence of 8 phenolic compounds was detected in the spectrum. The compounds, which were not separated but appeared as a single phenolic compound, were separated under ultraviolet light

Validation Procedure

Limit of Detection and Quantification

Achieving optimal levels of sensitivity and accuracy is crucial in any analytical method. With this in mind, it's important to note that limit of detection (LOD) and limit of quantification (LOQ) were established at Signal/Noise ratios of 3 and 10, respectively [29]. Table 1 displays the concentrations of each standard compound LOD and LOQ. Six injections of each standard compound experimentally verified LOD and LOQ concentrations.

Table 1. LOD and LOQ values for each standard compound

Standard compound	LOD concentration (µg/ml)	LOQ concentration (µg/ml)
Hyperosid	0.59	1.96
Isoquercetin	0.58	1.92
Isoorientin	0.13	0.42
Chlorogenic acid	0.21	0.71
Luteolin-7-*O*-*β*-glucoside	0.17	0.58
7-*O*-methylisoorientin	1.78	5.92
Orientin	0.32	1.06
Rutin	0.13	0.45
Swertisin	0.25	0.85
Vitexin	1.26	4.19
Hyperoside	0.59	1.96
Isoquercetin	0.58	1.92
Isoorientin	0.13	0.42

Preparation of the Extracts

To extract the plant material, 0.5 g of powdered aerial parts and roots were mixed with methanol: water (80:20) mixture (10 ml) in an ultrasonic bath for 30 minutes [30]. After extraction, each extract was filtered through filter paper and adjusted to 10 ml with the same solvent in a volumetric flask. Before injection, each extract was filtered again through a 0.22µ membrane filter.

HPLC Analysis

The HPLC analyses were expertly conducted on an Agilent LC 1100 model chromatograph, manufactured by the highly reputable Agilent Technologies based in California, USA. The diode array detector (DAD) was set to 254 nm wavelength. The peak areas were automatically integrated via Agilent software, and the resulting chromatograms were processed and plotted using the same software. The Supelcosil column (250 mm x 4.6 mm; 5 µm) was skillfully employed for separation, with acetonitrile (A) and water (B) expertly used as the mobile phase for gradient elution. The initial phase was A-B (8:92, v/v), which was followed by a linear change from A-B (8:92, v/v) to A-B (18:82) for 10 minutes. From 10 to 20 minutes, there was an isocratic flow of A-B (18:82), and the linear gradient elution was from A-B (18:82) to A-B (22:78) with a range of 20-45 minutes. From 45 to 55 minutes, A-B (22:78) was expertly changed and the flow rate was set to 0.7 ml/min. The column temperature was maintained at a precise 40°C and the sample injection volume was a carefully measured 10 µl.

RESULT AND DISCUSSION

The investigation aimed to examine the phenolic compounds present in *Scorzonera* species, utilizing both well-known flavonoids and pre-existing flavonoids extracted from plants within this genus and chlorogenic acid. The research revealed that the aerial parts of the plant exhibit a greater abundance of phenolic substances than the roots. Chlorogenic acid was detected in the aerial parts and roots of all the tested *Scorzonera* species and *S. kotschyi* Boiss. aerial parts were detected to contain the highest amount of this compound (1787.26±32.88 µg/g). Various tested species contained derivatives of quercetin such as hyperoside, isoorientin, isoquercetin, and orientin (namely the luteolin-C-glycoside). *S. aucheriana* Boiss. (572.93±0.04 µg/g), S. *laciniata* L. ssp. *laciniata* (524.07±5.06 µg/g), *S. tomentosa* L. (892.00±4.58 µg/g) and *S. cana* var. *jacquiniana* (W. Koch) Chamberlain (309.23±1.69 µg/g) aerial parts contain these mentioned compounds respectively at higher amounts compared to other tested species of *Scorzonera* genus. Vitexin, rutin and luteolin-7-*O*-*β*-glycoside were detected in a relatively small number of the tested *Scorzonera* species (Table 2).

The literature suggests that flavonoids can serve as taxonomic markers for Asteraceae. These compounds exhibit a broad range of structural diversity and have been isolated from many Asteraceae plants. Due to their wide structural diversity and abundance in Asteraceae species, flavonoids can serve as taxonomic markers at lower hierarchical levels [31]. However, the latest studies about flavonoids and their roles in the point of chemotaxonomy in Asteraceae, more specifically in Cichoridae which involves the *Scorzonera* genus, have reported that flavonoids are not very useful as markers on a higher level since rare compounds are found throughout the plant kingdom at various levels. However, flavonoids are the natural product class most widely employed for chemosystematic investigations. Flavonoids are frequently used in chemosystematics because they are easily separated and detected using simple techniques such as paper and thin-layer chromatography with UV-shift or spraying reagents [32].

Studies on the chemical composition of *Scorzonera* species have shown that they contain flavonoids and phenolic compounds. This study presents a simple and validated method for qualitative and quantitative analysis of some frequently isolated phenolic compounds from *Scorzonera* species. This study is of immense importance as it provides valuable insights into the phytochemical properties of *Scorzonera* species. By revealing their chemical compositions, this research can help develop new medicines and treatments that can significantly impact human health. The findings of this study can be used as a foundation for future research in this field.

Table 2. The amounts of the standard compounds in plant materials

Species		Standard compounds (µg/g plant material)									
		Chlorogenic acid	**Isoorientin**	**Orientin**	**7-*O*-methyl isoorientin**	**Isoquercetin**	**Rutin**	**Hyperoside**	**Vitexin**	**Luteolin-7-*O*-*β*-glucoside**	**Swertisin**
S. acuminata	AE	412.51±33.89	49.89±0.46	41.15±3.15		9.79±5.14	442.69±24.33				13.065±7.71
	R	327.16±30.34					0.23±2.79				
S. argyria	AE	756.65±28.97	127.08±0.93		110.81±1.93	13.521±0.11	107.22±0.47				57.51±2.44
	R	676.47±14.20									
S. aucherana	AE	551.01±3.99				170.77±0.05		572.93±0.05			
	R	244.68±4.03									
S. boissieri	AE	186.58±3.44	25.93±0.78	23.63±0.53		67.19±0.68	37.56±0.90	32.89±0.01		166.81±0.99	
	R	217.34±5.33									
S. cana var. *alpina*	AE	465.26±17.48	94.03±0.29	26.62±0.49					72.45±2.59		
	R	243.34±1.94									
S. cana var. *jacquiana*	AE	473.44±17.20	111.81±0.83	309.23±1.69					58.32±0.27	7.46±1.10	
	R	188.03±3.79									
S. cana var. *radicosa*	AE	334.52±56.79	263.11±3.19	71.69±7.74		18.51±3.81				116.29±2.47	130.92±2.74
	R	179.90±1.47									
S. cinerea	AE	218.75±5.36	34.92±0.12		61.79±0.20	53.11±0.34		153.99±0.58			28.38±0.11
	R	422.43±5.89									
S. ekimii	AE	732.29±35.26	38.91±3.23		260.64±1.43	48.31±2.06		136.03±1.75			175.75±2.13
	R	219.17±5.51									
S. elata	AE	201.22±3.33	154.40±0.86	70.91±0.24		0.74±0.08	1.39±0.18		12.79±0.03	3.52±0.11	
	R	150.34±2.18									
S. eriophora	AE	135.04±5.20	77.54±0.70			20.58±0.59	23.30±0.07	28.32±0.71			24.24±0.89
	R	306.45±3.92									
S. gokcheoglui	AE	363.06±14.38				50.69±0.28	31.78±0.41	117.19±0.42			
	R	361.26±5.88									
S. incisa	AE	653.06±14.59	137.04±11.24	76.26±20.71		25.58±0.31	182.44±0.03				
	R	122.66±25.77									
S. kotschyi	AE	1787.26±32.88				123.68±0.42	43.47±0.21	213.27±0.53			
	R	249.26± 0.96									
S. lacera	AE	1160.27±52.59				280.59±1.34		445.47±1.67		10.64±0.39	
	R	398.42±5.06						26.15±0.03			
S. laciniata ssp. *laciniata*	AE	333.72±11.91	524.07±5.06	130.58±0.42		2.92±0.04			17.91±0.03		
	R	98.83±0.76	42.71±0.07	130.58±0.16							
S. latifolia	AE	432.67±18.16	43.84±0.01		94.82±0.04	23.58±0.17	8.700±0.02	255.81±0.26			56.34±0.02
	R	1015.51±17.06									
S. mollis ssp. *szowitzii*	AE	1097.47±30.09	207.67±1.77	51.09±1.36		41.92±3.11	53.46 ±1.59	83.21±1.92		71.36±3.38	
	R	173.87±0.02									
S. parviflora	AE	438.49±8.38		38.29±2.23		74.58±0.32		30.72±0.14	19.2±0.13		
	R	549.01±9.08									
S. pisidica	AE	702.11±18.78	69.97±0.77			15.81±0.11	60.04±0.29	63.30±0.15			6.45±0.02
	R	752.10±11.59									

Table 2 (*continue*). The amounts of the standard compounds in plant materials

Species		Standard compounds (µg/g plant material)									
		Chlorogenic acid	**Isoorientin**	**Orientin**	**7-*O*-methyl isoorientin**	**Isoquercetin**	**Rutin**	**Hyperoside**	**Vitexin**	**Luteolin-7-*O*-*β*-glucoside**	**Swertisin**
S. pseudolanata	AE	379.37±12.84	125.63±0.27		123.07±1.49	23.26±0.71					61.76±0.25
	R	21.11±0.60									
S. sericea	AE	103.84±1.57						55.34±0.07			
	R	81.67±0.27									
S. suberosa ssp. *cariensis*	AE	612.53±21.47	38.33±0.15								
	R	500.71±13.47				122.76±0.44					
S. sublanata	AE	1207.77±37.56	339.99±6.21	382.88±7.65							
	R	391.89±0.45									
S. tomentosa	AE	365.73±4.89	49.68±1.24		71.06±0.91	213.89±1.23		892.00±4.58			32.41±0.01
	R	617.33±14.22									

AE: Aerial parts, R: Root

AUTHOR CONTRIBUTIONS

Concept: Ö.B.A.; Design: Ö.B.A.; Control: E.K.; Sources: Ö.B.A.; Materials: Ö.B.A.; Data Collection and/or Processing: S.E.; Analysis and/or Interpretation: S.E., E.K., Ö.B.A.; Literature Review: S.E., Ö.Y., Ö.B.A.; Manuscript Writing: E.K., Ö.Y., Ö.B.A.; Critical Review: S.E., E.K., Ö.Y., Ö.B.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1118-1127, 2024
Doi: 10.33483/jfpau.1436182

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF *N*-NITROSODIMETHYL AMINE (NDMA) AND *N*-NITROSODIETHYL AMINE (NDEA) IN MEDICINES CONTAINING SARTAN AND ITS DERIVATIVES

SARTAN VE TÜREVLERİNİ İÇEREN İLAÇLARDA N-NİTROSODİMETİL AMİN (NDMA) VE N-NİTROSODİETİL AMİN (NDEA) TAYİNİ

Şana SUNGUR[1]*, Muhammed ALJOUBASI[1], Zeki AYDIN[1]

[1]Hatay Mustafa Kemal University, Science and Letters Faculty, Department of Chemistry, 31024, Hatay, Türkiye

ABSTRACT

Objective: *In this study, it was aimed to determine the amounts of N-nitrosodimethyl amine (NDMA) and N-nitrosodiethyl amine (NDEA) in drugs containing sartan and its derivatives.*
Material and Method: *Medicines containing sartan and its derivatives as active ingredients such as valsartan, losartan, telmisartan, olmesartan, candesartan, irbesartan were purchased from pharmacies. NDMA and NDEA amounts of drugs were determined by headspace GC-MS.*
Result and Discussion: *The highest NDMA concentrations were found in drugs with losartan active ingredient (67.05±0.15mg kg^{-1}), and the highest NDEA concentrations were found in drugs with candesartan (22.48±0.06 mg kg^{-1}) and olmesartan (20.13 ± 0.06 mg kg^{-1}) active ingredient. The NDMA contents in each tablet were between $3.6x10^{-4}$- $1.7x10^{-2}$ mg and the NDEA contents were between $3.3x10^{-4}$ – $8.8x10^{-3}$ mg. The acceptable daily intake limits specified by the European Medicines Agency and the Food and Drug Administration are 96 ng/day for NDMA and 26.5 ng/day for NDEA. It was observed that the values obtained were above the acceptable intake limits even if one tablet was taken per day.*
Keywords: *Drug, headspace GC-MS, N-nitrosodiethyl amine (NDEA), N-nitrosodimethyl amine (NDMA), sartan*

ÖZ

Amaç: *Bu çalışmada, sartan ve türevlerini içeren ilaçların N-nitrosodimetil amin (NDMA) ve N-nitroso dietil amin (NDEA) miktarlarının belirlenmesi amaçlanmıştır.*
Gereç ve Yöntem: *Etken madde olarak valsartan, losartan, telmisartan, olmesartan, kandesartan, irbesartan gibi sartan ve türevlerini içeren ilaçlar eczanelerden satın alınmıştır. İlaçların NDMA ve NDEA miktarları headspace GC-MS ile belirlenmiştir.*
Sonuç ve Tartışma: *En yüksek NDMA konsantrasyonu losartan etken maddeli ilaçlarda (67.05 ± 0.15 mg kg^{-1}), en yüksek NDEA konsantrasyonu ise kandesartan (22.48±0.06 mg kg^{-1}) ve olmesartan (20.13 ± 0.06 mg kg^{-1}) etken maddeli ilaçlarda bulunmuştur. Her tabletteki NDMA içerikleri $3.6x10^{-4}$-$1.7x10^{-2}$ mg arasında, NDEA içerikleri ise $3.3x10^{-4}$- $8.8x10^{-3}$ mg arasında saptanmıştır. Avrupa İlaç Ajansı ve Gıda ve İlaç İdaresi tarafından belirlenen kabul edilebilir. Günlük alım limitleri NDMA için 96 ng/gün ve NDEA için 26.5 ng/gündür. Elde edilen değerlerin günde bir tablet alınsa bile kabul edilebilir alım limitlerinin üzerinde olduğu görülmüştür.*

* **Corresponding Author / Sorumlu Yazar:** Şana Sungur
e-mail / e-posta: sanasungur@hotmail.com, **Phone / Tel.:** +905354681551

Submitted / Gönderilme : 14.02.2024
Accepted / Kabul : 09.08.2024
Published / Yayınlanma : 10.09.2024

Anahtar Kelimeler: *Headspace GC-MS, ilaç, N-nitrosodietil amin (NDEA), N-nitrosodimetil amin (NDMA), sartan*

INTRODUCTION

Nitrosamines are chemically highly active amine derivatives with the R_1R_2N-N=O functional group. R_1 and R_2 denote alkyl or aryl groups. The vast majority of nitrosamines are classified as carcinogens by the International Agency for Research on Cancer (IARC). *N*-nitrosodiethylamine (NDEA) and *N*-nitrosodimethylamine (NDMA) are included in group 2A (probably carcinogenic to humans) [1].

N-nitrosamines are formed as a result of the reaction of an amine with a nitrosating agent. Nitrosing agents can be nitrous acid, nitrogen oxides, nitrosyl species and nitrite salts. When a nitrosating agent reacts with primary amines, the primary amine is rapidly converted to alcohol and nitrogen. When secondary amines react with the nitrosating agent, they form nitrosamines with the functional group R_1R_2N-N=O. When tertiary amines react with the nitrosating agent, nitrosamine formation does not occur [2].

Nitrosamines formed in drugs are caused by contaminated starting materials and intermediates, reagents, solvents, catalysts, errors made in the production process, recovered and recycled materials. For example, when azide is used as a reagent during tetrazole synthesis, hydrazoic acid is formed due to acidic conditions. The released hydrazoic acid can also lead to amine formation. In addition, the remaining azide can form gaseous by-products such as nitrogen and nitrogen oxides when sodium nitrite is added. Failure to maintain the proper pH or temperature and not adding the reagents in the proper order are examples of errors in the production process. The water used in production may also contain nitrosamines due to environmental pollution. If necessary precautions are not taken or equipment is not cleaned between customer orders, nitrosamine formation is inevitable.

In addition, nitrosamines can be formed after the drugs are produced, depending on the storage conditions, packaging materials and decomposition processes of the active substances [3].

In June 2018, the Food and Drug Administration (FDA) announced that *N*-nitrosodimethylamine impurity, which may have genotoxic potential, has been found in some drugs with the active ingredient valsartan in China. Then, drugs with valsartan active substance began to be studied not only in China but also in all European Union countries [4]. Studies have been extended for all sartans with tetrazole group such as losartan, irbesartan, candesartan, olmesartan. As a result of these studies, the presence of nitrosamines has been detected in many drugs. In 2019, the detection of *N*-nitrosodimethylamine impurity in ranitidine-containing drugs that do not contain sartan increased the concerns [5]. Studies have shown that nitrosamines can be formed both during and after the manufacture of drugs, depending on storage conditions. In November 2019, the Singapore government announced that N-nitrosodimethylamine impurity was found in drugs containing the active ingredient metformin [6]. In August 2022, the Food and Drug Administration reported that nitrosamines have been detected in drugs containing sitagliptin, which are commonly used in type 2 diabetes patients [7]. After all these explanations, studies have been started to determine whether drugs contain nitrosamines all over the world [6, 8-19]. However, there is no study done on this subject in our country. It is extremely important to know that how much nitrosamine, which is threaten human health, presence in which drugs.

The European Medicines Agency and the Food and Drug Administration have established acceptable daily intake limits for nitrosamines. These values are 96 ng/day for NDMA and 26.5 ng/day for NDEA [4].

Within the scope of this study, the NDMA and NDEA amounts of drugs containing sartan and its derivatives as active ingredient (valsartan, losartan, irbesartan, telmisartan, olmesartan and candesartan) sold in Turkey were determined.

MATERIAL AND METHOD

Materials

Certified standards of *N*-Nitrosodimethylamine and *N*-Nitrosodiethylamine were purchased from

Sigma Aldrich (Gillingham, UK). All chemicals used were of analytical reagent grade and were at least 99.5% pure. All drug samples were obtained from pharmacies in Turkey.

Preparation of Drug Samples for Analysis

0.1 g of the drug samples, which were crushed into powder, were weighed and placed in glass centrifuge tubes. 2 ml of dimethylsulfoxide was added to them and mixed using a vortex mixer until dissolved at room temperature. Then, the solutions were transferred to the headspace vial [20].

Headspace GC-MS Analysis

Headspace GC-MS analysis was performed using an Agilent 7890 A GC System with an Agilent 5975 C MS and an Agilent 7697A Headspace Auto-sampler. Headspace GC-MS operating conditions are shown in Table 1 [20].

Table 1. Headspace GC-MS operating conditions

Headspace Parameters			
Instrument Used	Agilent 7697 A		
Oven Temperature	120°C		
Sample Line Temperature	125°C		
Transfer Line Temperature	130°C		
Gas Pressure	103 kPa		
Equalization Time	15 min		
GC Parameters			
Instrument Used	Agilent 7890 A		
Column	DB WAX (50 m, 0.20 mm, 0.20 µm)		
Carrier Gas	Helium		
Flow Rate	2 ml/min		
Injector Temperature	240°C		
Detector Temperature	240°C		
Temperature Program	Ramp rate (°C/min)	Temperature (°C)	Hold time (min)
	-	40	2
	10	120	2
	25	230	5
MS Parameters			
Instrument Used	Agilent 5975 C		
Ionization Mode	EI (70 eV)		
Mode	SIM		
m/z values	NDMA	NDEA	
	74, 42, 43	102, 57, 56, 44, 42	

Quality Control and Quality Assurance

All analyses were repeated three times for each sample. Under the applied headspace GC-MS conditions, the retention times of NDMA and NDEA were 10.1 and 11.3 min, respectively. The peaks of nitrosamines are shown in Figure 1. For NDMA m/z 74, 42, and 43 were monitored (SIM), with m/z 74 used for quantitation; and for NDEA m/z 102, 57, 56, 44, and 42 were monitored, with m/z 102 used for quantitation (Figure 2a and 2b). Calibration standards were prepared in the range of 0.05 – 200 mg l^{-1} for NDMA and 0.05 – 20 mg l^{-1} for NDEA. In all cases, the correlation coefficients were found to be greater than 0.9995. The calibration curves were created from seven calibration standards and are shown in Figures 3a and 3b. The limit of detection (LOD) was determined to be three times the standard deviation of the blank test values. The limit of quantification (LOQ) was taken as three times the LOD. The obtained values are given in Table 2.

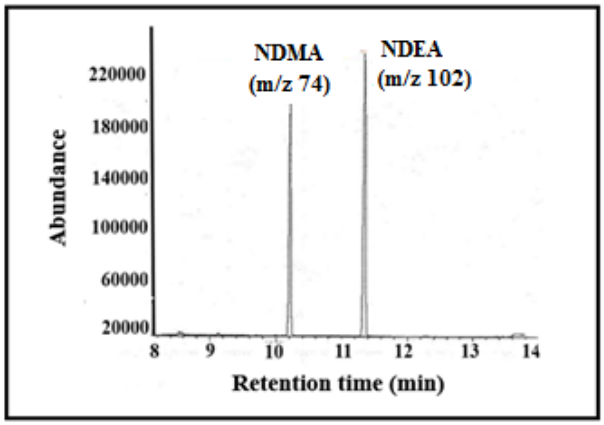

Figure 1. Under the applied headspace GC-MS conditions, peaks belonging to NDMA and NDEA standards

Table 2. The values of retention time, correlation coefficient, LOD and LOQ of NDMA and NDEA

Nitrosamines	Retention Time (min)	Correlation Coefficient (R^2)	LOD (mg / l)	LOQ (mg / l)
NDMA	10.1	0.9999	0.004	0.013
NDEA	11.3	0.9998	0.001	0.003

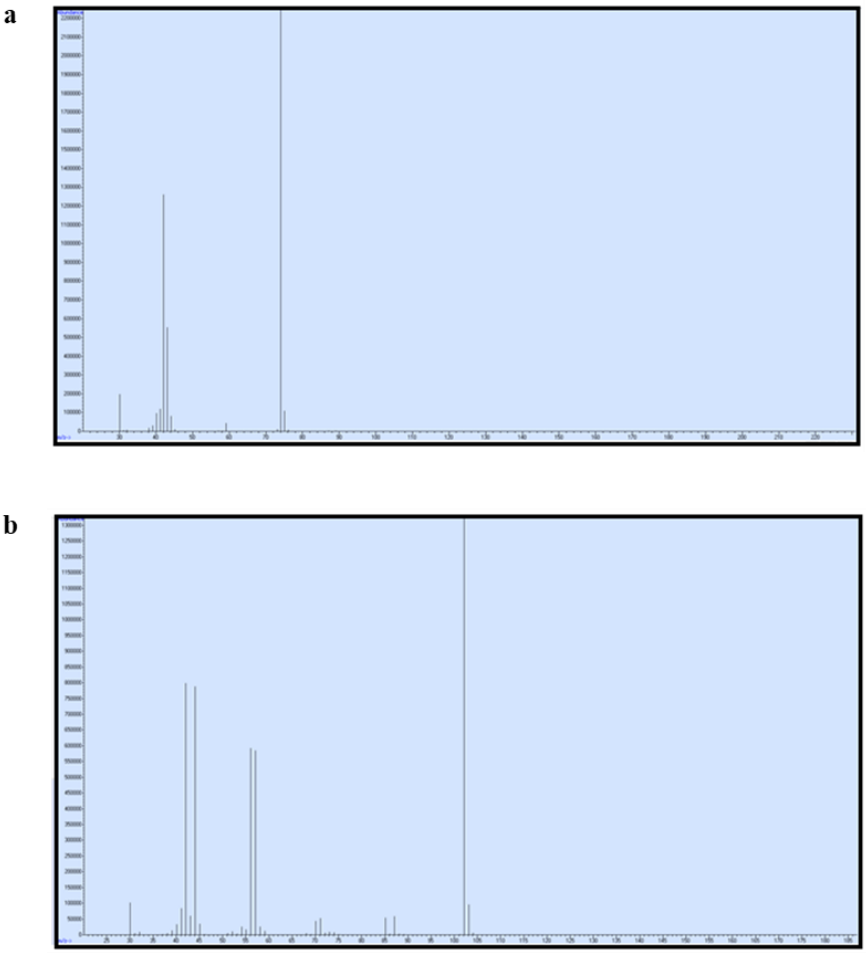

Figure 2. Mass spectrums of **(a)** NDMA and **(b)** NDEA

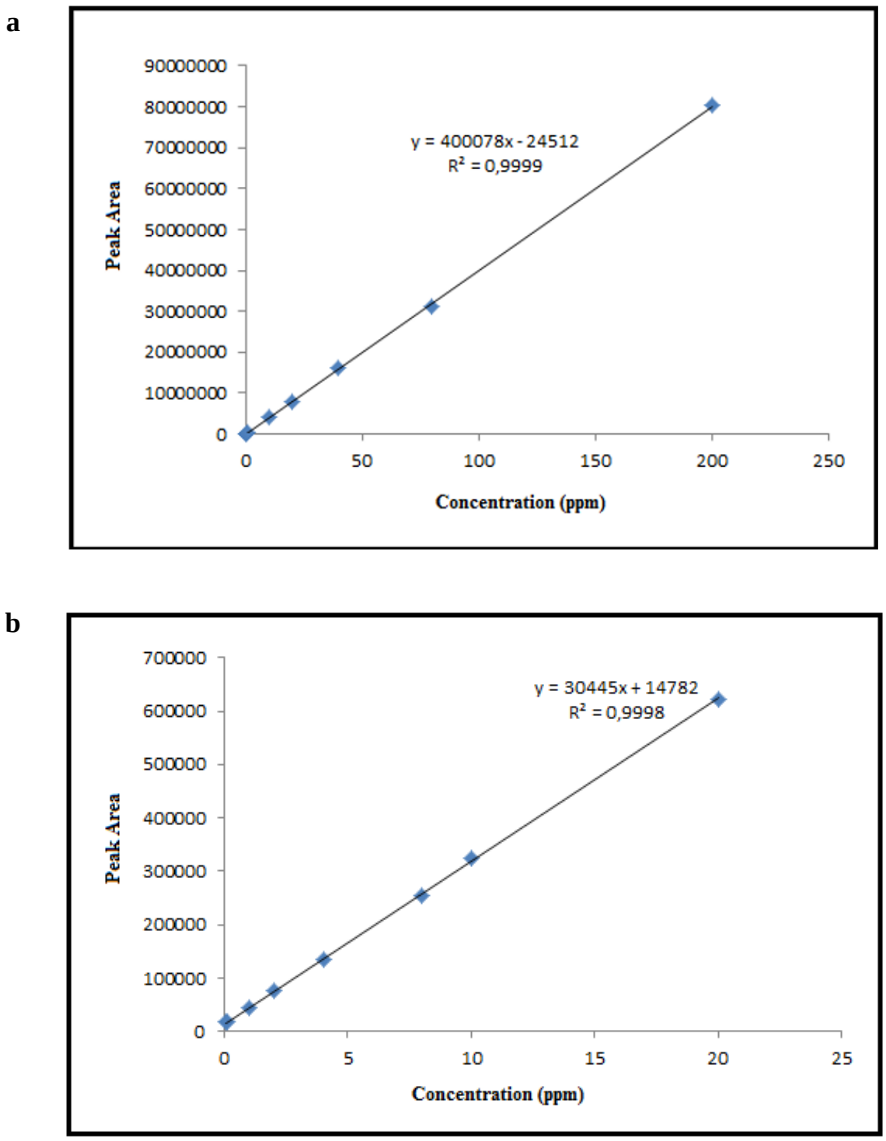

Figure 3. Calibration curves of **(a)** NDMA and **(b)** NDEA

RESULT AND DISCUSSION

The amounts of *N*-nitrosodimethylamine and N-nitrosodiethylamine detected in drug samples are shown in Table 3. Both NDMA and NDEA were detected in all of the drugs examined. The nitrosamine chromatograms of some drug samples were given in Figure 4a and 4b. The average NDMA concentrations were found ranging from 3.43 to 34.14 mg kg^{-1}, while NDEA levels were in the range of 1.09 to 6.78 mg kg^{-1} in drugs containing valsartan. The average NDMA concentrations determined in drugs containing losartan were between 18.41 and 67.05 mg kg^{-1}, and NDEA concentrations are between 2.96 and 16.05 mg kg^{-1}. The average concentrations of NDMA and NDEA in drugs containing irbesartan were in the range of 4.73 - 27.40 mg kg^{-1} and 1.94 - 5.88 mg kg^{-1}, respectively. A single drug with telmisartan active substance was examined, and the mean NDMA concentration was 1.71 mg kg^{-1} and the mean NDEA concentration was 0.62 mg kg^{-1}. It was observed that the mean NDMA concentrations ranged between 1.80 and 7.47 mg kg^{-1}, and the mean NDEA concentrations ranged between 1.53 and 20.13 mg kg^{-1} in drugs with olmesartan active ingredient. In drugs with candesartan active substance, mean NDMA contents were found between 11.10 and 31.73 mg kg^{-1}, and mean NDEA contents were between 10.46 and 22.48 mg kg^{-1}. While the highest NDMA concentrations were found in drugs with

losartan active ingredient, the highest NDEA concentrations were found in drugs with candesartan and olmesartan active ingredient.

Table 3. Amounts of NDMA and NDEA detected in drug samples (N=3)

Active Ingredient of the Drug	NDMA (mg kg^{-1})	NDEA (mg kg^{-1})
Valsartan	18.28 ± 0.06	6.27 ± 0.02
Valsartan	3.43 ± 0.01	6.78 ± 0.02
Valsartan	27.83 ± 0.09	1.09 ± 0.01
Valsartan	34.14 ± 0.11	1.39 ± 0.01
Valsartan	8.00 ± 0.02	5.61 ± 0.02
Valsartan	6.40 ± 0.02	1.18 ± 0.01
Losartan	18.41 ± 0.06	11.13 ± 0.03
Losartan	53.49 ± 0.13	13.06 ± 0.03
Losartan	29.56 ± 0.10	2.96 ± 0.01
Losartan	67.05 ± 0.15	16.05 ± 0.05
Irbesartan	4.73 ± 0.01	1.94 ± 0.01
Irbesartan	24.25 ± 0.08	3.62 ± 0.01
Irbesartan	27.40 ± 0.09	5.88 ± 0.02
Telmisartan	1.71 ± 0.01	0.62 ± 0.01
Olmesartan	6.64 ± 0.02	20.13 ± 0.06
Olmesartan	1.80 ± 0.01	4.20 ± 0.01
Olmesartan	7.47 ± 0.02	1.53 ± 0.01
Kandesartan	16.14 ± 0.05	22.48 ± 0.06
Kandesartan	11.10 ± 0.03	13.21 ± 0.03
Kandesartan	31.73 ± 0.10	10.46 ± 0.03

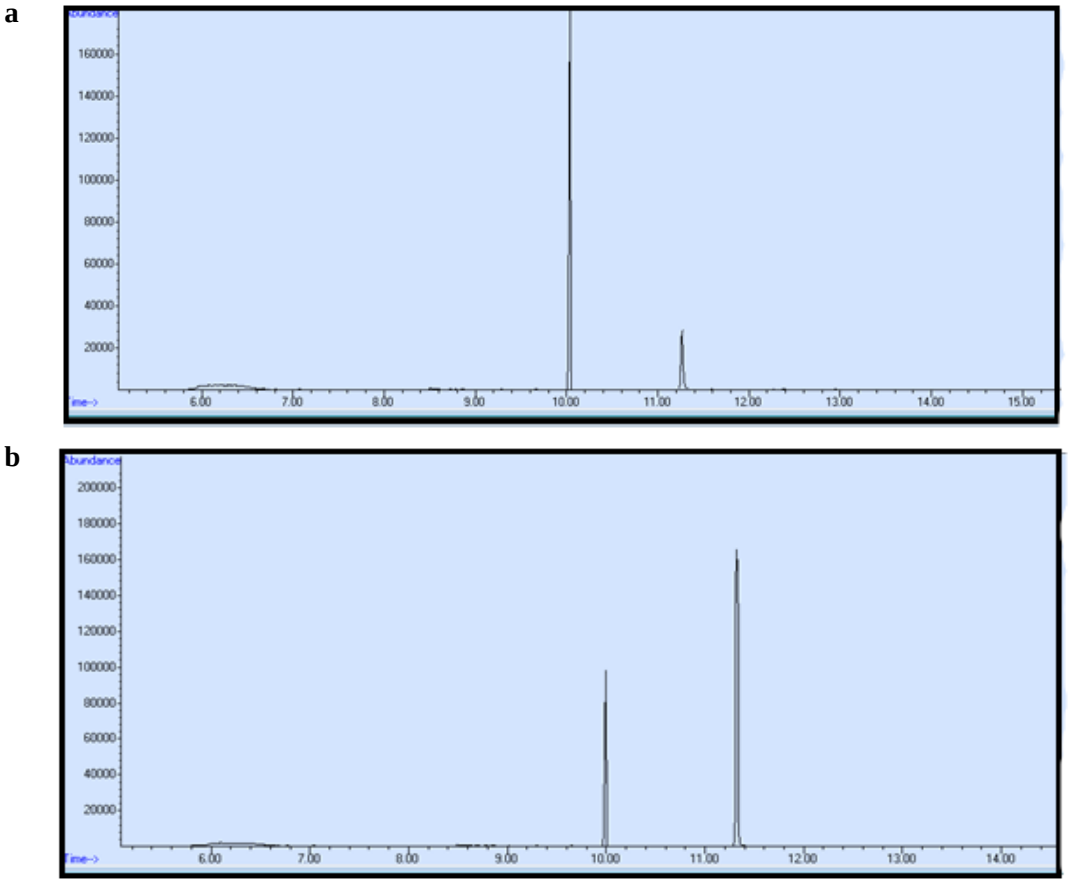

Figure 4. The nitrosamine chromatograms of drugs containing **(a)** valsartan and **(b)** olmesartan

The NDMA and NDEA amounts in each tablet of the examined drug samples are given in Table 4. The amounts of NDMA in each tablet of drugs with valsartan active ingredient were between $1.1x10^{-3}$ and $1.4x10^{-2}$ mg, the amounts of NDEA were between $4.4x10^{-4}$ and $2.9x10^{-3}$ mg, the amounts of NDMA in each tablet of drugs with losartan active ingredient were between $6.9x10^{-3}$ and $1.7x10^{-2}$ mg, NDEA amounts were between $6.9x10^{-4}$ and $5.1x10^{-3}$ mg, the amounts of NDMA in each tablet of drugs with irbesartan active ingredient were between $2.5x10^{-3}$ and $8.5x10^{-3}$ mg, NDEA amounts were between $9.6x10^{-4}$ and $1.8x10^{-3}$ mg, the amounts of NDMA in each tablet of drugs with olmesartan active ingredient were between $3.6x10^{-4}$ and $2.9x10^{-3}$ mg, NDEA amounts were between $3.3x10^{-4}$ and $8.8x10^{-3}$ mg, the amounts of NDMA in each tablet of drugs with candesartan active ingredient were between $1.4x10^{-3}$ and $4.2x10^{-3}$ mg, NDEA amounts were between $1.4x10^{-3}$ and $2.9x10^{-3}$ mg. The amount of NDMA in each tablet of the drug with the active ingredient of telmisartan was determined as $1.2x10^{-3}$ and the amount of NDEA was determined as $4.3x10^{-4}$. The acceptable daily intake limits specified by the European Medicines Agency and the Food and Drug Administration are 96 ng/day for NDMA and 26.5 ng/day for NDEA. When these values are expressed in milligrams, they will be $9.6x10^{-5}$ mg for NDMA and $2.65x10^{-5}$ mg for NDEA. When Table 4 is examined, it is seen that the values obtained are above the acceptable intake limits even if one tablet was taken per day.

Table 4. Amounts of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in each tablet of the drug samples examined

Active Ingredient of the Tablet	Amount of Active Ingredient in the Tablet (mg)	NDMA (mg/tablet)	NDEA (mg/tablet)
Valsartan	160	$6.5x10^{-3}$	$2.2x10^{-3}$
Valsartan	160	$1.1x10^{-3}$	$2.2x10^{-3}$
Valsartan	160	$1.4x10^{-2}$	$5.3x10^{-4}$
Valsartan	160	$1.1x10^{-2}$	$4.4x10^{-4}$
Valsartan	160	$4.2x10^{-3}$	$2.9x10^{-3}$
Valsartan	160	$3.2x10^{-3}$	$5.8x10^{-4}$
Losartan	100	$8.5x10^{-3}$	$5.1x10^{-3}$
Losartan	100	$1.7x10^{-2}$	$4.1x10^{-3}$
Losartan	50	$6.9x10^{-3}$	$6.9x10^{-4}$
Losartan	50	$1.2x10^{-2}$	$2.9x10^{-3}$
Irbesartan	300	$2.5x10^{-3}$	$1.0x10^{-3}$
Irbesartan	150	$6.4x10^{-3}$	$9.6x10^{-4}$
Irbesartan	150	$8.5x10^{-3}$	$1.8x10^{-3}$
Telmisartan	80	$1.2x10^{-3}$	$4.3x10^{-4}$
Olmesartan	40	$2.9x10^{-3}$	$8.8x10^{-3}$
Olmesartan	40	$3.6x10^{-4}$	$8.5x10^{-4}$
Olmesartan	20	$1.6x10^{-3}$	$3.3x10^{-4}$
Kandesartan	16	$2.1x10^{-3}$	$2.9x10^{-3}$
Kandesartan	8	$1.4x10^{-3}$	$1.7x10^{-3}$
Kandesartan	8	$4.2x10^{-3}$	$1.4x10^{-3}$

Table 5 was created in order to compare the amounts of nitrosamines detected in the examined samples with the values in the literature. When Table 5 is examined, it can be seen that the values we obtained for drugs containing the active ingredient valsartan are close to the values in the literature. However, it seems that the values we obtained for drugs containing the active ingredients losartan and irbesartan are higher than the studies in the literature. Since there is no information in the literature about the NDMA and NDEA contents of drugs containing the active ingredients telmisartan, olmesartan and candesartan, no comparison could be made. As mentioned above, there are many factors that contribute to the formation of nitrosamines in drugs. Contaminated starting materials and intermediates, reagents, solvents, catalysts, errors made in the production process, recovered and recycled materials, and manufacturers without sufficient process knowledge are among these. In addition, nitrosamines may

form after the drugs are produced, depending on storage conditions, packaging materials and degradation processes of active substances. Taking all these factors into consideration, in many countries, necessary precautions have begun to be taken and strict controls have begun to be carried out during and after the production stages. It is thought that the lower amounts of nitrosamines detected in studies in the literature compared to our study are due to the reasons mentioned above.

Table 5. Comparison of the amounts of nitrosamines detected in the analyzed samples with the values in the literature

Active Ingredient of the Drug	NDMA (mg kg^{-1})	NDEA (mg kg^{-1})	Reference
Valsartan	3.43 – 34.14	1.09 – 6.78	This study
Valsartan	0.004	----	[11]
Valsartan	0.10 – 137.60	0.07 – 6.90	[12]
Valsartan	0.06 – 99.79	0.11 – 8.84	[22]
Valsartan	17.57	----	[16]
Valsartan	0.01518 – 59.3	----	[18]
Losartan	18.41 – 67.05	2.96 – 16.05	This study
Losartan	----	0.08 – 0.23	[12]
Losartan	----	0.07 – 0.20	[22]
Irbesartan	4.73 – 27.40	1.94 – 5.88	This study
Irbesartan	----	0.11 – 0.12	[12]
Irbesartan	----	0.10 – 0.14	[22]

Today, it is known that nitrosamines are mutagenic, carcinogenic and teratogenic. While nitrosamines, which are extremely harmful to human health, were previously found in water, processed foods and cosmetic products, they began to be found in medicines after 2018 [21]. Since these drugs are used in the treatment of high blood pressure and type 2 diabetes, they are used by many people over a certain age. Therefore, it is of great importance to take the necessary precautions to prevent the formation of nitrosamine impurities.

Within the scope of this study, 20 different drugs containing sartan and its derivatives were examined, and both NDMA and NDEA were detected in all of the tablets examined. The FDA's recommended method was used to determine NDMA and NDEA levels, and as a result, it was seen that the amounts of both impurities determined were above acceptable limit values. Thus, the nitrosamine contents of some drugs containing sartan and its derivatives used in Turkey have been revealed. It is of great importance as it is the first study conducted on this subject in our country. Considering that the presence of nitrosamines has been found not only in sartan group drugs but also in many drugs with different active ingredients in various countries, it is obvious that similar studies should be carried out. In this context, it is thought that the study carried out will shed light on the conduct of similar studies and set an example.

ACKNOWLEDGMENTS

This research (22.YL.014) was supported by the Scientific Research Projects Coordination Unit of Hatay Mustafa Kemal University. The authors would like to thank the Scientific Research Projects Coordination Unit of Hatay Mustafa Kemal University for financial support.

AUTHOR CONTRIBUTIONS

Concept: Ş.S., M.A., Z.A.; Design: Ş.S.; Control: Ş.S.; Sources: Ş.S., M.A., Z.A.; Materials: Ş.S., M.A.; Data Collection and/or Processing: Ş.S., M.A., Z.A.; Analysis and/or Interpretation: Ş.S., M.A.; Literature Review: Ş.S., M.A.; Manuscript Writing: Ş.S.; Critical Review: Ş.S., M.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1128-1134, 2024
Doi: 10.33483/jfpau.1514714

ÖZGÜN MAKALE / ORIGINAL ARTICLE

PİROKSİKAM DİLALTI POLİMERİK FİLMLERİNİN HAZIRLANMASI VE *İN VİVO* DEĞERLENDİRİLMESİ

PREPARATION AND IN VIVO EVALUATION OF PIROXICAM SUBGLINGUAL POLYMERIC FILMS

Okan Ali AKSOY[1]*, Merve Zanbak ÇOTAOĞLU[2], Gizem Rüya TOPAL[3], Berk Alp GÖKSEL[1], Özgür EŞİM[2], Cansel KÖSE ÖZKAN[2], Ayhan SAVAŞER[2], Yalçın ÖZKAN[2]

[1]Sağlık Bilimleri Üniversitesi, Gülhane Sağlık Bilimleri Enstitüsü, 06010, Ankara, Türkiye
[2]Sağlık Bilimleri Üniversitesi, Gülhane Eczacılık Fakültesi, Farmasötik Teknoloji ABD, 06010, Ankara, Türkiye
[3]Sağlık Bilimleri Üniversitesi, Gülhane Eczacılık Fakültesi, Farmasötik Biyoteknoloji ABD, 06010, Ankara, Türkiye

ÖZ

Amaç: *Farklı ilaç uygulamalarının etken maddelerin etkinliğini arttırabildiği bilinmesine rağmen hangi ilaç salım sisteminin daha iyi olduğu konusunda birçok çalışma bulunmaktadır. Bunun nedeninin etken maddenin özelliklerine göre bu uygulamaların daha büyük avantaj sağlamasıdır. Bu çalışmada piroksikam dilaltı polimerik film uygulamasının potansiyel kullanımı araştırılmıştır.*
Gereç ve Yöntem: *Çalışmada piroksikam dilaltı maltodekstrin ve pullulan içeren filmler hazırlanmış ve sağlıklı tavşan in vivo modelinde ilacın plazma düzeyleri araştırılmıştır.*
Sonuç ve Tartışma: *İn vivo sonuçlar, tek doz uygulamadan sonra 10 saat içinde piroksikamın en yüksek plazma konsantrasyonlarına ulaştığını göstermiştir. Ayrıca plazma ilaç profilinde olası gastrointestinal emilime karşılık gelen ikinci bir tepe noktası oluşmamıştır. Özetle, piroksikam yüklü dilaltı polimerik film uygulamasının analjezik etkinin sağlanması amacıyla kullanılabilir özellikte olduğu tespit edilmiştir.*
Anahtar Kelimeler: *Farmakokinetik, film, maltodekstrin, piroksikam, pullulan*

ABSTRACT

Objective: *Although it is known that different drug applications can increase the effect of drugs, there are many studies that shows the superiority of different dosage forms. The reason of these different results is due to the different properties of used active ingredients. In this study, the potential use of piroxicam sublingual polymeric film application was investigated.*
Material and Method: *In the study, maltodextrin and pullulan sublingual films containing piroxicam were prepared, and plasma levels of the drug were investigated in a healthy rabbit in vivo model.*
Result and Discussion: *In vivo results showed that piroxicam reached its highest plasma concentrations within 10 hours after a single dose administration. Additionally, a second peak corresponding to possible gastrointestinal absorption did not occur in the plasma drug profile. In summary, it has been determined that piroxicam-loaded sublingual polymeric film application can be used to provide an analgesic effect.*
Keywords: *Film, maltodextrin, pharmacokinetic, piroxicam, pullulan*

* **Sorumlu Yazar / Corresponding Author:** Okan Ali Aksoy
e-posta / e-mail: okanali.aksoy@sbu.edu.tr, **Tel. / Phone:** +903123045118

Gönderilme / Submitted : 12.07.2024
Kabul / Accepted : 09.08.2024
Yayınlanma / Published : 10.09.2024

GİRİŞ

İlaçların ağız yoluyla uygulanması uzun zamandır tercih edilen yöntem olmuştur. Ancak gastrointestinal sistemdeki pH dalgalanmaları, aktif farmasötik bileşenin enzimatik bozunma riski ve hepatik ilk geçiş metabolizması etken maddelerin etkinliği açısından önemli zorluklar teşkil etmekte ve bu yolu ilaçlar için önemli bir zorluk haline getirmektedir. Sonuç olarak, bu engelleri aşan alternatif ilaç uygulama yolları gün geçtikçe daha popüler hale gelmektedir [1].

Oromukozal ilaç uygulamaları, bu istenmeyen yan etkilerden arınma için cazip bir yol sunar [2]. Bu yol, lubrikasyon görevi gören ancak aynı zamanda etken maddelerin çözünürlüğünü ve permeabilitesini etkileme potansiyeline sahip olan tükürük gibi farklı koruyucu bariyerlere sahiptir [1]. Ayrıca, çözünürlüğü ve/veya geçirgenliği düşük olan ilaçların, oral mukoza difüzyonu engellenmektedir [3].

Piroksikam (PRX), ankilozan spondilit, osteoartrit, romatoid artrit, dismenore gibi akut ve kronik kas-iskelet sistemi ve eklem sorunlarını ve bu durumlarla ilişkili rahatsızlıkları tedavi etmek için yaygın olarak kullanılan, non-steroid yapılı bir anti-inflamatuar ilaçtır. Oral uygulama sonucu istenmeyen gastrointestinal yan etkiler gösteren etken maddeler için oral mukozadan ilaç emilimi sağlayacak yeni formülasyonların geliştirilmesine büyük ilgi vardır. Ayrıca PRX'ın suda çözünürlüğü sınırlı olduğundan, yavaş bir emilim profiline sahiptir [4] ve mukoadezif ilaç şekilleri geniş yüzey alanına sahip formülasyonları ile düşük çözünürlüğe sahip etken maddelerin biyoyararlanımını artırmaya uygun bir teknolojik stratejidir [5].

Hızlı çözünen dilaltı polimerik filmler kullanılarak, ilacın hızlı emilimi sağlanabilir ve bu da sonuçta ilacın etkisinin hızlı bir şekilde başlamasına yol açar. Yeni oral ilaç taşıyıcı sistemlerden olan dilaltı polimerik filmler, ağıza yerleştirildikten sonra birkaç saniye içinde hızla çözünür veya dağılır. Dozaj formu anında veya birkaç saniye içinde parçalanarak tükürükte çözünen veya dağılan ilaçları serbest bırakır. Dil altı mukozası ince membran ve geniş damarlar nedeniyle nispeten geçirgendir. Yüksek kan akışı nedeniyle ilaçların hızlı emilimini ve anında biyoyararlanımını sağlar [6].

Bu çalışmada, PRX dil altı polimerik filmlerinin hazırlanarak etkisinin arttırılması hedeflenmiştir. Dilaltı polimerik filmler polisakkarit yapıları kullanılarak formüle edilmiş ve fizikokimyasal özellikleri karakterize edilmiştir. Hazırlanan polimerik filmler tavşanlara uygulanmış ve farmakokinetik davranışları belirlenmiştir.

GEREÇ VE YÖNTEM

PRX Dilaltı Filmlerinin Hazırlanması

Dilaltı filmleri oluşturmak için solvent dökme yöntemi kullanılmıştır [7]. Ön çalışmalara göre, film oluşturucu polimerler olarak pullulan ve maltodekstrin, plastikleştirici olarak propilen glikol, PEG 400 ve gliserol kullanılmıştır. Polimerik çözelti hazırlamak için %1.5 pullulan ve %2 maltodekstrin, 10 ml su içerisinde çözülmüş ve ardından %5 plastikleştirici ile birleştirilmiştir. Plastikleştirici içerisine manyetik karıştırma altında 100 mg PRX ilave edilmiş ve polimerik çözelti ile manyetik karıştırıcıda (400 rpm) (MS-H280-Pro, DLAB, Çin) karıştırılmıştır. İnce bir film oluşturmak için karışım önceden temizlenmiş bir cam plaka üzerine dökülmüş ve 60°C'de 4 saat etüvde (Heraeus, Thermo, Almanya) kurutulmuştur. Filmler cam plakalardan dikkatlice çıkarılmış ve 1 cm x 1 cm boyutlarında kesilerek kullanılmıştır.

İn Vitro Piroksikam Salım Çalışmaları

Dilaltı polimerik filmlerden PRX salımı, USP palet yöntemi (Sotax, İsviçre) kullanılarak belirlenmiştir. Palet dönüşü 50 rpm'ye ayarlanmıştır. Sıcaklık 37 ± 0.5°C'de tutulmuştur. 5 mg PRX içeren polimerik filmlerin salım özellikleri, 500 ml distile su kullanılarak incelenmiştir. 0, 10, 20, 30, 60, 90, 120, 180, 240, 360, 480, 960, 1200 ve 1440 dakikalarda ortamdan 1 ml'lik çözünme numuneleri toplanmış ve yerine aynı hacimde ortam sıvısı eklenmiştir. Salınan ilacın miktarı, bir DAD dedektörü (Agilent 1100, Agilent Technologies, ABD) kullanılarak 360 nm'de HPLC ile belirlenmiştir [4].

Farmakokinetik Çalışmalar

Çalışmalar Sağlık Bilimleri Üniversitesi Etik Kurulu tarafından onaylanmıştır (Onay No: 2021-23). Dilaltı polimerik filmler, erkek Yeni Zelanda tavşanlarına (n= 3) (vücut ağırlığı 2000 ± 200 g) 20 mg'lık tek bir dozda uygulanmıştır. Tedavi sonrasında t=0, 0.5, 1, 1,5, 2, 3, 4, 6, 10, 24, 36 ve 48. saatlerde seri kan örnekleri alınarak önceden heparinize edilmiş santrifüj tüplerine aktarılmıştır. Numuneler 4000 g'de 10 dakika boyunca santrifüjleme yoluyla ekstrakte edilmiştir. Numunelerin analizi için bir HPLC sistemi kullanılmıştır. Plazma örneklerinde PRX konsantrasyonunu belirlemek için kullanılan HPLC parametreleri şunlardır: C18 kolonu (250 mm x 3 mm id, 5 µm), 0.8 ml/dak akış hızı, dalga boyu: 360 nm, 20 µl enjeksiyon hacmi, mobil faz: 0,04 M KH_2PO_4: asetonitril. metanol (50:40:10 h/h/v) (pH 3.8). İlaç konsantrasyonu-zaman eğrisinin (AUC) altındaki alanı belirlemek için trapez yöntemi kullanılmıştır.

İstatistiksel Analiz

Tüm deneysel sonuçlar, en az üç ölçümün ortalama değeri ± standart sapması olarak gösterilmiştir.

SONUÇ VE TARTIŞMA

Polimerik Filmlerin Hazırlanması

Ön formülasyon çalışmalarında; toksisite göstermemesi dikkate alınarak distile su kullanılmıştır. Öncelikle pullulan (%1.5) ve maltodekstrin (%2) su içerisinde çözülmüştür. Çözeltiden 4.5 ml alınarak üzerine 0.5 ml plastikleştirici eklenmiştir. Plastikleştiricinin etkisinin incelenmesi ve uygulama için en uygun plastikleştiricinin seçilmesi amacıyla 3 farklı polimer (propilen glikol, gliserin ve PEG 400) incelenmiştir (Tablo 1). Maltodekstrin-pullulan çözeltisi üzerine 20 mg PRX içeren 0.5 ml plastikleştirici eklenmiş ve oluşan süspansiyon kalıplara dökülerek hava kabarcıklarının çıkması için kalıplar 12 saat oda sıcaklığında bekletilmiştir. Hazırlanan formülasyon daha sonra etüvde 60°C'de kurumaya bırakılmıştır. Kurutma numuneleri her saatte uygun filmin oluşması açısından değerlendirilmiş ve optimum kuruma süresi 4 saat olarak belirlenmiştir (Şekil 1).

Tablo 1. Ön formülasyon çalışmalarında kullanılan formüller

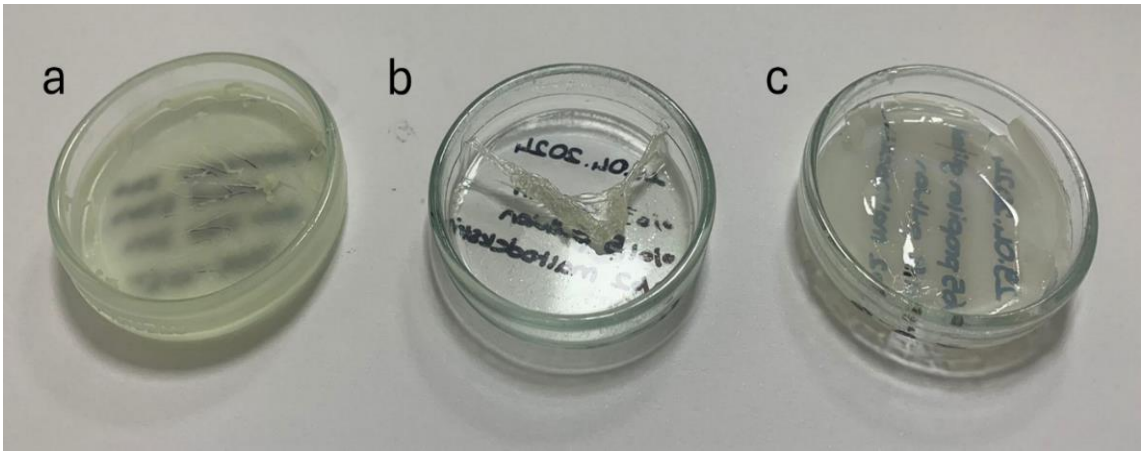
			Plastikleştirici		
PRX	**Pullulan**	**Maltodekstrin**	**Propilen Glikol**	**Gliserin**	**PEG 400**
20 mg	% 1.5	% 2	% 5		
20 mg	% 1.5	% 2		% 5	
20 mg	% 1.5	% 2			% 5

Hazırlanan formülasyonlar incelendiğinde PEG 400 içeren filmlerin uygulama için uygun olmadığı tespit edilmiştir. Söz konusu formülasyona ait elde edilen filmler, dilaltı uygulama için kabul edilemeyecek kadar opak ve kırılgan bulunmuştur (Şekil 1a) Ayrıca elde edilen filmler incelendiğinde sarı-yeşil renk gözlenmiş ve bunun nedeninin PRX'ın polimorfik dönüşüme uğraması olduğu belirtilmiştir [8]. Amorf PRX'in sarı rengi, moleküller arası proton transferi ile oluşan zwitteriyonik PRX moleküllerine atfedilir. Polar çözücüler kullanılarak hazırlanan PRX çözeltileri de sarıdır ve zwitteriyonik PRX molekülleri içerir. Zitteriyonik PRX molekülü, anyonik enolat ve katyonik piridinyum fonksiyonel gruplarından kaynaklanan çeşitli rezonans formlarına sahiptir. Çeşitli kristalli bileşiklerin ve PRX çözeltilerinin sarı rengi, zwitteriyonik PRX moleküllerinin uzun süreli konjugasyonuna atfedilir [9].

Gliserinin plastikleştirici olarak kullanıldığı filmler şeffaf fakat yapışkan bir yapı oluşturduğu tespit edilmiştir. Hassas dozlama gerektiren formülasyonlarda elde edilen yapışkanlık uygun büyüklükte kullanılabilir yapılar elde edilmesine engel olmaktadır. Bu halile elde edilen gliserin filmler de kullanım için uygun bulunmamıştır (Şekil 1b). Propilen glikol içeren filmlerin ise uygun adezif özellikte ve

uygulanabilir yapıda olduğu tespit edilmiştir. Sonuç olarak plastikleştirici olarak propilen glikol seçilmiştir (Şekil 1c).

Metilen mavisi ile yapılan çalışmada, plastikleştirici olarak PEG 400 kullanılan filmler, bukkal uygulama için kabul edilemeyecek kadar opak ve kırılgan bulunmuştur. Çalışmamızda olduğu gibi gliserol plastikleştirici olarak kullanıldığında şeffaf fakat yapışkan bir film oluştuğu ve propilen glikolün plastikleştirici olarak kullanılması ile filmlerin yeterli mekanik özelliklere sahip olduğu belirtilmiştir [10].



Şekil 1. a) PEG 400, (b) gliserol ve (c) propilen glikol ile hazırlanan PRX dilaltı filmlerine ait görseller

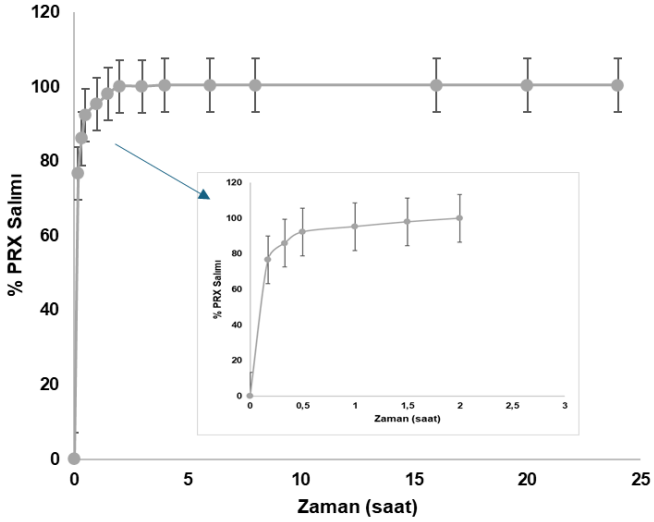
İn Vitro Piroksikam Salım Çalışmaları

PRX gibi sınıf II ilaçlar için (zayıf çözünürlük, yüksek geçirgenlik) çözünme hızı sıklıkla oral absorpsiyon hızını kontrol eder [11]. PRX dissolüsyon testi USP'ye göre distile su içerisinde gerçekleştirilmektedir [12]. PRX içeren ağızda dağılan tablet formülasyonları şu anda akut ağrı tedavisi için 20 mg dozunda piyasada kullanılmaktadır. Bu nedenle çözünme hızı çalışmalarında 20 mg PRX içeren ağızda dağılan film formülasyonları kullanılmıştır. Çözünme hızı sonuçları incelendiğinde PRX'ın önce hızlı bir şekilde %80'inin çözündüğü daha sonra ise 2 saat içerisinde etken maddenin tamamının çözündüğü tespit edilmiştir. Polimerik film hazırlanmasında plastikleştirici olarak propilen glikol kullanılmaktadır. Formülasyona eklenen propilen glikol aynı zamanda çözünürlük arttırıcı etki göstermektedir. Yüksek ve hızlı çözünürlüğün nedeninin eklenen plastikleştirici olduğu düşünülmektedir (Şekil 2) [13]. Noyes-Whitney çözünme modelinde öngörüldüğü gibi, PRX çözünme hızındaki iyileşmenin temel olarak film oluşum süreci nedeniyle artan yüzey/hacim oranından kaynaklandığı sonucuna varılabilir [5].

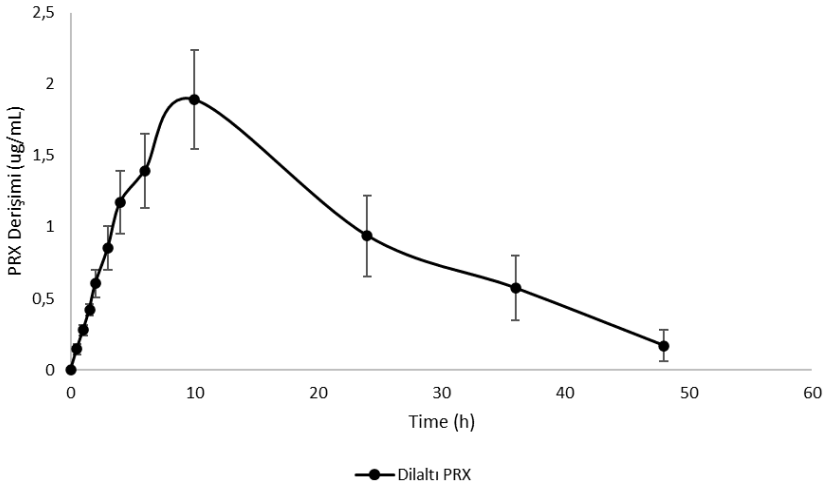
Farmakokinetik Çalışmalar

Tek doz uygulamadan sonra, dil altı PRX filminin plazma konsantrasyonları 30 dakika içinde elde edilmiştir (Şekil 3). Ayrıca dil altı filmin olası gastrointestinal emilimine karşılık gelen ikinci bir tepe noktası tespit edilmemiştir [14]. Bu profil emilimin yüksek oranda dilaltından gerçekleştiğini göstermektedir. Formüle edilen film, Yüksek biyoyararlanım göstermesi ve emilimin dilaltından olması nedeniyle sadece PRX uygulaması için değil dilaltı uygulamanın avantaj sağladığı diğer etken maddeler için de yüksek potansiyel göstermektedir.

Dil altı PRX filminin uygulanmasının ardından tespit edilen farmakokinetik parametreler Tablo 2'de verilmiştir. Emilimin tüm farmakokinetik parametreleri, yani Cmax, Tmax ve AUC, dil altı film formülasyonu olarak uygulandığında PRX'ın yüksek biyoyararlanımını gösterdiği tespit edilmiştir. Plazma ilaç seviyelerinin karşılık gelen kan seviyeleriyle aynı olduğu varsayılarak ilaçların toplam vücut klirensi (Cl) tahmin edilebilir. PRX uygulamalarından hesaplanan Cl 0.51±0.01 (mg)/(µg/ml)/saattir. Ayrıca dil altı PRX uygulaması sonrası literatüre göre yüksek bir seviye olan [AUC] 0-48 saat 36.74±0.59 µg/ml/saat olarak tespit edilmiştir [15].



Şekil 2. PRX dilaltı filmlerine ait çözünme hızı grafiği



Şekil 3. PRX dilaltı filmlerine ait farmakokinetik profil

Tablo 2. PRX dilaltı filmlerine ait farmakokinetik parametreler

Parametre	Birim	Dilaltı PRX
Plazma doruk süresi (Tmax)	h	10.00±0.00
Plazma doruk derişimi (Cmax)	µg/ml	1.52±0.14
Eğri Altında Kalan Alan 0-t zamanı (AUC 0-t)	µg/ml*h	36.74±0.59
Eğri Altında Kalan Alan 0-∞ zamanı (AUC 0-∞)	µg/ml*h	39.13±0.05
İlk an konsantrasyon-zaman eğrisinin altındaki toplam alan (AUMC 0-∞)	µg/ml*h^2	774.31±45.29
Ortalama yaşam süresi (MRT 0-∞)	H	19.78±1.13
Dağılım hacmi/ Gözlenen emilim oranı (Vz/F_obs)	(mg)/(µg/ml)	7.52±0.81
Klirens/ Gözlenen emilim oranı Cl/F_obs	(mg)/(µg/ml)/h	0.51±0.01

Sonuç olarak bu çalışmada, PRX dilaltı polimerik filmleri başarıyla hazırlanmıştır. Hazırlanan polimerik filmlerin yüksek çözünme hızı gösterdiği tespit edilmiştir. Ayrıca hazırlanan filmlerin

tavşanlarda farmakokinetik davranışları incelenmiş ve gastrointestinal emilimi gösteren ikincil bir tepe noktası oluşmadığı tespit edilmiştir. Özellikle gastrointestinal sistemde ilk geçiş etkisine uğramaları ve hızlı etki istenmesi nedeniyle non-steroidal anti inflamatuvar ilaçların dilaltı uygulanması umut verici bir yaklaşımdır. Çalışmamız sonucunda hazırlanan filmlerin PRX uygulaması amacıyla kullanılabilir olduğu tespit edilmiştir.

TEŞEKKÜR

Bu çalışma Sağlık Bilimleri Üniversitesi tarafından (BAP: 2022/060) desteklenmiştir.

YAZAR KATKILARI

Kavram: O.A.A., Y.Ö.; Tasarım: M.Z.Ç., Ö.E.; Denetim: G.R.T., A.S.; Kaynaklar: O.A.A., B.A.G.; Malzemeler: C.K.Ö., Y.Ö.; Veri Toplama ve/veya İşleme: C.K.Ö., B.A.G., G.R.T.; Analiz ve/veya Yorumlama: O.A.A., Ö.E.; Literatür Taraması: O.A.A., Ö.E., M.Z.Ç.; Makalenin Yazılması: O.A.A., A.S.; Kritik İnceleme: O.A.A., M.Z.Ç., Ö.E., G.R.T., B.A.G., C.K., A.S., Y.Ö.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Sağlık Bilimleri Üniversitesi Gülhane HADYEK tarafından 2021-23 sayılı etik kurul izni alınmıştır.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1135-1141, 2024
Doi: 10.33483/jfpau.1525060

ORIGINAL ARTICLE / ÖZGÜN MAKALE

TURKISH EXAMPLE OF PRIORITIZATION AND RESTRICTION DECISIONS IN MEDICINE ACCESS: EVALUATION BASED ON TWO INNOVATIVE DRUGS

İLAÇ ERİŞİMİNDE ÖNCELİKLENDİRME VE KISITLAMA KARARLARINA İLİŞKİN TÜRKİYE ÖRNEĞİ: İKİ YENİLİKÇİ İLACA DAYALI DEĞERLENDİRME

Elif Hilal VURAL[1,2]*, Ensar Korkut KILIÇ[3], Enver Kağan ATİKELER[4], Bülent GÜMÜŞEL[1,5]

[1]Lokman Hekim University, Health Economics and Health Technology Assessment Application and Research Center, 06510, Ankara, Türkiye
[2]Lokman Hekim University, Faculty of Medicine, Department of Medical Pharmacology, 06510, Ankara, Türkiye
[3]Abbott EPD Turkiye, Health Policy Department, 06460, Ankara, Türkiye
[4]Turkish Health Economics and Policy Association, 06530, Ankara, Türkiye
[5]Lokman Hekim University, Faculty of Pharmacy, Department of Pharmacology, 06510, Ankara, Türkiye

ABSTRACT

Objective: *Drug licensing, price, and reimbursement are essential for medical access. This study examines US, EU, and Turkish reimbursement for innovative medicines and evaluates Türkiye's recent licensing and reimbursement decisions.*
Material and Method: *Nivolumab, an anticancer medicine, and Evolocumab, a hyperlipidemia treatment, were studied. Web-based searches of FDA, EMA, and TİTCK offical websites revealed authorized indications and approval dates for chosen medications.*
Result and Discussion: *Nivolumab has been authorized for 11 indications by the FDA and 10 by the EMA, although it is only approved for 8 in Türkiye. Evolocumab has been authorized for three indications by the FDA, three by the EMA, and two in Türkiye. Nivolumab was approved in Türkiye an average of 24.0 months after the FDA and 20.4 months after the EMA. In Türkiye, the indications for this medicine were reimbursed 27.6 and 25.2 months later, respectively. The FDA and EMA authorized the indications for evolocumab in Türkiye 10 months and 13.2 months later, respectively. The FDA and EMA authorized evolocumab's single reimbursement indication in Türkiye after 72.0 and 74.4 months, respectively. Our investigation found that some patient groups were given priority by limiting pharmaceuticals with high budget expectations, and these prioritizing decisions were made to secure patients' access to therapy.*
Keywords: *Drug access, innovative drugs, licensing, reimbursement*

ÖZ

Amaç: *İlaca erişimde ilaç ruhsatlandırması, fiyatlandırması ve geri ödenmesi kritik öneme sahiptir. Bu çalışmadaki amaç; Amerika Birleşik Devletleri, Avrupa Birliği ve Türkiye arasında seçilmiş yenilikçi ürünlerin geri ödeme koşullarını karşılaştırmak ve Türkiye'nin son yıllardaki ruhsat ve geri ödeme kararlarını değerlendirmektir.*
Gereç ve Yöntem: *Bu çalışmada onkoloji ve hiperlipidemi tedavileri çalışma alanı olarak*

* **Corresponding Author / Sorumlu Yazar:** Elif Hilal Vural
e-mail / e-posta: elif.vural@lokmanhekim.edu.tr, **Phone / Tel.:** +905333639140

Submitted / Gönderilme : 30.07.2024
Accepted / Kabul : 11.08.2024
Published / Yayınlanma : 10.09.2024

belirlendi. Onkoloji alanında birden fazla endikasyonu olan Nivolumab ve hiperlipidemi alanında kullanılan Evolokumab çalışma ilaçları olarak seçildi. Seçilen ilaçların onaylı endikasyonları ve endikasyon onay tarihleri web tabanlı taranan FDA, EMA ve TİTCK resmi internet sitelerinden elde edildi

Sonuç ve Tartışma: *Nivolumabın FDA tarafından 11, EMA tarafından 10 endikasyonu onaylıyken, Türkiye'de 8 endikasyonu onaylıdır. Evolokumabın FDA tarafından 3, EMA tarafından 3 endikasyonu onaylıyken, Türkiye'de 2 endikasyonu onaylıdır. Nivolumabın, Türkiye'de ruhsat alması, ortalama FDA'den 24.0 ve EMA'dan 20.4 ay sonra gerçekleşmiştir. Bu ilacın endikasyonlarının Türkiye'de geri ödenmesi de sırasıyla 27.6 ve 25.2 ay sonra gerçekleşmiştir. Evolokumab'ın Türkiye'deki endikasyonları FDA ve EMA'dan sırasıyla 10 ay ve 13.2 ay sonra onaylanmıştır. Evolokumab'ın Türkiye'de geri ödenen tek endikasyonu, FDA ve EMA tarafından onaylandıktan sonra sırasıyla 72.0 ve 74.4 ay sonra ödemeye girmiştir.*

Çalışmamızda incelenen ilaçlar üzerinden bütçe beklentisi yüksek olan ilaçlara yapılan kısıtlamalarla belirli hasta gruplarına öncelik verilmiş olduğu ve bu önceliklendirme kararları ile hastaların tedaviye erişimi sağlanmaya çalışıldığı görülmektedir.

Anahtar Kelimeler: *Geri ödeme, ilaç erişimi, ruhsatlandırma, yenilikçi ilaçlar*

INTRODUCTION

Different interventions are made by public decision makers all over the world to effectively use limited resources in health economics. Restriction and prioritization decisions aim to distribute financial resources equally to target patient groups and ensure early access to medicine. However, over time, the scope of priority areas is being expanded with reimbursement agreements that include new evidence of effectiveness and safety. The demographic pyramid, disease incidence, and health economic findings influence the scope of each country's prioritization decision for the new molecule. Due to the continuous increase in health expenditures and the increasing cost of health services, policy makers all over the world have had to implement various mechanisms. To ensure access to innovative medicines, risk-sharing agreements are made with financial or performance-based models in different countries [1,2]. Countries might prioritize distinct therapeutic drug classes to provide effective access to medicines with limited resources. A study investigating the access to antineoplastic drugs in various European countries shows that access to antineoplastic drugs is prioritized through practices in reimbursement and pricing [3]. Another study evaluating ten country examples revealed that country authorities use budget impact, clinical effectiveness, disease burden, ethics, evidence and many other parameters for determining priorities of medicine access in the health technology assessments [4].

Drug licensing, pricing, and reimbursement are critical for access to medicine. Licensing and pricing activities of drugs in Türkiye are carried out by the Turkish Medicines and Medical Devices Agency (TITCK). The retail sales price of drugs is determined by the external reference pricing system [5]. Countries followed by external reference pricing systems are France, Spain, Italy, Portugal, and Greece. However, if any drug is imported from outside these countries, the price in the country where that drug is manufactured or imported can also be taken as a reference. The sales price to the cheapest warehouse in these monitored countries is taken in euros as the reference price. Then, this value is multiplied by the periodic Euro value, and the sales price to the warehouse is determined in Turkish Lira. Periodic euro value; Each year, it is determined as 60% of the average euro sales value of the previous year, based on the euro sales value announced by the Central Bank of the Republic of Türkiye. [6].

Reimbursement of medications is regulated by the Social Security Institution (SSI). Companies apply to SSI for drugs that receive licenses and prices. Taking into account the discount rates specified in the Health Practice Communiqué (SUT), public prices of drugs are determined and a reimbursement list, which is a positive list, is created [7]. Medicines that have been licensed and priced are added to the reimbursement list according to their characteristics, by the Institution or by the commissions whose secretariat is managed by the Institution (Medical and Economic Evaluation Commission, Drug Reimbursement Commission). Positive decisions taken by the commissions are published in the SSI Health Implementation Communiqué [8]. In order for agreements similar to risk-sharing agreements to be made in Türkiye, an alternative reimbursement commission (AGÖK) within the SSI was established

in 2016 and its legislation was published [9]. Medicines included in the reimbursement are made available to insurance patients with indications approved by the Ministry of Health and with the discount rates specified in Article 4.4.1 of the Health Practice Communiqué. For drugs whose use requires special regulation, the reimbursement criteria are explained in the article "Regulations regarding some special diseases and drugs". This article explains at what stage of treatment or under what conditions the drug can be accessed under insurance [8].

In Canada, a study that evaluated the reimbursement assessment times of some antineoplastic drugs demonstrated that delays caused losses in life years and QALY values [10]. Another study investigating the group of innovative antineoplastic drugs reimbursement processes in Australia and the United Kingdom stated that using risk sharing or manage entry agreements in the reimbursement processes could provide early and easy access for patients to these medicines [11]. Nivolumab, one of the innovative drugs evaluated in the above studies, is an antineoplastic medicine and a human immunoglobulin monoclonal antibody. Nivolumab binds to the programmed death-1 (PD-1) receptor and blocks the binding of PD-1 to PD-L1 and PD-L2 ligands, thereby exerting antitumor activity [12]. There are also innovative drugs used in the treatment of different diseases including a human IgG2 monoclonal antibody evolocumab. Evolocumab binds to circulating human proprotein convertase subtilisin kexin type 9 (PCSK9). It inhibits PCSK9 binding to the low-density lipoprotein receptor (LDLR) and reduces the LDL levels in the blood [13]. A study reported the high cost of evolocumab in Italy and stated that using the drug in high-risk subgroups could help reduce costs [14].

As the years progress, the number of drugs on the reimbursement list and the number of paid indications for drugs currently on the reimbursement list increase. In order to prevent the unforeseen budget burden that these increases will create, some limitations are imposed. The aim of this study is; To compare the reimbursement conditions of selected innovative products between the United States of America (USA), the European Union, and Türkiye and to evaluate Türkiye's reimbursement decisions in recent years.

MATERIAL AND METHOD

It is predicted by IQVIA that the oncology and immunology treatment fields will grow at a CAGR of 9-12% and 6-9% by 2026 [15]. Therefore, oncology and hyperlipidemic treatments were determined as the study area in this study. Both direct and indirect health costs of these two health problems continue to increase day by day. In recent years, the market shares and budget burdens of drugs produced by biotechnological methods have been increasing rapidly all over the world. Therefore, drugs with a non-biosimilar monoclonal antibody structure used in these indications have been examined. As a result of all these examinations, Nivolumab, which has multiple indications in the field of oncology [12, 16], and Evolocumab, used in the field of hyperlipidemia, were selected as study drugs [13,17].

Approved indications and indication approval dates of the selected drugs were obtained from the web-based scanned EMA, FDA, and TITCK official websites [16-23]. Reimburst indications and reimbursement dates in Türkiye; It was obtained from the Communiqué on Amendments to the Health Implementation Communiqué published in the Official Gazette and its annexes, as well as from the web-based scanned SSI official website [24].

RESULT AND DISCUSSION

There are 70 headings in the Health Practice Communiqué in the section on regulations regarding some special diseases and drug use. These topics include many topics such as antineoplastic drugs, enteral and parenteral nutrition products, and congenital metabolic diseases.

In the Health Practice Communiqué, regulations regarding Nivolumab are included under the title "4.2.14 - Principles of drug use in cancer treatment", while Evolocumab is included under the title "4.2.28 - Principles of use of lipid-lowering drugs".

While Nivolumab is reimbursed in 4 indications, Evolocumab is reimbursed in 1 indication. The approved indications of these drugs in the USA (FDA) and Europe (EMA) are shared in the Table 1 below.

Table 1. Number of indications for which Nivolomab and Evolocumab are licensed

	FDA	**EMA**	**TİTCK**
Nivolumab	11	10	8
Evolocumab	3	3	2

Nivolumab; After being approved by the FDA and EMA, it took an average of 24.0 and 20.4 months for it to be licensed in Türkiye, respectively. In addition, the reimbursement of the indications of this drug in Türkiye took place after 27.6 and 25.2 months, respectively (Table 2).

Evolocumab has two approved indications in Türkiye, and these indications were approved in Türkiye 10 months and 13.2 months after the FDA and EMA, respectively. The only reimbursed indication of evolocumab in Türkiye was 72 months and 74.4 months after approval by the FDA and EMA, respectively (Table 2).

Table 2. Registration dates of Nivolumab and Evolocumab indications in FDA, EMA, and Türkiye

		FDA	**EMA**	**Türkiye**	
	Indications			**Approval**	**Reimbursement**
Nivolumab	Malignant melanoma	12.2014	04.2015	04.2017	04.2018
	Non-small cell lung cancer	09.2015	09.2015	07.2018	02.2022
	Renal cell cancer	11.2015	02.2016	04.2017	04.2018
	Hodgkin Lymphoma	05.2016	10.2016	07.2018	04.2018
	Head and neck squamous cell carcinoma	11.2016	03.2017	07.2018	-
	Urothelial carcinoma	02.2017	04.2017	-	-
	Colorectal cancer	07.2017	05.2021	-	-
	Hepatocellular carcinoma	09.2017	-	-	-
	Esophageal cancer	06.2020	10.2020	02.2022	-
	Pleural malignant mesothelioma	10.2020	04.2021	12.2022	-
	Gastric cancer	04.2021	09.2021	-	-
Evolocumab	Hypercholesterolemia and mixed dyslipidemia	12.2017	05.2015	-	-
	Homozygous familial hypercholesterolemia	08.2015	05.2015	06.2016	08.2021
	Established atherosclerotic cardiovascular disease	08.2015	03.2018	06.2016	-

Program Budgeting and Marginal Analysis, Health Technology Assessments, and Multi-Criteria Decision Analysis methods are frequently used in high-income countries to determine priority decisions regarding resource allocation in health. The aim is to provide transparent and accurate justification of the decisions taken within the healthcare system [25].

In determining which services to prioritize in determining health technologies, disease burden, clinical impact, alternative treatments, budget impact, economic impact, and the evidence obtained stand out as important topics that determine the decisions of decision makers [26].

Pricing of medicines in Türkiye is carried out by TITCK in accordance with the Decision on Pricing of Human Medicinal Products and the Communiqué on Pricing of Medicinal Products for Human Use. In 2004, the external reference system was introduced in drug pricing and the price of the drug in France, Spain, Italy, Portugal, Greece and the country where the drug was manufactured or imported can be taken as reference [27]. The external reference system is also widely used in European countries [28]. A recent study found that in countries where the external reference system is implemented, there are larger annual decreases in the list prices of medicines compared to other countries. However, it has been observed that in these countries, the launch of drugs on the market is

significantly delayed after they are licensed. In that study, it was calculated that in countries that apply the external reference system in pricing, the rate of drugs being released to the market 9 months after receiving a license decreases by over 70% compared to countries that do not [29]. While 4 of the 8 indications of nivolumab approved in Türkiye are reimbursed, the earliest of these indications to be reimbursed was 7 months after approval, and the latest to be reimbursed was 41 months after receiving approval. Only 1 out of 3 indications of evolocumab is reimbursed, and the time it takes for that indication to be reimbursed after it is licensed is more than 5 years. Access to expensive drugs, especially oncology drugs, is limited in many countries. A study found that strict price controls and reimbursement mechanisms in different countries such as India and Poland caused obstacles in access to medicine. On the other hand, it is stated that in the USA and Brazil, high drug prices and limited negotiations for reimbursement make access to medicine difficult [30]. For this reason, the interest of payers and companies in risk-sharing agreements has been increasing in developed countries since the early 2000s [2]. The aim of risk-sharing agreements is to limit the budget impact and access to innovative medicines, especially for patients who are more likely to benefit. It is stated that the companies aim to supply their drugs to the market faster with these agreements [1].

In Türkiye, AGÖK was established within the scope of SGK in 2016 and started to operate. The legislation of this commission is updated in line with needs. In this study, the registration and reimbursement dates of the two drugs were compared and it was determined that both drugs were licensed and reimbursed later in our country compared to both the USA and Europe. In addition, at the time of review of this study, some of the indications approved in the USA and Europe have not yet been added to the licenses of drugs in our country. As mentioned above, previous studies have shown that strict price controls, especially the external reference system, delay access to medicine in many different country examples [29,30]. In addition, a recent study has shown that strict pricing policies in Türkiye are a barrier to access to both innovative and generic drugs used in cancer treatment [31]. On the other hand, in order to solve the problems of access to medicines, access is provided from abroad on a prescription basis, with the permission of TITCK, for medicines that are not licensed or not available on the market in our country. However, this pathway constitutes a small proportion of public pharmaceutical expenditures [32].

The continuous increase in health expenditures and the increasing cost of health services all over the world are seen as important problems. In addition, the aging population and the increase in life expectancy increase the burden on healthcare systems both today and in the future. Health expenditures have been increasing in our country, as in the rest of the world, in recent years.

In conclusion, it can be seen that certain patient groups were prioritized through restrictions on drugs with high budget expectations over the drugs examined in our study, and these prioritization decisions attempted to ensure patients' access to treatment. Access to drugs can be prioritized in areas where they are the most effective among alternative treatments, where their cost effectiveness is highest, or where the budget impact is manageable. On the other hand; In the examples examined, it is seen that, similar to other countries, strict price controls delay access to innovative medicines in our country. Further studies are needed to evaluate access to new treatments and focus on the potential benefit to patient access to medicines.

AUTHOR CONTRIBUTIONS

Concept: E.H.V., E.K.K., E.K.A., B.G; Design: E.H.V., E.K.K., E.K.A., B.G.; Control: E.H.V., B.G.; Sources: E.H.V., B.G.; Materials: - ; Data Collection and/or Processing: E.H.V., E.K.K.; Analysis and/or Interpretation: E.H.V., E.K.K., E.K.A.; Literature Review: E.H.V., E.K.K.; Manuscript Writing: E.H.V., E.K.K.; Critical Review: E.H.V., E.K.K., E.K.A., B.G.; Other:-

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1142-1154, 2024
Doi: 10.33483/jfpau.1465242

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION CORRELATION BETWEEN THE ANTIOXIDANT, ANTIMICROBIAL, ANTI-FUNGI ACTIVITIES AND CONTENT OF PHENOLIC COMPOUNDS OF RASPBERRY LEAF (*RUBUS IDAEUS* L.) EXTRACTS

AHUDUDU YAPRAĞI (RUBUS IDAEUS L.) SIVI EKSTRELERİNİN ANTİOKSİDAN, ANTİMİKROBİYAL, ANTİ-FUNGAL AKTİVİTELERİ İLE FENOLİK BİLEŞİKLERİNİN İÇERİĞİ ARASINDAKİ KORELASYONUN İNCELENMESİ

Olexander MASLOV[1]*, Mykola KOMISARENKO[2], Svitlana PONOMARENKO[3], Tetiana OSOLODCHENKO[3], Sergii KOLISNYK[1]

[1]National University of Pharmacy, Faculty of Pharmacy, Department of General Chemistry, 61001, Kharkiv, Ukraine
[2]National University of Pharmacy, Faculty of Pharmacy, Department of Pharmacognosy and Nutriciology, 61001, Kharkiv, Ukraine
[3]Mechnikov Institute of Microbiology and Immunology of the NAMS of Ukraine, Laboratory of Biochemistry and Biotechnology, 61001, Kharkiv, Ukraine

ABSTRACT

Objective: *The purpose of work was study the total content of some biologically active substances (BAS), determine antimicrobial, anti-fungi and antioxidant activities of obtained raspberry leaf extracts, and study a correlation analysis between the content of natural compounds and antimicrobial/antifungal and antioxidant activities.*
Material and Method: *The quantity of phenolic compounds, catechins, flavonoids and hydroxycinnamic acids was determined by spectrophotometric method of analysis, whereas organic acids by alkalimetric method; antioxidant activity of obtained extracts was evaluated by potentiometric method, antimicrobial and anti-fungi wad determined by method of "well".*
Result and Discussion: *Results demonstrates the highest amount of polyphenols, flavonoids, catechins and hydroxycinnamic acids were 1.85±0.02, 1.01±0.02, 0.33±0.01 and 0.24±0.005% in 60% ethanolic extract, respectively. The organic acids were dominated in aqueous extract (1.02±0.02%). The most potent antioxidant property possessed 60% extract of raspberry leaf. There is a high correlation between the content of polyphenols, catechins and antioxidant activity, in the case of inhibition of S. aureus, P. aeruginosa, B. subtillis depends on polyphenols, catechins and antioxidant activity, whereas fungi C. albicans significantly depends only on the content of hydroxycinnamic acids as well as E. coli is not depend on any BAS. These findings show the great potential in the development and creation of new medicines with antimicrobial, antioxidant and antifungal effects that are not inferior to, and even superior to, the effects of synthetic analogues.*
Keywords: *Antibacterial activity, anti-fungi, antioxidant activity, correlation analysis, polyphenols, raspberry leaf*

* **Corresponding Author / Sorumlu Yazar:** Olexander Maslov
e-mail / e-posta: alexmaslov392@gmail.com, **Phone / Tel.:** +380664257224

Submitted / Gönderilme : 02.05.2024
Accepted / Kabul : 12.08.2024
Published / Yayınlanma : 10.09.2024

ÖZ

Amaç: *Çalışmanın amacı, bazı biyolojik olarak aktif maddelerin (BAS) toplam içeriğini incelemek, elde edilen ahududu yaprağı ekstrelerinin antimikrobiyal, anti-fungal ve antioksidan aktivitelerini belirlemek ve doğal bileşiklerin içeriği ile antimikrobiyal/antifungal ve antioksidan aktivitleri arasındaki korelasyon analizini incelemektir.*

Gereç ve Yöntem: *Fenolik bileşikler, kateşinler, flavonoitler ve hidroksisinamik asitlerin miktarı spektrofotometrik analiz yöntemiyle, organik asitlerin miktarı ise alkalimetrik yöntemle belirlenmiştir. Elde edilen ekstrelerin antioksidan aktiviteleri potansiyometrik yöntemle değerlendirilmiş, antimikrobiyal ve anti-fungal aktivite ise "kuyu" yöntemiyle belirlenmiştir.*

Sonuç ve Tartışma: *Sonuçlar; en yüksek polifenol, flavonoit, kateşin ve hidroksisinnamik asit miktarının sırasıyla %1.85±0.02, 1.01±0.02, 0.33±0.01 ve %0.24±0.005 olarak %60 etanolik ekstrede olduğunu göstermiştir. Sulu ekstrede organik asitler baskın bulunmuştur (%1.02±0.02). En güçlü antioksidan özelliği %60'lık ahududu yaprağı ekstresi göstermiştir. Polifenol ve kateşin içeriği ile antioksidan etki arasında yüksek korelasyon tespit edilmiştir; S. aureus, P. aeruginosa, B. subtillis'in inhibisyonunun polifenoller ile kateşinlerin içeriğine ve ve antioksidan aktiviteye bağlı olduğu görülürken; C. albicans mantarlarına karşı etkinin yalnızca hidroksisinnamik asit içeriği ile önemli ölçüde ilişkili olduğu tespit edilmiştir. E. coli üzerine etkinin ise herhangi bir BAS'a bağlı olmadığı gözlenmiştir. Bu bulgular; sentetik analogların etkilerinden daha düşük olmayan ve hatta onlardan daha üstün etkinlik göstermesi dolayısıyla, antimikrobiyal, antioksidan ve anti-fungal etkilere sahip yeni ilaçların geliştirilmesi ve yaratılmasındaki büyük potansiyeli göstermektedir.*

Anahtar Kelimeler: *Ahududu yaprağı, antibakteriyel aktivite, anti-fungal, antioksidan aktivite, korelasyon analizi, polifenoller*

INTRODUCTION

Nowadays, the problem of bacterial infection is still relevant. According to recent statistical studies, it has been found that every year 13.7 million people per year die from bacterial infections in the world. The mortality rate for all ages was 99.6 deaths per 100.000 population. Of the pathogens studied, *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumonia, Escherichia coli,* and *Pseudomonas aeruginosa* accounted for 54.9% of the 7.7 million deaths, with *S aureus* being associated with more than 1.1 million deaths. *S. aureus* was the leading bacterial cause of death in 135 countries and was associated with the largest number of deaths among people over 15 years of age (940.000) [1]. In addition, this problem is compounded by the emergence of resistance in bacteria to widely used antibiotics, which makes treatment more complex, time-consuming and expensive [2]. In addition, an important threat to human populations is fungal infection. According to the latest statistics, every year 1.433.000 people suffer from systemic candidal infections, of which approximately 611 thousand people die annually [3]. Thus, the search for new antimicrobial natural compounds is perspective for today.

Scientific community has paid a high attention to research of pharmacological activity of natural compounds such as derivatives of flavon-3-ols and flavonols. According to literature sources natural compounds have certain advantages over synthetic compounds. Above all, natural are safe as they possess limited number of side effects, secondly, natural compounds are more effective, and thirdly, it is a cheap production of compounds [4,5].

In the world exist 700 species of raspberry (*Rubus idaeus* L.), most of them occurring on the territory of East and West Europe, Russia and the North America. The chemical composition of *R. idaeus* is represented by derivatives of flavon-3-ols (catechins), flavonols, ellagic and organic acids. The main constituent of leaf is ellagic acid and its derivatives [6]. There are exist of three forms: Free ellagic acid, ellagic acid glycosides, and ellagotannins. In folk medicine *R. idaeus* leaf applied tin the treatment of influenza, diabetes mellitus, respiratory and renal disorders [7].

There are a lot of scientific researches about determination a level antioxidant activity of *R. idaeus* leaf extracts [8-10]. However, there is no date about assessing antioxidant\antimicrobial\anti-fungi activities and its correlation with content of BAS by potentiometric method. So, the aim of the study was to determine the total content of polyphenols, flavonoids, hydroxycinnamic and organic acids, catechins, moreover study antimicrobial activities against *Staphylococcus aureus, Proteus vulgaris,*

Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa and fungi *Candida albicans*. In addition, it was aimed to study a correlation analysis between the content of BAS in *R. idaeus* leaf extracts and antimicrobial and antioxidant activities.

MATERIAL AND METHOD

Plant Material

Rubus idaeus leaf were the object of the study, which were collected in the places of its cultivation. The material was collected in 2021 during the fruiting period in the vicinity of the village of Ternova, Kharkiv region.

Equipment

The pH meter HANNA 2550 (Germany) with a combined platinum electrode EZDO 50 PO (Taiwan) was applied for potentiometric measurements. Quantitative analysis of biological active compounds was carried out on UV-spectrophotometer UV – 1000 (China) with matched 1 cm quarts cells. Weighing was carried out using digital analytical balance AN100 (AXIS, Poland) with d = 0.0001 g.

Extraction Procedure

A six samples of 10.0 g (exact mass) of *R. idaeus* leaf had the size of particles 1-2 mm. The extraction was conducted with distilled water, 20%, 40%, 60%, 96% ethanol at 80º C within 1 hour with a condenser, ratio raw material/solvent – 1/20. The extraction technique was completed twice to provide totally extract all BAS, then the filtrates were joint and evaporated by vacuum rotary to ratio of extract to raw material 1:2. The five extracts of 96, 60, 40, 20% ethanol and aqueous were obtained. The extraction was carried by a little correction [11]. The green tea (*Camellia sinensis* L.) extract was obtained by the mentioned above method with 60% ethanol.

Quantitative Analysis

The total content of phenolic compounds was measured by the Folin-Ciocaltau assay, the absorbance was measured at 760 nm [11].

The vanillin reagent assay was applied to find out the total catechins [12], the absorbance was measured at 505 nm.

The total flavonoids were determined using assay of complex formation with $AlCl_3$, the absorbance was measured at 415 nm [13].

The total hydroxycinnamic acids derivatives content was measured by assay of complex formation with $NaNO_2$-Na_2MoO_4, the absorbance was measured at 505 nm [14].

The total organic acids content was determined by acid-base titration with the fixation end-point by potentiometric method [14,16].

Antioxidant Activity Assay

Antioxidant activity of extract was evaluated by potentiometric method [17,18]. The standardized green tea leaf 60% extract was used as the reference drug.

Test Organisms

Strains of *Staphylococcus aureus ATCC 25923*, *Pseudomonas aeruginosa ATCC* 27853, *Escherichia coli ATCC 25922, Bacillus subtillis ATCC 6538, Proteus vulgaris NTCS 4636*, and *Candida albicans ATCC 885/653* were used in accordance with the recommendations for the assessment of antimicrobial activity of drugs**.**

Antimicrobial Activity Assay

The method of diffusion of the drug into agar carried out using the method of "wells" [19,20]. Gentamycin, and fluconazole were used as reference drugs for assessing antimicrobial activity.

Correlation Analysis

Pearson's (r) correlation coefficient was used to analyze the correlation between antioxidant activity (AOA) and the amount of phenolic, catechins-, flavonoid, hydroxycinnamic acids derivatives and organic acids. The correlation coefficient to takes a value in the range of -1 to +1. Correlation is very high if it is within the range from 0.90 to 1.00; from 0.70 to 0.90 is a high correlation; from 0.50 to 0.70 is a moderate correlation; from 0.30 to 0.50 is a low correlation; from 0.00 to 0.30 negligible correlation [21].

RESULT AND DICUSSION

According to obtained results shown in Table 1, the 60% extract (1.85±0.02%) had the most significant amount of polyphenols, followed by 40% extract (1.53±0.02%), whereas the lowest one – ethanolic extract (0.38±0.01%).

The content of catechins increasing in the following order 96% extract (0.20±0.01%) > aqueous extract (0.73±0.01%) > 20% extract (0.74±0.01%) > 40% extract (0.96±0.02%) > 60% extract (1.01±0.02%). The percentage of catechins out of total of polyphenols was 53.1, 54.2, 63.0, 50.1 and 54.3% for 96%, 60%, 40%, 20% and aqueous extracts, respectively. The highest percentage of catechins was in 40% extract, whereas the lowest in 20% extract (Table 1).

Table 1 demonstrates that the most significant content of flavonoids was found in 60% extract (0.33±0.01%), whereas in the aqueous extract (0.20±0.002%) was the lowest one. The percentage of flavonoids out of total of polyphenols was 63.2, 18.1, 43.2, 15.3 and 15.0% for 96%, 60%, 40%, 20% and aqueous extracts, respectively. The highest percentage of flavonoids was in 96% extract, whereas the lowest in aqueous and 20% extract.

The content of hydroxycinnamic acids increasing in the following order aqueous and 20% extract (0.10±0.005%) > 40% extract (0.15±0.005%) > 96% extract (0.11±0.005%) > 60% extract (0.24±0.005%). The percentage of hydroxycinnamic acids out of total of polyphenols was 29.0, 13.1, 10.2, 7.1 and 6.2% for 96%, 60%, 40%, 20% and aqueous extracts, respectively. The highest percentage of hydroxycinnamic acids was in 96% extract, whereas the lowest in 6% extract. (Table 1)

The highest amount of organic acids was determined in aqueous extract (1.02±0.02%), followed by 20% extract (1.00±0.02%), whereas the lowest one in 96% extract (0.42±0.01%). The total content of organic acids was lower 153.3%, 110.2%, 48.1%, 32.1% than content polyphenols in 96%, 60%, 40%, 20% extracts, respectively (Table 1).

Table 1. The sum of phenolic compounds, flavonoids, catechins, hydroxycinnamic acids and organic acids in *R. idaeus* leaf liquid extracts

Sample	Amount of polyphenols, %±SD[a]	Amount of catechins	Amount of flavonoid		Amount of hydroxycinnamic acids		Amount of organic acids, %± SD[a]
		% ±SD[a]	% ±SD[a]	Part out polyphenols	% ±SD[a]	Part out polyphenols	% ±SD[a]
96% EtOH extract	0.38±0.01	0.20±0.01	0.24±0.01	63.2	0.11±0.005	29.0	0.42±0.01
60% EtOH extract	1.85±0.02	1.01±0.02	0.33±0.01	18.1	0.24±0.005	13.1	0.73±0.01
40% EtOH extract	1.53±0.02	0.96±0.02	0.25±0.01	43.2	0.15±0.005	10.2	0.73±0.01
20% EtOH extract	1.48±0.02	0.74±0.01	0.22±0.01	15.3	0.10±0.005	7.1	1.00±0.02
Aqueous extract	1.35±0.02	0.73±0.01	0.20±0.01	15.0	0.10±0.005	6.2	1.02±0.02

[a]Standard deviation, n=4

A potentiometric method for determining antioxidant activity was used to evaluate the effect of the obtained extracts of *R. idaeus* leaf. Table 2 shows that the level of antioxidant activity increases in the following order: 96% extract (28.9±0.3 mmol-eqv./$m_{dry\ res.)}$ > aqueous extract (54.3±0.5 mmol-eqv./$m_{dry\ res.}$) > 20% extract (60.8±0.6 mmol-eqv./$m_{dry\ res.}$) > 40% extract (69.9±0.7 mmol-eqv./$m_{dry\ res.}$) > 60% extract (76.1±0.8 mmol-eqv./$m_{dry\ res.}$). In light of the data obtained, it can be established that the 60% extract has the highest level of antioxidant activity. According to the modern classification of antioxidant activity, which was previously developed in our previous research [22], it was found that all extracts obtained have a high level of antioxidant activity. Moreover, a comparative analysis of the "strength" of antioxidant activity was carried out with the gold standard 60% extract of *C. sinensis* leaf. The *C. sinensis* leaf extract was obtained by the same technological method as *R. idaeus* leaf extracts. The obtained extracts were significantly inferior in antioxidant effect to *C. sinensis* leaf extract. Further, a 0.03 mol/l solutions (in terms of the amount of polyphenols expressed as gallic acid) of extracts of *v* and *C. sinensis* leaf were prepared. As a result of the study, it was found that when compared at the same concentrations, the 96% extract had the highest antioxidant effect, and the least - 20% extract (Table 3).

Table 2. The level of antioxidant activity of *R. idaeus* leaf liquid extracts

Sample	Antioxidant activity, mmol-eqv./$m_{dry\ res.}$ ±SD[a]	Conditional term of antioxidant level
96% EtOH extract	28.9±0.3	High level
60% EtOH extract	76.1±0.8	High level
40% EtOH extract	69.9±0.7	High level
20% EtOH extract	60.8±0.6	High level
Aqueous extract	54.3±0.5	High level
Green tea leaf 60% EtOH extract	548.8±5.5	Very high level

[a]Standard deviation, n=4

The potentimetric assay was chosen for evaluation antioxidant activity for several reasons: above all, the potentiometric assay is expressive secondly, cheap and moreover, this assay accurate and precise. To compare the antioxidant effect, we used the obtained green tea leaf extract, the results showed that green tea extract inactivates free radicals significantly better than *R. idaeus* leaf extracts. The 60% extract was found to have the highest level of antioxidant activity than other *R. idaeus* extracts. After, we decided to compare the antioxidant effect of extracts at the same concentration of phenolic compounds, as a result, it was shown that green tea extract works lower than in 96%, 60%, 40% extracts of *R. idaeus* leaf. In addition, it was found that the order of levels of antioxidant activity of the extracts changed dramatically. At different concentrations of phenolic compounds, the 60% extract had the highest level of antioxidant activity, and when compared at the same concentration, the ethanolic extract was the best.

Table 3. Comparing the value of antioxidant activity of *R. idaeus* leaf liquid extracts with *C. sinensis* leaf 60% extract at the concentration 0.03 mol/l expressed in the total phenolic compounds as gallic acid

Sample	Concentration of polyphenols, mol/l	Antioxidant activity, mmol-eqv./$m_{dry\ res.}$ ±SD[a]
96% EtOH extract	0.03	43.3±0.4
60% EtOH extract		20.7±0.2
40% EtOH extract		23.3±0.2
20% EtOH extract		20.3±0.2
Aqueous extract		20.3±0.2
Green tea leaf 60% EtOH extract		27.9±0.3

[a]Standard deviation, n=4

In this research work, the antimicrobial activity of the obtained *R. idaeus* leaf extracts was investigated against the following strains of *S. aureus*, *B. subtilis*, *E. coli*, *P. vulgaris*, *P. aeruginosa*, as well as a strain of the fungus *C. albicans*. According to the obtained results, all extracts obtained from the *R. idaeus* leaf had an effective antimicrobial effect (Table 4).

S. aureus was most sensitive to the 60% extract (25.0 ± 0.20 mm) and least sensitive to the 96% extract (20.0 ± 0.4 mm). When comparing the results of the gentamicin standard and the 60% extract, it was found that the 60% extract was 12.1% better at inhibiting the growth of the *S. aureus* strain of bacteria. According to the results presented in Table 3, it was found that *B. subtilis*, as well as *S. aureus*, was highly sensitive to the 60% extract (25.0 ± 0.3 mm), followed by 40% extract (22.0 ± 0.4 mm), and the aqueous and ethanolic extracts inhibited the growth of the bacterial strain the least. (21.0+0.5 mm). The most resistant strains of bacteria to the action of *R. idaeus* leaf extracts was *P. aeruginosa*. *E. coli* were most sensitive to the action of 20% extract, in second place – 60% extract, whereas *P. vulgaris* were most sensitive to the action of 20% extract (Table 4).

When studying antifungal activity against *C. albicans*, the results showed that 60% extract of *R. idaeus* leaf was the most actively inhibited the growth of the fungus, whereas the ethanolic, 20% and aqueous extracts were least active inhibited the growth of fungi. When compared with the fluconazole standard, it was found that the 60% extracts inhibited fungal growth 10.2% better than fluconazole (Table 4).

The studied *R. idaeus* leaf extract showed antimicrobial activity against the following strains of *S. aureus*, *P. aeruginosa*, *P. vulgaris*, *B. subtillis* and *C. albicans*. According to the obtained data, at first glance it can be considered that the antimicrobial activity of *R. idaeus* leaf extracts is significantly inferior to the action of gentamicin and fluconazole, because their concentration of solutions was significantly lower than the content of polyphenols in the extract. However, we would like to note that gentamicin has serious toxicity to the auditory nerve, kidneys and liver, which can lead to serious complications of the disease [22]. Comparing the antifungal effects of fluconazole and *R. idaeus* leaf extract, it was found that they inhibited the growth of the fungal strain at the same level, while the concentration of fluconazole was also lower, like gentamicin. We can declare that fluconazole is a leader as anti-fungi medicine, but at the same time it weakly inhibits the growth of gram-negative and gram-positive bacteria, but to *R. idaeus* leaf extracts both strains of bacteria and fungus are sensitive. Thus, *R. idaeus* leaf extracts is a combined pharmaceutical that affects different mechanisms of vital activity of bacteria and fungi, thereby having a wide spectrum of action against different strains of bacteria and fungi, and at the same time not possessing serious toxicity.

Table 4. The value of antimicrobial activity of *R. idaeus* leaf liquid extracts

Sample	**Concentration mmol/l, (expressed in total polyphenols as gallic acid)**	**Diameter of the growth retardation zone, mm±SD[a]**					
		Gram-positive		**Gram-negative**			**Fungi**
		S. aureus	*B. subtilis*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*C. albicans*
96% EtOH extract	0.006	20.0±0.4	21.0±0.4	20.0±0.4	20.0±0.4	20.0±0.4	20.0±0.4
60% EtOH extract	0.033	25.0±0.3	25.0±0.3	21.0±0.4	20.0±0.4	18.0±0.6	23.0±0.4
40% EtOH extract	0.027	22.0±0.4	23.0±0.4	20.0±0.5	20.0±0.4	18.0±0.6	21.0±0.4
20% EtOH extract	0.027	25.0±0.3	22.0±0.5	22.0±0.4	23.0±0.3	18.0±0.6	20.0±0.4
Aqueous H_2O extract	0.024	23.0±0.4	21.0±0.5	20.0±0.5	21.0±0.4	18.0±0.6	20.0±0.4
Gentamycin	0.003	22.0±0.5	24.0±0.3	25.3±0.3	25.0±0.3	25.7±0.2	12.0±0.8
Fluconazole	0.003	18.0±0.5	12.0±0.6	14.3±0.8	12.3±0.8	10.0±0.8	20.0± 0.4

[a]Standard deviation, n=4

The dependence of antioxidant, antimicrobial activity on the content of different groups of BAS was studied using the method of linear regression. In Figure 1 shows that the correlation between the antioxidant effect and the content of polyphenols was very high (R=0.9789), in the case of catechins was very high (R=0.9809), flavonoids was moderate (R=0.5168), in the case of hydroxycinnamic acids was moderate (R=0.6512), and the lowest correlation value was observed for organic acids. The results

obtained are similar from those obtained by other Ispiryan et al. [23], they reported about the correlation between antioxidant and the polyphenols content in extracts of raspberry shoots, leaves, seeds and fruits. In their research correlation coefficient was 0.845, 0.854, and 0.700 beetwen antioxidant and polyphenols, flavonoids, and hydroxycinnamic acids content.

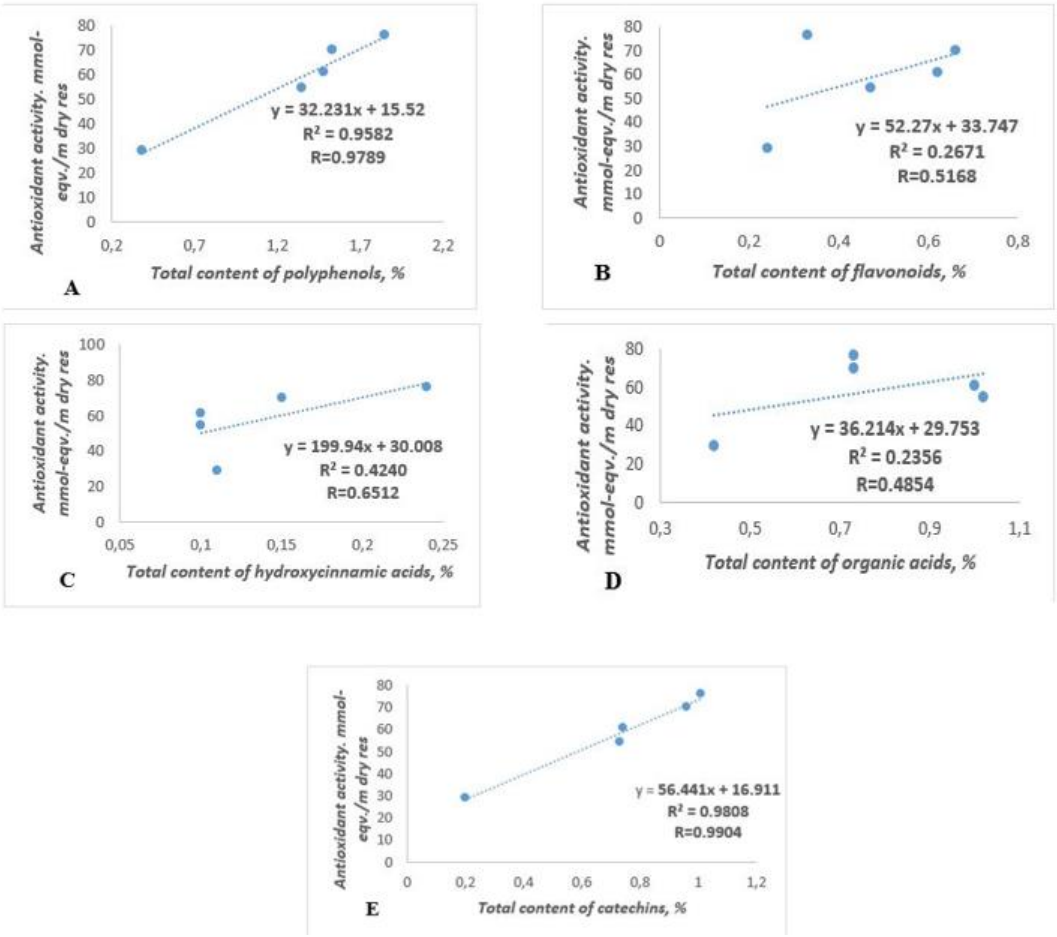

Figure 1. Correlation relationship between value of antioxidant activity and total content of polyphenols (A), flavonoids (B), hydroxycinnamic acids (C), organic acids (D) and catechins (E)

According to the research results presented in Figure 2 it was found that there is a high correlation between phenolic compounds (R=0.8462), catechins (R=0.7122), organic acids (R=0.7064), antioxidant activity (R=0.7554) and inhibition of the growth of *S. aureus*, in the case of flavonoids (R=0.3387) and hydroxycinnamic acids (R=0.3936) – low correlation. Comparing with results obtained of research of Ispiryan *et al.* [23], they are different as in the research of Ispiryan et al. correlation coefficient was 0.300, 0.315, 0.380 and 0.370 between antimicrobial activity against *S. aerous* and polyphenols, flavonoids, hydroxycinnamic acids and antioxidant effect.

In Figure 3 shows that the antimicrobial effect against *B. subtilis* is very highly dependent on the content of hydroxycinnamic acids (R=0.9528), high dependent on polyphenols (R=0.7297), flavonoids (R=0.7465) and antioxidant activity (R=0.8088), in turn, the content of organic acids and flavonoids there is no dependence. Stagos et al. [24] investigated antibacterial activity of *Mentha*, *Salvia* and *Sideritis* leaf extracts, results were shown that there was observed a low correlation between antibacterial and polyphenols contenr.

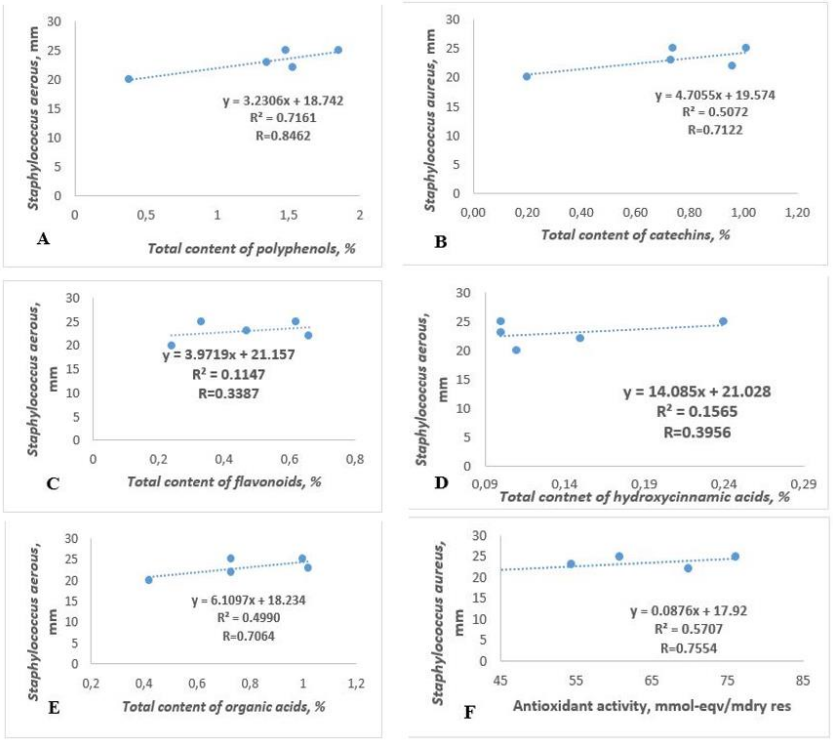

Figure 2. Correlation relationship between value of antimicrobial activity against *Staphylococcus aureus* and total content of polyphenols (A), catechins (B) flavonoids (C), hydroxycinnamic acids (D), organic acids (E) and antioxidant activity (F)

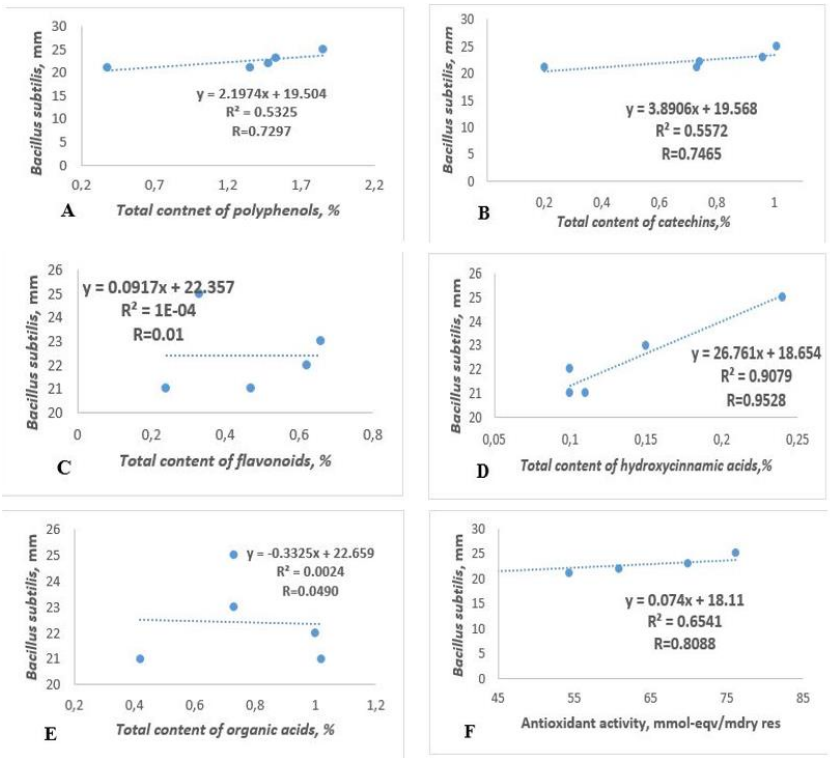

Figure 3. Correlation relationship between value of antimicrobial activity against *Bacillus subtilis* and total content of polyphenols (A), catechins (B), flavonoids (C), hydroxycinnamic acids (D), organic acids (E), and antioxidant activity (F)

The study showed that there is a low correlation between phenolic compounds (R=0.4306), organic acid (R=0.4445), antioxidant effect (R=0.3622) and inhibition of *E. coli* growth, while flavonoids, hydroxycinnamic acids and catechins are not effect on the growth inhibition of *E. coli*. (Figure 4). Cirovic *et al.* [25] investigated antibacterial activity of *Sanguisorba minor* root extracts against *E. coli*, results were shown that there was observed a low correlation between antibacterial and polyphenols, flavonoids content. Comparing results with our, there are similar.

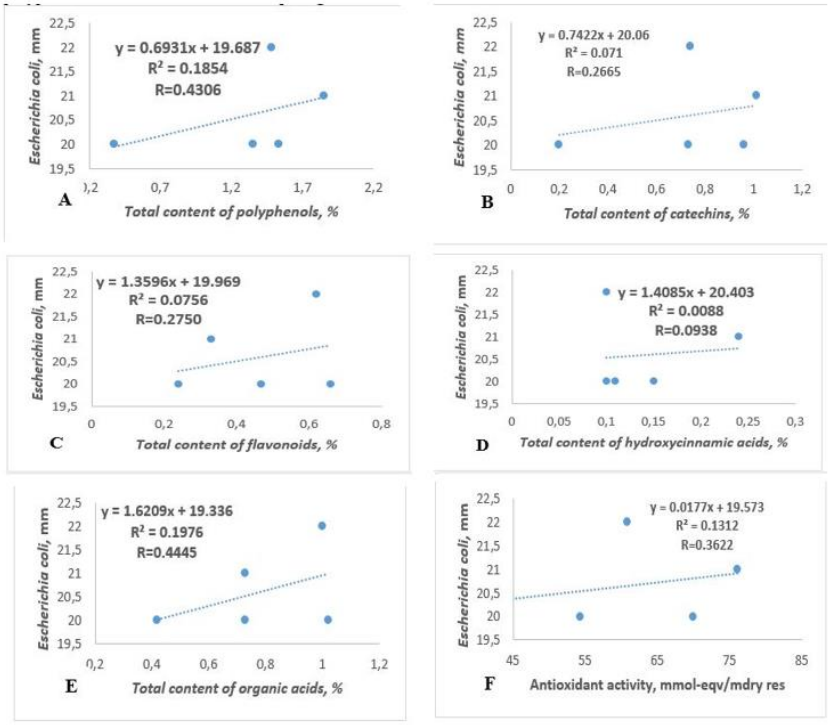

Figure 4. Correlation relationship between value of antimicrobial activity against *Escherichia coli* and total content of polyphenols (A), catechins (B) flavonoids (C), hydroxycinnamic acids (D), organic acids (E), and antioxidant activity (F)

When studying the relationship between inhibition of growth of *P. vulgaris* and the content of different groups of BAS, it was found that there is a very high dependence of antimicrobial activity on the amount of polyphenols (R=0.8838), flavonoids (R=0.7262) and hydroxycinnamic acid (R=0.7347), in turn, the antioxidant effect had a moderate correlation, while catechins, organic acids had not correlation at all. (Figure 5) Cirovic *et al.* [25] investigated antibacterial activity of *Sanguisorba minor* root extracts against *P. vulgaris*, results were shown that there was observed a high correlation between antibacterial and polyphenols, flavonoids and hydroxycinamic acid content. Comparing results with our, there are similiar.

Figure 6 shows that the correlation between the growth inhibition of *P. aeruginosa* and the sum of polyphenols (R=0.9437), catechins (R=0.9194) is very high, with the sum of organic acids and antioxidant activity was high, in the case of flavonoids it was found moderate correlation. Whereas, the total content of hydroxycinnamic acids was not effect on the inhibition of growth *P. aeruginosa*. The results obtained are similar from those obtained by other Katalinic *et al.* [26], they reported about the correlation between antioxidant and the polyphenols content in *Vitis vinifera* leaf extracts against *P. aeruginosa*. In their research correlation coefficient was 0.745, 0.600 beetwen antibacterial effect and polyphenols content, antioxidant effect, respectively.

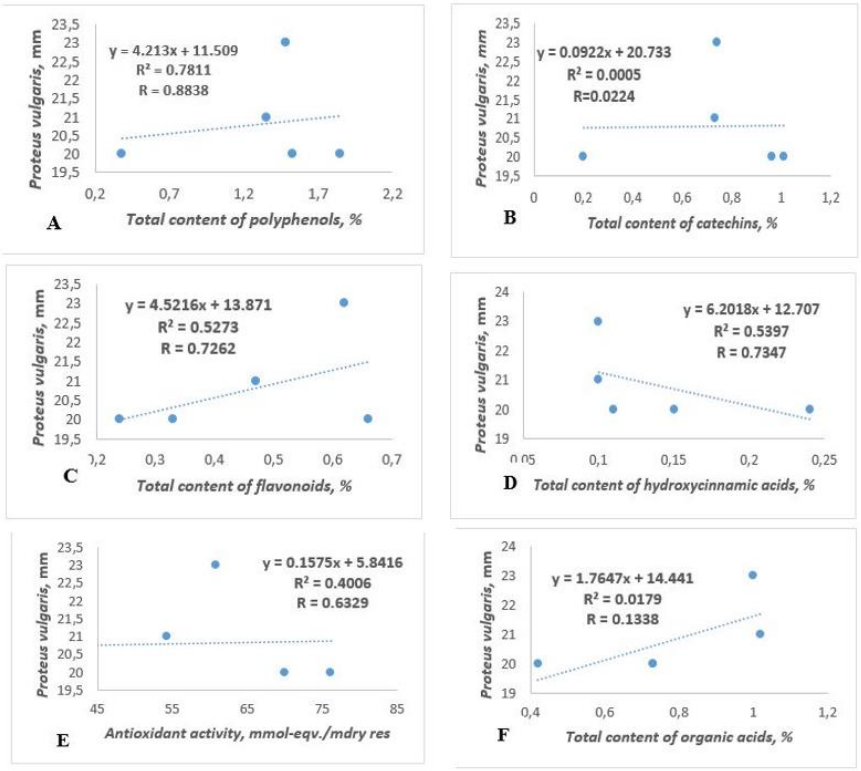

Figure 5. Correlation relationship between value of antimicrobial activity against *Proteus vulgaris* and total content of polyphenols (A), catechins (B), flavonoids (C), hydroxycinnamic acids (D), organic acids (F), and antioxidant activity (E)

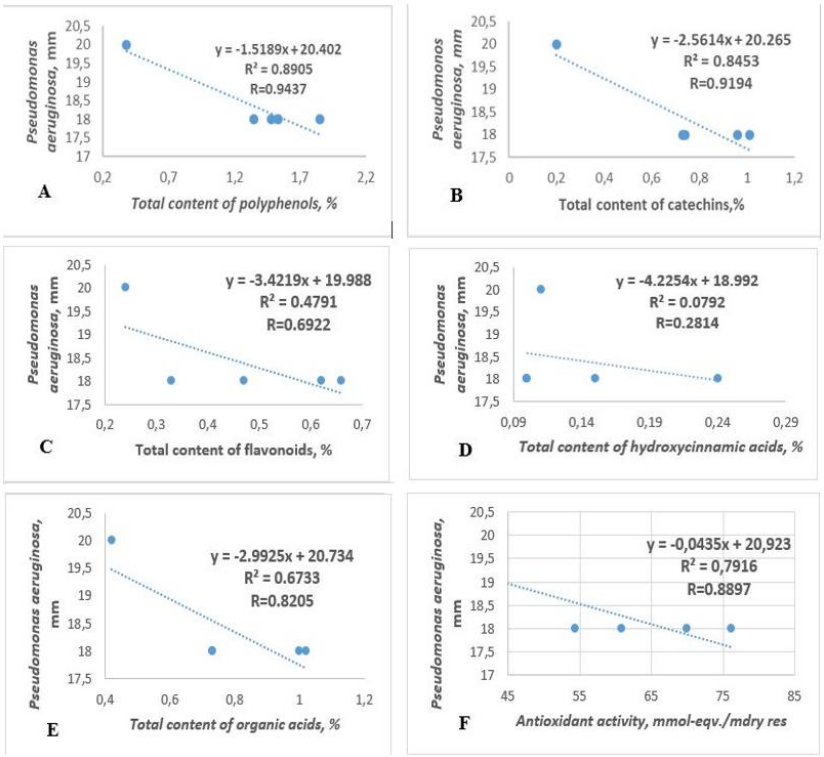

Figure 6. Correlation relationship between value of antimicrobial activity against *Pseudomonas aeruginosa* and total content of polyphenols (A), catechins (B), flavonoids (C), hydroxycinnamic acids (D), organic acids (F), and antioxidant activity (E)

In Fig. 7 shows a significant high correlation between inhibition of the growth of *C. albicans* and the content of hydroxycinnamic acids (R=0.9976), in turn, with the sum of polyphenols (R=0.6239), catechins (R=0.6438) and antioxidant activity (R=0.6940) was found a moderate dependence, and in the case of organic acids and flavonoids there was no correlation at all. The results obtained are similar from those obtained by other Chen *et al.* [27], they reported about the correlation between antifungi and the polyphenols content in *Curcuma longa* extract against *Fusarium graminearum*.

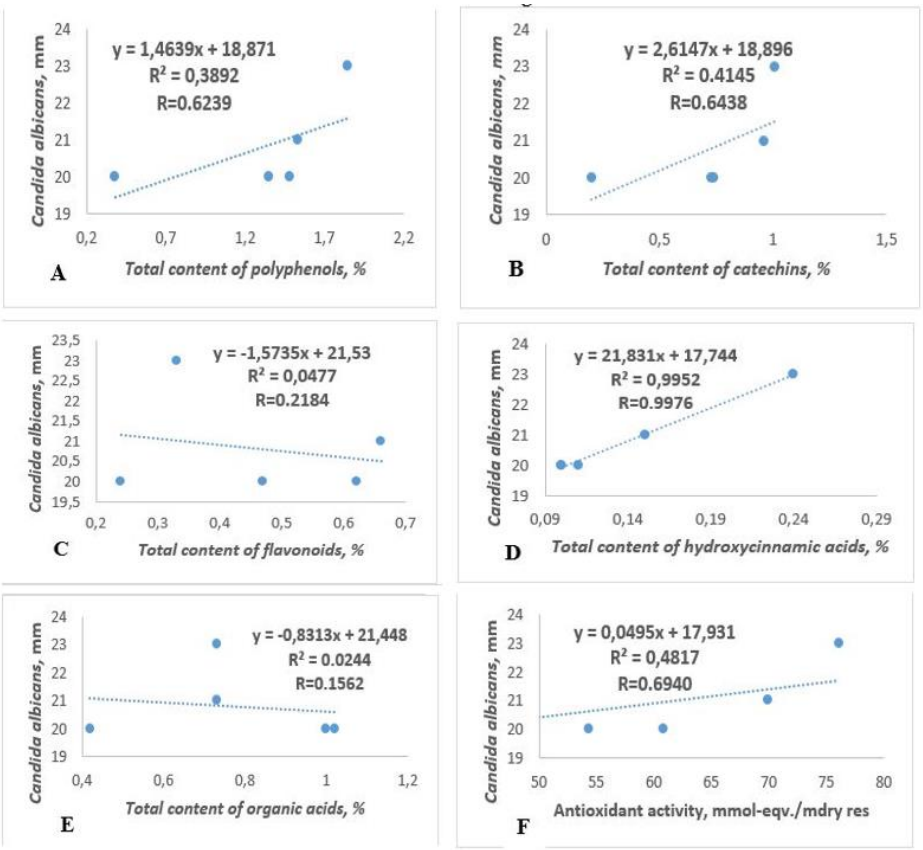

Figure 7. Correlation relationship between value of antimicrobial activity against *Candida albicans* and total content of polyphenols (A), catechins (B), flavonoids (C), hydroxycinnamic acids (D), organic acids (E), and antioxidant activity (F)

When studying the dependence of antioxidant action on the content of different groups of BAS, it was found that phenolic compounds and catechins most strongly influence on the level of antioxidant action. The investigation of correlation between inhibition of the growth of bacteria strains *S. aureus*, *B. subtillis*, *P. aeruginosa*. and the content of BAS, it was shown that the growth of microorganisms is most strongly influenced by phenolic compounds, catechins and antioxidant effects, in addition, the inhibition of the growth of *S. aureus* and *P. aeruginosa* depends on the presence of organic acids, in turn, the growth of the strain of *B. subtillis* is not dependent, but, on the contrary, is highly correlated with content of hydroxycinnamic acids. The growth of *E. coli* does not depend on the content of BAS or on the antioxidant effect. In our opinion, this may be due to the fact that bacterial growth depends on another group of compounds, for example, ellagitannins, which were not represented in our research. The growth of fungi *C. albicans* significantly depends only on the content of hydroxycinnamic acids.

Conclusion

In the research, it has been determined the content of BAS, antioxidant, antimicrobial activity of the obtained extracts of *R. idaeus* leaf. The dominant content of the sum of polyphenols, flavonoids,

catechins was observed in 60% extract, whereas the organic acids in aqueous extract. The 60% extract has a high level of antioxidant activity, all obtained extracts actively inhibits the growth of all studied Gram-positive, Gram-negative strains of bacteria and the fungus *C. albicans* in the range from 18 to 25 mm (diameter of growth inhibition). We have shown that there is a high correlation between the content of polyphenols, catechins and antioxidant activity, in the case of inhibition of *S. aureus*, *P. aeruginosa*, *B. subtillis* depends on polyphenols, catechins and antioxidant activity, whereas fungi *C. albicans* significantly depends only on the content of hydroxycinnamic acids as well as E. coli is not depend on any BAS. These findings show the great potential in the development and creation of new medicines with antimicrobial, antioxidant effects that are not inferior to, and even superior to, the effects of synthetic analogues.

AUTHOR CONTRIBUTIONS

Concept: O.M., M.K., S.P., T.O., S.K.; Design: O.M., M.K., S.P., T.O., S.K.; Control: T.O., S.K.; Sources: O.M., M.K., S.P.; Materials: O.M., M.K., S.P.; Data Collection and/or Processing: O.M., M.K., S.P.; Analysis and/or Interpretation: O.M., M.K., S.P.; Literature Review: O.M., M.K., S.P.; Manuscript Writing: O.M., M.K., S.P.; Critical Review: T.O., S.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1155-1162, 2024
Doi: 10.33483/jfpau.1530342

ÖZGÜN MAKALE / ORIGINAL ARTICLE

BAZI UÇUCU YAĞLARIN ORAL BAKTERİLER ÜZERİNDE ANTİBAKTERİYEL ETKİNLİĞİNİN DEĞERLENDİRİLMESİ

EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF SOME ESSENTIAL OILS ON ORAL BACTERIA

Murat Sefa KARAASLAN[1,2], Nurten ALTANLAR[1]*

[1]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Mikrobiyoloji Anabilim Dalı, 06560, Ankara, Türkiye
[2]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, 06110, Ankara, Türkiye

ÖZ

Amaç: *Ağız sağlığı ve genel sağlık arasında önemli bir ilişki bulunmaktadır. Ağızda görülen hastalıklar bireylerin günlük hayatını etkilemekte ve yaşam kalitesini düşürmektedir. Diş çürükleri ve periodontal hastalıklar ağız sağlığını bozan başlıca nedenler arasındadır. Diş fırçalarının bakterilerin kolonize olduğu bölgelere ulaşamaması, kimyasal içerikli gargara kullanımında görülen yan etki gibi nedenlerden dolayı güvenli ve etkili ürünlere ihtiyaç duyulmaktadır. Bu çalışmada bazı uçucu yağların oral bakteriler üzerindeki antibakteriyel etkinliğinin araştırılması amaçlanmıştır.*
Gereç ve Yöntem: *Çalışmada Enterococcus faecalis ATCC 29212, Streptococcus mutans ATCC 25175, Lactobacillus acidophilus ATCC 11975 ve L. casei RSKK 06029 test bakterileri olarak kullanılmıştır. Uçucu yağların antibakteriyel aktiviteleri disk difüzyon ve agar kuyu difüzyon yöntemleriyle araştırılmıştır.*
Sonuç ve Tartışma: *Her iki yöntemde de test bakterilerine karşı en yüksek antibakteriyel aktiviteyi kekik uçucu yağı göstermiştir. Kekik uçucu yağının disk difüzyon yönteminde L. acidophilus'a karşı ve agar kuyu difüzyon yönteminde S. mutans'a karşı inhibisyon zon çapları sırasıyla 57.6±0.8 ve 58±0.9 mm olarak belirlenmiştir. Disk difüzyon yönteminde okaliptüs uçucu yağının E. faecalis, L. acidophilus ve S. mutans test bakterilerine karşı inhibisyon zon çapı saptanırken, kuyu difüzyon yönteminde zon çapı gözlemlenmemiştir. Yöntem bulguları literatürle kıyaslandığında gözlemlenen aktivite farklılıkları uçucu yağların farklı bileşenler içermesi, izole edildikleri bitkilerin toplanma zamanına göre değişkenlik gösterebilmektedir. Gözlemlenen aktivite sonuçlarındaki farklılığın daha iyi aydınlatılabilmesi için uçucu yağların karakterizasyonunun yapılması önem taşımaktadır.*
Anahtar Kelimeler: *Ağız sağlığı, antibakteriyel aktivite, kekik, uçucu yağ*

ABSTRACT

Objective: *There is a significant relationship between oral health and general health. Oral diseases affect the daily lives of individuals and reduce the quality of life. Dental caries and periodontal diseases are among the primary causes that deteriorate oral health. Safe and effective products are needed due to reasons such as the inefficient of toothbrushes to reach areas where bacteria are colonized and the side effects seen when using chemical mouthwash. This study aims to investigate the antibacterial activity of the essential oils against oral bacteria.*
Material and Method: *In this study, Enterococcus faecalis ATCC 29212, Streptococcus mutans ATCC 25175, Lactobacillus acidophilus ATCC 11975 and L. casei RSKK 06029 were used as test*

* **Sorumlu Yazar / Corresponding Author:** Nurten Altanlar
e-posta / e-mail: naltanlar@ankara.edu.tr, **Tel. / Phone:** +905324424312

Gönderilme / Submitted : 08.08.2024
Kabul / Accepted : 13.08.2024
Yayınlanma / Published : 10.09.2024

bacteria. Antibacterial activities of the essential oils were investigated by disk diffusion and agar well diffusion methods.

Result and Discussion: *In antibacterial activity methods, thyme essential oil showed the highest activity against the test bacteria. The inhibition zone diameters of thyme essential oil against L. acidophilus in the disk diffusion method and against S. mutans in the agar well diffusion method were determined as 57.6±0.8 and 58±0.9 mm, respectively. While the inhibition zone diameters of eucalyptus essential oil against E. faecalis, L. acidophilus and S. mutans test bacteria were determined in the disk diffusion method, the zone diameters were not observed in the well diffusion method. The methods findings may differ between literatures due to reasons such as the essential oils contain different components and the difference in the collection time of the plants which they are isolated. It is important to characterize essential oils to better explain differences in observed activity results.*

Keywords: *Antibacterial activity, essential oil, oral health, thyme*

GİRİŞ

İnsanlarda genel sağlık ile ağız sağlığı doğrudan ilişkilidir. Ağızda görülen hastalıklar bireylerin çalışma kapasitesini ve yaşam kalitesini düşürmektedir. Ayrıca kötü ağız sağlığında görülen ağrı ve rahatsızlık hissi bireyleri fiziksel, duygusal ve sosyal olarak etkilemektedir [1]. Aynı zamanda ağız sağlığı osteoporoz, romatoid artrit ve kardiyovasküler sistemi etkileyen sistemik hastalıklarla da ilişkilendirilmiştir [2].

Dünya Sağlık Örgütü (DSÖ), dünya nüfusunun yaklaşık yarısının ağız hastalıklarından etkilendiğini bildirmektedir. 2022 Küresel Ağız Sağlığı Durum Raporu'nda, ağız sağlığını bozan hastalıkların başlıca nedenleri diş çürükleri, diş kayıpları, periodontal hastalıklar ve ağız-dudak kanserleri olarak belirtilmiştir [3].

Diş çürükleri en yaygın ağız sağlığı problemidir ve tedavi edilmeyen çürüklerin dünya genelinde 2 milyar yetişkini ve 520 milyon çocuğu etkilediği düşünülmektedir [3,4]. Biyofilm oluşturma yeteneğiyle diş çürüklerinin en önemli etiyolojik ajanı *Streptococcus mutans* kabul edilmektedir. İnsanda diğer önemli karyojenik mikroorganizma, çürük lezyonlarının gelişiminden sorumlu olan *Lactobacillus casei* ve *L. acidophilus* gibi *Lactobacillus* türleridir [5].

Ağız boşluğunda bulunan *Enterococcus faecalis* ise endodontik enfeksiyonlarla ilişkilendirilmektedir [5,6]. Endodontik tedavi sonrası yeniden enfeksiyon ve ağrıyla raporlanan vakalarda yaklaşık %90 prevalansla tespit edilmektedir [6].

Ağızdaki enfeksiyon riskinin düşürülmesinde buradaki mikroorganizma yükünün sınırlanması önemlidir. Günlük hijyenin bir parçası olan diş fırçalamayla uygulanan mekanik temizlik bakterilerin kolonize olduğu bölgelere ulaşmada yetersiz kalabilmektedir. Mekanik temizlemenin yanında kullanılan kimyasal içerikli gargaraların mukozal emilimi bunların uzun süreli kullanımını sınırlamakta ek olarak dil ve diş rengini değiştirmesi ve alerjik reaksiyonlar gibi yan etkilere neden olabilmektedir [7,8]. Ağız içi hijyenin sağlanması ve ağız sağlığının sürdürülmesinde güvenli ve etkili ürünlere ihtiyaç duyulduğu görülmektedir.

Binlerce yıldır hastalıkların tedavisinde bitkiler kullanılmakta ve bu bitkilerden elde edilen doğal ürünler farmasötik endüstri için temel oluşturmaktadır [9]. Uçucu yağlar bitkilerin oda sıcaklığında buharlaşabilen ve kokulu ikincil metabolitleri olarak tanımlanmaktadır [10]. Yapılan birçok çalışmada uçucu yağların antibakteriyel etkinliği bildirilmiştir [10-12].

Uçucu yağların gerek antibakteriyel etkileri gerekse hoş kokuları sayesinde ağız hijyen ürünlerinde kullanımları yaygındır. Bunların yanında ekonomik uygunluk ve düşük toksisite nedeniyle öne çıkmaktadır [9].

Çalışmamızda toplumda sıklıkla kullanılan ve ticari olarak temin edilen *Eucalytus globulus* Labill. (okaliptüs), *Mentha piperita* L. (tıbbi nane), *Syzygium aromaticum* L. (karanfil) ve *Thymus vulgaris* L. (kekik) uçucu yağların, *E. faecalis* ATCC 29212, *S. mutans* ATCC 25175, *L. acidophilus* ATCC 11975 ve *L. casei* RSKK 06029 standart test bakterilerine karşı antibakteriyel etkinliklerinin disk difüzyon ve agar kuyu difüzyon testleriyle değerlendirilmesi amaçlanmıştır.

GEREÇ VE YÖNTEM

Kullanılan Uçucu Yağlar

Çalışmada ticari olarak temin edilen *E. globulus* (okaliptüs), *M. piperita* (tıbbi nane), *S. aromaticum* (karanfil) ve *T. vulgaris* (kekik) bitkilerinin uçucu yağları kullanılmıştır.

Test Bakterileri ve Kültür Koşulları

Çalışmada *E. faecalis* ATCC 29212, *S. mutans* ATCC 25175, *L. acidophilus* ATCC 11975 ve *L. casei* RSKK 06029 standart bakteri suşları kullanılmıştır. *S. mutans* ATCC 25175 ve *L. acidophilus* ATCC 11975, *L. casei* RSKK 06029 test bakterileri sırasıyla Brain Heart Infusion (BHI) (Merck, Almaya) agar ve De Man-Rogosa-Sharpe (MRS) (Merck, Almanya) agar besiyerlerinde 37°C'de %5 CO_2'li ortamda 24-48 saat inkübe edilmiştir. *E. faecalis* ATCC 29212 test bakterisi ise BHI agar besiyerinde 37°C'de aerob ortamda 24 saat inkübe edilmiştir.

Antibakteriyel Aktivite

Disk Difüzyon Testi

Uçucu yağların antibakteriyel aktivitesi Kirby-Bauer disk difüzyon yöntemine göre yapılmıştır. Bazı eklemeler ile çeşitli modifikasyonlar gerçekleştirilmiştir. Tüm test bakterileri uygun koşullarda üretilerek taze kültürleri hazırlanmıştır. Bu kültürler steril serum fizyolojik çözeltisi ile 0.5 McFarland yoğunluğuna ayarlanmıştır. Steril eküvyonlar ile bakteri süspansiyonları *E. faecalis* için Mueller Hinton Agar (MHA) (Merck, Almanya), *S. mutans* için BHI agar, *L. acidophilus* ve *L. casei* için ise MRS agar besiyerlerine tatbik edildikten sonra daha önce uçucu yağların 6 mm çaplı steril boş disklere (20 µl emme kapasitesine sahip) eşit hacimde emdirilerek hazırlanmış diskleri petrilere yerleştirilmiştir. İnkübasyon süresi ardından zon çapları kumpas ile ölçülerek kaydedilmiştir [13,14]. Kontrol olarak siprofloksasin (5µg, Oxoid) ve teikoplanin (30 µg, Bioanalyse) antibiyotik diskleri kullanılmıştır.

Agar Kuyu Difüzyon Testi

Tüm test bakterileri belirtilen koşullara göre kültürlenmiş ve taze kültürler steril serum fizyolojik çözeltisi içerisinde 0.5 MacFarland yoğunluğuna göre ayarlanmıştır. Bakteri süspansiyonlarından steril eküvyonlar yardımıyla alınarak *E. faecalis* için MHA, *S. mutans* için BHI agar, *L. acidophilus* ve *L. casei* için ise MRS agar, besiyerlerine tatbik edilmiştir. Ardından petrilere 6 mm çapında kuyular açılıp, dip kısımlarına 45 dereceye kadar soğutulmuş steril besiyerlerinden 20 µl damlatılmış ve kuyulara 50 µl uçucu yağ örnekleri ilave edilmiştir. Petriler *E. fecalis* için aerobik ortamda 18-24 saat, *S. mutans, L. acidophilus, L. casei* bakterileri için %5 CO_2'li ortamda 24-48 saat inkübasyona bırakılmıştır. İnkübasyon sonunda kuyuların etrafında oluşan inhibisyon zon çapları ölçülerek kaydedilmiştir [15].

SONUÇ VE TARTIŞMA

Antibakteriyel Aktivite Sonuçları

Uçucu yağların test bakterilerine karşı disk difüzyon test sonuçları Tablo 1'de verilmiştir. İnhibisyon zon çaplarının görseli Şekil 1'de gösterilmiştir.

Disk difüzyon yönteminde en yüksek aktiviteyi kekik uçucu yağı *L. acidophilus*'a karşı 57.6±0.8 mm zon çapıyla göstermiştir. En düşük aktivite okaliptüs uçucu yağında görülmüştür.

Agar kuyu difüzyon testi sonuçları Tablo 2'de verilmiştir. İnhibisyon zon çaplarının görseli Şekil 2'de gösterilmiştir.

Kuyu difüzyon yönteminde en yüksek aktiviteyi kekik uçucu yağı *S. mutans*'a karşı 58±0.9 mm zon çapıyla göstermiştir. Kuyu difüzyon yönteminde okaliptüs uçucu yağının test bakterilerine karşı zon oluşumu gözlenmemiştir.

Çalışmamızda uçucu yağların antibakteriyel etkinlikleri disk difüzyon ve agar kuyu difüzyon yöntemleriyle değerlendirilmiştir. *S. mutans*'ın MHA'da zayıf üremesi sonucu BHI agar besiyeri kullanılmıştır [16,17]. *L. acidophilus* ve *L. casei* test bakterileri için ise MRS agar besiyeri kullanılmıştır [18,19].

Tablo 1. Disk difüzyon test sonuçları (mm)

		Test Bakterileri			
		E. faecalis ATCC 29212	*L. acidophilus* ATCC 11975	*L. casei* RSKK 06029	*S. mutans* ATCC 25175
Uçucu Yağlar	*T. vulgaris* (kekik)	22±0.3	57.6±0.8	48.7±0.9	47.5±1.3
	M. piperita (tıbbi nane)	10.2±0.2	32.4±0.5	27±0.5	38.2±0.8
	S. aromaticum (karanfil)	10.3±0.3	18.9±0.2	23.2±0.4	22.8±0.7
	E. globulus (okaliptüs)	9.9±0.2	9.0±0.4	-	13.2±0.8
Kontrol	Siprofloksasin	18±0.2	-	26.1±0.4	20±0.4
	Teikoplanin	TE	28.7±0.3	-	TE

*TE: Test edilmedi, -:zon yok., ±Standart Sapma

Tablo 2. Agar kuyu difüzyon test sonuçları (mm)

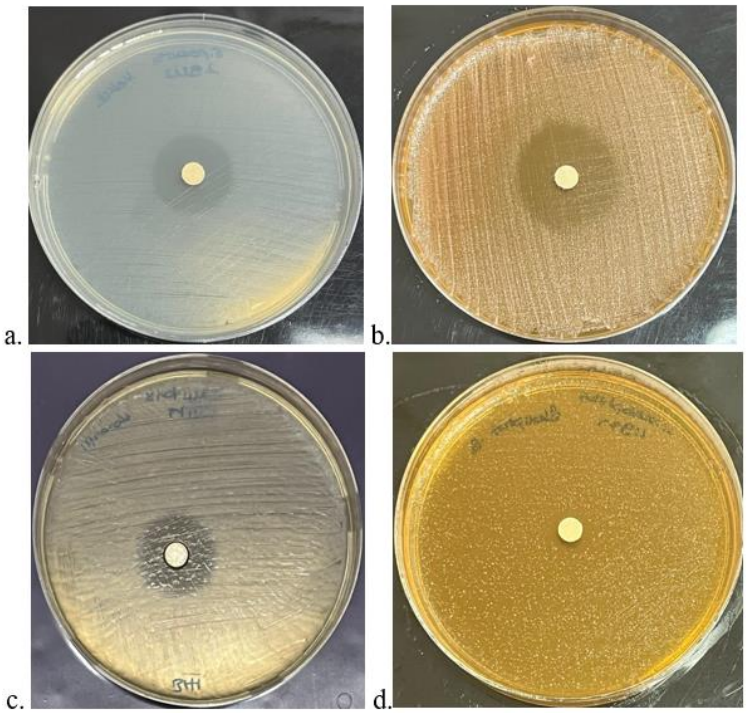
		Test Bakterileri			
		E. faecalis ATCC 29212	*L. acidophilus* ATCC 11975	*L. casei* RSKK 06029	*S. mutans* ATCC 25175
Uçucu Yağlar	*T. vulgaris* (kekik)	25±0.2	39±0.3	27±0.6	58±0.9
	M. piperita (tıbbi nane)	-	12±0.5	18±0.4	30±0.5
	S. aromaticum (karanfil)	11.5±0.5	17±0.1	16±0.3	21±0.3
	E. globulus (okaliptüs)	-	-	-	-

*-:zon yok, ± Standard Sapma

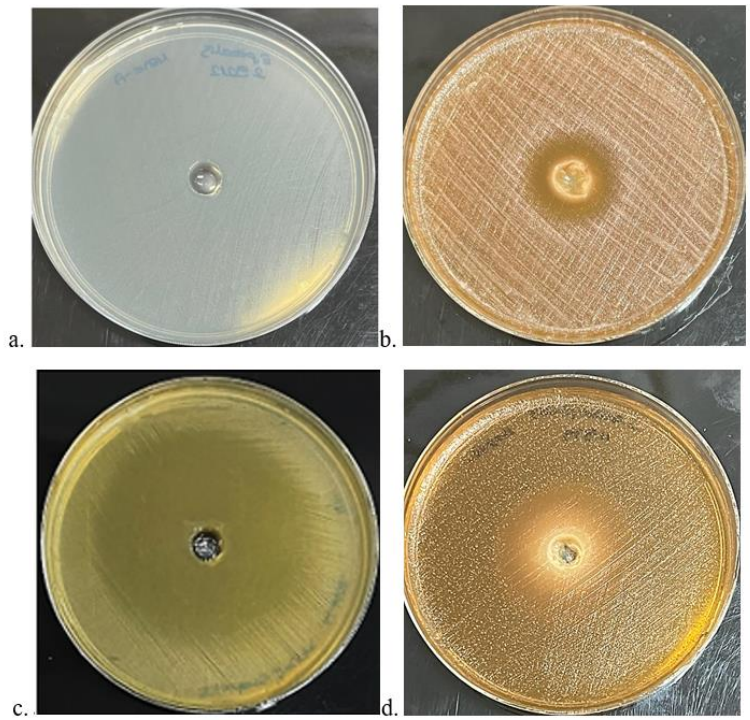
Maddelerin antimikrobiyal aktivite açısından taranmasında disk difüzyon yöntemi sıklıkla kullanılmaktadır. Yöntem basit uygulama şekli ve düşük maliyetli olmasıyla avantaj sağlamaktadır. Ancak kullanılacak besiyeri seçimi, ortam pH'sı, agar derinliği, nem içeriği ve inkübasyon koşulları gibi parametreler önemlidir. Yöntemde test edilen maddenin çözünürlüğü, difüzyon özellikleri, buharlaşma ya da maddenin disk üzerinde çökmesi inhibisyon çapını etkileyebilmektedir [20,21]. Farklı difüzyon hızlarına sahip bileşenleri içeren karışımların incelenmesi yöntemi sınırlayabilmektedir [20].

Agar kuyu difüzyon testi bitki kaynaklı maddelerin aktivitesinin test edilmesinde sıklıkla kullanılan tarama testlerinden biridir [20]. Özellikle katyonik özelliklere sahip maddelerin diske adsorbsiyonu sonucu ortama yayılamaması nedeniyle agar kuyu difüzyonu yöntemi tercih edilmektedir [22].

Disk difüzyon yönteminde boş kağıda emdirilmiş örneğin agar ortamına difüzyonu olurken agar kuyu difüzyon yönteminde örnek doğrudan ortamla temas ederek difüze olmaktadır. Her iki yöntemde de görülen difüzyon süreci moleküllerin yüksek konsantrasyon alanından düşük konsantrasyon alanına geçmesidir. Bu süreç moleküllerin sayısı, ağırlığı, yüzey alanı ve şekli gibi çok sayıda faktöre bağlı olarak değişmektedir [22]



Şekil 1. Uçucu yağların disk difüzyon yöntemiyle test bakterilerine karşı etkisi
a- *T. vulgaris* (kekik) uçucu yağı ve *E.faecalis*, b- *M. piperita* (tıbbi nane) uçucu yağı ve *L. casei*, c- *S. aromaticum* (karanfil) uçucu yağı ve *S. mutans*, d- *E. globulus* (okaliptüs) uçucu yağı ve *L. acidophilus*



Şekil 2. Uçucu yağların agar kuyu difüzyon yöntemiyle test bakterilerine karşı etkisi
a- *M. piperita* (tıbbi nane) ve *E. faecalis*, b- *S. aromaticum* (karanfil) uçucu yağı ve *L. casei*, c- *T. vulgaris* (kekik) uçucu yağı ve *S. mutans*, d- *T. vulgaris* (kekik) uçucu yağı ve *L. acidophilus*

Bir bitki ekstresinin antimikrobiyal aktivitesinin disk difüzyon ve agar kuyu difüzyon yöntemleri kullanılarak karşılaştırıldığı bir çalışmada ölçülen zon çaplarında farklılık olduğu gözlemlenmiştir [23]. Kawhena ve ark. (2021) yaptıkları çalışmada uçucu yağın antifungal aktivitesi disk difüzyon ve agar kuyu difüzyon yöntemleriyle değerlendirilmiştir. Zon çapları arasındaki değişkenlik uçucu yağın diske emdirilen hacmi ile ve kuyuya eklenen haciminin farklı olmasına dayandırılmıştır [24].

Çalışmamızda kullanılan her iki yöntemde test bakterilerine karşı en yüksek aktiviteyi kekik uçucu yağı göstermiştir. Disk difüzyon yönteminde *L. acidophilus*'a karşı 57.6±0.8 zon çapı kaydedilirken, agar kuyu difüzyon yönteminde *S. mutans*'a karşı 58±0.9 mm zon çapı belirlenmiştir. Disk difüzyon yönteminde okaliptüs uçucu yağının *E. faecalis*, *L. acidophilus* ve *S. mutans* test bakterilerine karşı zon çapı belirlenebilirken kuyu difüzyon yönteminde zon çapı gözlemlenmemiştir.

Park ve Yoon (2018) uçucu yağların oral patojen bakterilere karşı aktivitelerini disk difüzyon yöntemiyle değerlendirdikleri bir çalışmada nane ve okaliptüs uçucu yağının *S. mutans*'a karşı inhibisyon çaplarını sırasıyla; 16.10±0.44 ve 14.75±1.25 mm olarak bildirmişlerdir [25]. Çalışmamızda bu değerler 38.2±0.8 ve 13.2±0.8 olarak saptanmıştır.

Galovičová ve ark. (2021) kekik uçucu yağının çeşitli bakterilere karşı etkinliğini değerledirdiği bir çalışmada uçucu yağın *E. faecalis*'e karşı disk difüzyon yönteminde 10.22±1.30 zon çapı bildirilmiştir [26]. Çalışmamızda bu değer 22±0.3 mm olarak bulunmuştur.

Çalışmamız sonucunda test edilen uçucu yağların oral patojen bakterilere karşı antibakteriyel etki gösterdiği saptanmıştır. Yöntem bulguları literatürle kıyaslandığında değişkenlik göstermektedir. Bu değişkenlik, uçucu yağların çok bileşenli karışımlar olmasına, uçucu yağların izole edildiği bitkilerin coğrafyası ve toplanma zamanına göre değişkenlik gösterebileceğine dayandıralabilir. Etkiden sorumlu bileşenler ve bu bileşenlerin miktarlarının tespit edilebilmesi ve içerdiği maddelerin fizikokimyasal özelliklerinin saptanabilmesi için yağların karakterizasyonunun yapılması aktivite sonuçlarındaki değişkenliğin daha iyi aydınlatılabilmesi açısından önem taşımaktadır.

TEŞEKKÜR

Bu çalışma TYL-2023-2776 proje kodu ile Ankara Üniversitesi Bilimsel Araştırma Projeleri Koordinatörlüğü tarafından desteklenmiştir. Çalışmanın yürütülmesi için proje desteği sunan Ankara Üniversitesi Bilimsel Araştırma Projeleri Koordinatörlüğü'ne teşekkür ederiz.

YAZAR KATKILARI

Kavram: M.S.K, N.A.; Tasarım: M.S.K., N.A.; Denetim: M.S.K., N.A.; Kaynaklar: M.S.K., N.A.; Malzemeler: M.S.K., N.A.; Veri Toplama ve/veya İşleme: M.S.K., N.A.; Analiz ve/veya Yorumlama: M.S.K., N.A.; Literatür Taraması: M.S.K., N.A.; Makalenin Yazılması: M.S.K., N.A.; Kritik İnceleme: M.S.K., N.A.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1163-1179, 2024
Doi: 10.33483/jfpau.1456868

DERLEME MAKALE / REVIEW ARTICLE

İLAÇLARDA SANAL BİYOEŞDEĞERLİK UYGULAMALARI

VIRTUAL BIOEQUIVALENCE APPLICATIONS IN DRUGS

Tuğba GÜLSÜN[1], Huriye DEMİR[2]*, Levent ÖNER[1]*

[1]Hacettepe Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 06100, Ankara, Türkiye
[2]Ondokuz Mayıs Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 55000, Samsun, Türkiye

ÖZ

Amaç: *Sanal biyoeşdeğerlik çalışmaları hem yeni ilaçların hem de jenerik ilaçların geliştirme süreçlerini kolaylaştırma ve optimize etmede kritik rol oynamaktadır. Bu yaklaşım, ilaçların insan vücudundaki davranışlarını taklit etmek ve kestirebilmek için matematiksel hesaplamalara dayanmaktadır. Sanal biyoeşdeğerlik çalışmaları ile in vitro, in siliko ve in vivo veriler kullanılarak, test ve referans formülasyonlar arasındaki farmakokinetik ve klinik performans değerlendirebilir. Bu modeller, ilaçların vücutta nasıl dağıldığını, metabolize olduğunu ve atıldığını daha duyarlı bir şekilde tahmin edebilir. Bu sayede ilaçların etkilerinin kestirilebilmesi ve dozun optimize edilmesine olanak sağlar.*
Sonuç ve Tartışma: *Sanal biyoeşdeğerlik çalışmalarının yasal düzenlemelerdeki yeri henüz tam olarak belirlenememiştir, bu nedenle ilaçla ilgili yasal otoriteler, ilaç endüstrisi, üniversiteler ve araştırma kuruluşlarının iş birliği yapması oldukça önemlidir. Özellikle ağız yolu ve diğer uygulama yolları ile kullanılan sistemik etki gösteren ilaçların, fizyolojik temelli farmakokinetik ve biyofarmasötik modelleme çalışmalarının çerçevesinin belirlenmesi, in vivo klinik çalışmalardan muafiyetin ve optimizasyonunun desteklenmesi için sanal biyoeşdeğerlik çalışmaları önemlidir. Sanal biyoeşdeğerlik çalışmaları, ilaç geliştirme süreçlerini iyileştirmek, süreyi kısaltmak ve maliyetleri düşürmek için önemli bir araç olabilir, ancak bu alandaki ilerlemelerin devam etmesi ve bu yöntemlerin ilaçla ilgili yasal düzenleme süreçlerine daha fazla entegre edilmesi gerekmektedir.*
Anahtar Kelimeler: *Biyomuafiyet, fizyolojik temelli farmakokinetik modelleme, güvenli alan, in siliko, sanal biyoeşdeğerlik, simülasyon, yapay zekâ*

ABSTRACT

Objective: *Virtual bioequivalence studies play a critical role in facilitating and optimizing drug development processes of new drugs and generic drugs. This approach relies on mathematical calculations to mimic and predict the behavior of drugs in the human body. Virtual bioequivalence studies can assess the pharmacokinetic and clinical performance between test and reference formulations by utilizing in vitro, in silico and in vivo data. This enables the prediction of drug effects and optimization of dosage.*
Result and Discussion: *The regulatory position of virtual bioequivalence studies has not yet been fully determined, making collaboration among regulatory authorities, the pharmaceutical industry, universities, and research institutions crucial. Particularly for drugs administered orally or through*

* **Sorumlu Yazar / Corresponding Author:** Levent Öner
e-posta / e-mail: loner@hacettepe.edu.tr, **Tel. / Phone:** +903123101524

* **İkinci Sorumlu Yazar / Second Corresponding Author:** Huriye Demir
e-posta / e-mail: huriye.demirkabas@omu.edu.tr, **Tel. / Phone:** +903623121919-2085

Gönderilme / Submitted : 22.03.2024
Kabul / Accepted : 16.05.2024
Yayınlanma / Published : 10.09.2024

other systemic routes, determining the framework of physiologically-based pharmacokinetic and biopharmaceutical modeling studies through virtual bioequivalence is important to support exemptions and optimization from in vivo clinical trials. Virtual bioequivalence studies can be a significant tool in improving drug development processes, reducing time, and cutting costs. However, continued progress in this field and further integration of these methods into drug-related regulatory processes are necessary.
Keywords: *Artificial intelligence, biowaiver, physiologically based pharmacokinetic modeling, in silico, safe space, simulation, virtual bioequivalence*

GİRİŞ

Aynı etkin maddeyi, aynı molar dozda içeren, farmasötik eşdeğer veya farmasötik alternatif iki ilaç, biyoyararlanımları (emilme hız ve dereceleri) önceden belirlenmiş kabul sınırları içerisinde ise, biyoeşdeğer olarak kabul edilir [1-3]. Jenerik farmasötik ürünler, aynı etkin maddeyi, aynı molar dozda içeren referans ürüne terapötik olarak eşdeğer ise piyasaya sunulur [1]. Dünya Sağlık Örgütü (World Health Organization, WHO) ve Amerika İlaç ve Gıda Ajansı'na (Food and Drug Administration, FDA) göre terapötik eşdeğerlik, referans ilacın piyasaya sunulmuş olması koşulu ile, ilaç etkin maddesi ve dozaj şeklinin özelliklerine göre, biyoeşdeğerlik, *in vitro* çözünme, farmakodinamik veya klinik çalışmalardan uygun olanın yapılması koşulu ile sağlanır [4]. Bu çalışmalar herhangi bir yeni ilaç geliştirilmesi sürecinde de (formülasyon değişikliği, klinik deneme formülasyonunda yapılan değişiklerde, ölçek büyütmelerde) uygulanır. İki formülasyon arasındaki biyoeşdeğerliği belirlemek için, klinik çalışmalar genellikle önceden belirlenen ölçütleri sağlayan sağlıklı gönüllülerde gerçekleştirilir [2]. Bu çalışmalar oldukça sıkı yasal düzenlemeler nedeniyle uzun zaman alır ve maliyeti de yüksektir. İlaç geliştirme ve üretiminde kalite, etkililik ve güvenlilikten ödün vermemek koşulu ile maliyetlerin azaltılması ve zamandan tasarruf sağlanması, son yıllarda yapay zekanın da ilaç geliştirme süreçlerine dahil olması ile çok fazla önem kazanmıştır. Yeni etkin madde içeren yeni ilaç, bilinen etkin madde içeren yeni ilaç ve jenerik ilaç geliştirme süreçlerinde, terapötik eşdeğerliğin sağlanması için, bilimsel olarak gerekçelendirilerek uygulanan biyoeşdeğerlik çalışmalarından muafiyet, 1995 yılından itibaren yoğun şekilde tartışılmakta ve uygulanmaktadır [3]. Uluslararası Uyum Konseyi (International Council for Harmonisation, ICH), 2020 yılında biyoeşdeğerlik çalışmalarından muafiyet ölçütlerini, etkin madde ve bitmiş ürün özellikleri yönünden açıklayan oldukça kapsamlı bir rehber yayımlamıştır [5]. Bu rehbere göre biyoeşdeğerlik çalışmalarından vazgeçme kriterlerinin yerine getirilebilmesi için öncelikle etkin madde ve dozaj şekline ilişkin sağlanması gereken ölçütlerin yerine getirilmesi gerekir. Bu ölçütlerden farmasötik dozaj şekli için en önemlisi mide bağırsak kanalı pH'sını taklit eden ortamlarda yapılan çözünme yönteminden elde edilen sonuçlardır. Biyofarmasötik spesifikasyon için çözünme yönteminin uygulanması, ölçek büyütme ve onay sonrası değişikliklerde (Scale-Up and Post-Approval Changes, SUPAC) ilk kez FDA tarafından önerilmiştir [6]. Daha sonra bu öneri, ağız yolu ile uygulanan ve hemen salım sağlayan jenerik ürünleri içerecek şekilde genişletilmiştir. Biyoeşdeğerlik çalışmalarından vazgeçme düşüncesi, özellikle ölçek büyütme ve onay sonrası değişikliklerde, düşük dozlarda ve bazı durumlarda yüksek dozlarda yenilikçi ve jenerik ilaç üreticileri tarafından benimsenmiş, Biyofarmasötik Sınıflandırma Sistemine (Biopharmaceutics Classification System, BCS) göre biyomuafiyet kriterlerini sağlayan ilaçlarda başarıyla uygulanmaktadır. Etik yönden insan çalışmalarına olan gereksinimi azaltmak, zaman ve maliyet tasarrufu sağlamak amacıyla biyoeşdeğerlik çalışmalarından vazgeçme önem kazanmaktadır.

Fizyolojik Temelli Farmakokinetik Modelleme

Son yıllarda *in vitro*, *in vivo* ve klinik yönden ilişkilendirilen modelleme ve simülasyon uygulamalarını içeren, *in vitro in vivo* korelasyon (*In Vitro-In Vivo* Correlations, IVIVC) [7], fizyolojik temelli emilim modellemesi (Physiologically Based Absorption Modeling, PBAM) [7], fizyolojik temelli farmakokinetik modelleme (Physiologically Based Pharmacokinetic Model, PBPK), fizyolojik temelli biyofarmasötik modelleme (Physiologically Based Biopharmaceutics Model, PBBM) [8,9] yöntemleri ilaç araştırmalarında yaygın olarak kullanılmaktadır. Son 15 yılı kapsayacak şekilde yapılan bir literatür taraması, klinik yönden ilişkilendirilmiş çözünme spesifikasyonları, çözünme

spesifikasyonlarına bağlı PBPK veya PBBM uygulamaları ve formülasyon köprüleme ile bağlantılı PBPK veya PBBM uygulamaları ile ilgili yayınların 2011 yılında başladığını ve sayılarının katlanarak arttığını ortaya koymaktadır [10]. İlaç ürünlerinin farmakolojik veya biyofarmasötik yönden yapılandırılan mekanistik modellerinde, ilacın klinik öncesi çalışmaları, klinik çalışmaları ve *in vitro* testlerde geçirdiği süreçler, ilacın kimyasal, fiziksel ve biyolojik/fizyolojik özellikleriyle ilişkilendirilir. Bunlardan bazıları, etkin maddenin ilaç ürününden salımı, ilacın emilimi, dağılımı, metabolizması, atılımı ve farmakodinamik özellikleridir. Mekanistik modeller, etkin maddenin fizikokimyasal özelliklerine, ilaç ürününün formülasyon özelliklerine ve etkin madde ile formülasyonun fizyolojik sistemle etkileşimine dayalı olarak ürünün güvenliliği ve etkililiğiyle ilgili bilgi sağlamayı amaçlayan bütünleştirici sistemlerdir [11]. Bu sistemler, sistemik olarak kanda veya belirli bir dokuda ilaca maruziyetin kestirimini yapabilir. Bu tür bir mekanistik modelleme yaklaşımında, etkin maddenin fizikokimyasal özelliklerini ve *in vitro* çözünme ve partikül büyüklüğü dağılımı gibi bilgilerini, fizyolojik faktörlerle bütünleştiren kompartman tabanlı bir modelleme yaklaşımı olan PBPK modellemesinin uygulanmasıyla, etkin maddenin sistemik ve farklı dokulardaki lokal maruziyetinin kestirimi yapılabilir. Biyoeşdeğerlikle ilgili uygulamalarda, modeldeki formülasyon farklılıklarını tanımlayabildikleri için, çoğunlukla modellerin mekanistik emilim bileşenlerine odaklanılmıştır [12]. Diğer mekanistik modelleme yaklaşımı da sıvı ve partikül taşınmasını izlemek için uygulanan, fizik tabanlı bir modelleme yaklaşımı olan hesaplamalı akışkanlar dinamiğidir (computational fluid dynamics). Bu modelleme yaklaşımı, inhalasyon yolu ile yapılan ilaç uygulamalarında aerosollerin birikim yerini izlemek veya ağız yolu ile uygulanan ilaçlarda mide bağırsak kanalı boyunca katı dozaj şeklinin hareketini ve değişimini belirlemek için kullanılabilir [13,14]. PBBM ise, *in vitro-in vivo* ilişkinin kurulması için biyofarmasötik modellemenin kullanımına odaklanmaktadır. Oral absorpsiyonu tahmin etmek, formülasyon geliştirmeyi desteklemek ve klinik öncesinden kliniğe geçişte PBAM uygulanması ile ilgili çok sayıda çalışma mevcuttur. Daha sonraki aşamalarda PBAM modelleri, üretim prosesi veya formülasyon bileşimindeki değişiklik üzerine formülasyonların performansını değerlendirebilir. Onay sonrası değişiklikler de sanal klinik araştırmalarla ve biyofarmasötik güvenli alanın oluşturulmasıyla desteklenebilir [15,16]. Ayrıca bu modeller yasal otoritelerin inceleme ve karar alma süreçlerinde rutin olarak kullanılmaya başlanmıştır [17].

Sanal (Virtual) Biyoeşdeğerlik

Son yıllarda, ilaç sektöründeki hızlı gelişim ve verimlilik arayışı, araştırmacıları ilaç geliştirme sürecinde yenilikçi ve geliştirilebilir yaklaşımlara yönlendirmiştir. Bu yaklaşımlardan birisi de ilaçların insan vücudundaki davranışlarının taklit edilmesi ve kestiriminin yapılmasında çeşitli matematiksel hesaplamalara dayanan bir uygulama olan sanal biyoeşdeğerlik çalışmalarıdır. Jenerik ve yenilikçi ilaç üreticilerinin ilaç geliştirme sürecinde karşılaştıkları süreci hem zaman hem de ekonomik yönden etkileyen kritik aşamalardan birisi de biyoeşdeğerliğin kanıtlanmasıdır. Bu nedenle, klinik çalışmaların sayısını azaltmak için sanal biyoeşdeğerlik, modelleme ve simülasyon kullanımına ilişkin çalışmalar, son yıllarda üzerinde en çok araştırma yapılan konulardandır. Sanal biyoeşdeğerlik çalışmaları; *in vitro, in siliko* ve *in vivo* arasındaki translasyonel ilişkilere dayanarak test ve referans formülasyonlar arasındaki farmakokinetik ve klinik performanstaki benzerlik ve potansiyel farklılıkları değerlendirebilir. Sanal biyoeşdeğerlik, ilaçların biyoeşdeğerliğinin kestiriminde hesaplama yöntemlerinden yararlanan alternatif bir yaklaşım sunmaktadır, böylece klinik deneylere olan gereksinimi azaltır. İlaç emilim, dağılım, metabolizma ve atılım (ADME) süreçlerini taklit eden matematiksel modellerin geliştirilmesi ve uygulanması sanal biyoeşdeğerlik çalışmalarının temelini oluşturur. Özellikle *in vitro* verilere dayanan fizyolojik temelli farmakokinetik (PBPK) çalışmaları, *in vivo* karşılaştırmalı klinik sonlanım noktalı biyoeşdeğerlik çalışmalarının yerine geçen uygun maliyetli bir seçenektir [18]. Ayrıca, sanal biyoeşdeğerlik, belirli durumlarda BCS Sınıf 1 ve 3 ilaçlara uygulanan BCS tabanlı biyomuafiyeti, daha fazla etkin maddeye uygulama (genişletme) kabiliyetine sahiptir [18]. Fizyolojik tabanlı popülasyon farmakokinetik modellemesi, *in vitro* biyofarmasötik araçlarla birleştiğinde, sanal biyoeşdeğerlik oluşturmak ve klinik olarak ilgili spesifikasyonları belirlemek için güçlü bir araçtır. Sanal biyoeşdeğerlik uygulaması, geleneksel biyoeşdeğerlik yöntemlerine kıyasla hem zaman hem de ekonomik yönden tasarruf sağlayarak hastalara daha hızlı ve güvenli ilaçlar ulaştırmayı amaçlamaktadır.

Yenilikçi ve jenerik ilaçların hızlı bir şekilde geliştirilmesine katkı sağlayacak önemli ölçüde klinik araştırmalar verisi sanal biyoeşdeğerlik araştırmaları ile elde edilebilir. Dolayısıyla, sanal biyoeşdeğerlik, klinik olarak ilgili spesifikasyonları belirlemek ve sağlıklı veya hasta popülasyonlarda, ayrıca çocuk hastalar, proton pompası inhibitörlerinin birlikte uygulandığı ilaçlar ile yapılan çalışmalar gibi özel durumlarda da beklenen klinik sonuçları tahmin etmek için güçlü bir araç olarak karşımıza çıkmaktadır.

PBPK Modeli ve Sanal Biyoeşdeğerlik Modeli Arasındaki İlişki

Sanal biyoeşdeğerlik modeli, *in vitro* çözünme verileri ile bütünleştirilen, PBPK modelinin genişletilmiş bir uygulaması olarak düşünülebilir. Ağız yolu ile uygulanan ilaçlar için sanal biyoeşdeğerlik modeline özgül durumlar aşağıda belirtilmiştir [19]:

i) Emilim modeli, yani bir ilaç etkin maddesinin formülasyondan salımını ve ardından sistemik dolaşıma geçişini tanımlayan modeldir. Referans ve test ürünleri arasında atılım süreçlerinde bir fark olmaması bu modelde önemlidir [20];

ii) Sanal biyoeşdeğerlik çalışmalarında, ürünler arasındaki *in vivo* farmakokinetik davranışın benzerliklerini ve farklılıklarını değerlendirmek için, *in vitro* çözünme yaklaşımı PBPK modeli ile bütünleştirilmiştir [21,22]. *İn vitro* çözünme ölçütü, mide bağırsak kanalında *in vivo* çözünme ölçütü ile ilişkilendirilir ve formülasyonlar arasındaki farklılıkları değerlendirmeye yardımcı olur [21,23].

iii) Popülasyonun değişkenliği, biyoeşdeğerlik değerlendirmesinde kritik rol oynadığından, test ve referans ürünler arasındaki farklılıkların değerlendirilmesinde oldukça önemlidir [20,24].

PBPK modellemesi bir ilacın tüm *in vivo* emilim, dağılım, metabolizma ve atılım davranışını taklit etmek için uygulanır, sanal biyoeşdeğerlik modeli ise, özellikle ağız yolu ile kullanılan ilaçlar için emilim modellemesine odaklanır ve test ile referans ürünler arasındaki farkları değerlendirmeyi amaçlar.

IVIVC ve Sanal Biyoeşdeğerlik Çalışması Arasındaki İlişki

IVIVC kavramı, *in vitro* salım (çözünme profili) ile *in vivo* yanıt (plazma ilaç konsantrasyonu veya emilen ilaç miktarı) arasındaki nicel bağlantıyı ifade eder. Bu terim, öncelikle çözünme spesifikasyonlarını belirlemek ve ağız yolu ile uygulanan uzatılmış salım sağlayan ilaçlar için *in vivo* biyoeşdeğerlik değerlendirmelerine aracılık etmek amacıyla ortaya atılmıştır. İlk yıllarda IVIVC, *in vitro* çözünme verileri kullanılarak *in vivo* çözünme davranışını taklit etmek için uygulanmıştır [19,23,25]. Bir ilacın emiliminin ilk adımı, formülasyondan salımı ve *in vivo* çözünmesidir. Bu nedenle, ilacın emilimi bir ilacın *in vivo* farmakokinetiğini tahmin etmek için önemli bir gösterge olarak kabul edilebilir. Ancak, farmakokinetik davranış, sitokrom P450 enzimleri, taşıyıcılar ve diğer birçok faktörlerden de etkilenir. Bu faktörler, özellikle *in vivo* çözünme davranışı dikkate alınarak geliştirilen IVIVC'lerde yer almamaktadır [19,26]. Son zamanlarda, çözünme, emilim, metabolizma ve ileri kompartmantal emilim geçişi gibi PBPK emilim modelleri, bir ilacın mide bağırsak kanalındaki çözünme, permeasyon ve translasyon süreçlerini kantitatif olarak tanımlayan fizyolojik temelli IVIVC oluşturmak için kullanılmaktadır [19,27,28]. Bu modeller, fizyolojik koşulları taklit edebildikleri gibi *in vivo* farmakokinetiği tahmin etmek üzere sanal biyoeşdeğerliğin temelini oluştururken sürece çözünme bilgilerini de dahil edebilir [19].

Sanal Biyoeşdeğerlik ve Yasal Yönü

Fizyolojik temelli farmakokinetik modelleme, mükemmele yakın tahmin yetenekleri nedeniyle giderek artan bir ilgi görmektedir. Web of Science Core Collection veri tabanında PBPK modelleme ile ilgili makalelerin tarandığı çalışmada 1999'dan 2023'e kadar toplam 4.649 kayıt tespit edilmiş ve en fazla sayıda makalenin 2018-2023 yılları arasında yayımlandığı bildirilmiştir [29]. FDA'ya 2008-2013 yılları arasında 112 adet PBPK başvurusu yapılmıştır. 2014 yılında yapılan PBPK başvuru sayısı ise 45'tir [30]. 2008-2017 yılları arasında ise, PBPK analizler içeren 130 adet araştırma amaçlı yeni ilaç başvurusu ve 94 adet yeni ilaç başvurusu yapılmıştır [31]. Bu başvurularda genellikle klinik sorunları ele almak, intrinsik veya ekstrinsik etkenlerin ilaç farmakokinetiği üzerindeki etkilerini değerlendirmek için çeşitli modeller uygulanmıştır. PBPK modellerinin ruhsat dosyalarına dahil edilmesi ve resmi otoritelere başvuru sayısının artması ile, akademi ve endüstri arasında bilgi ve uygulamalara ait paylaşımı artırmak için çeşitli çalıştaylar düzenlenmeye başlanmıştır. Devam eden süreç içerisinde

PBPK doz seçimleri, model tahmin performansları, *in vitro-in vivo* bağlantı kurmaya yönelik çözünme ve translasyonel modelleme stratejileri, hasta merkezli ürün geliştirme ve olgu çalışmaları kullanılarak PBPK modelleme uygulamaları, düzenleyici karar verme, ürün etiketleme, biyokestirimsel çözünme yöntemleri (*in vitro* çözünme test koşulları ile farmakokinetik profilleri tahmin etme yeteneği), model bilgilendirmeli formülasyon seçimi, klinik performansın tahmin edilmesi, jenerikler için biyoeşdeğerlik güvenli alanının oluşturulması gibi konular zaman zaman ilaç otoritelerinden temsilcilerin katıldığı platformlarda tartışılmıştır [28,32-35]. Bu çalıştaylardan elde edilen raporlarda otoriteler tarafından modellerin reddedilme nedenleri "modelin amaca uygun olmaması", "test edilen uyarlamaların sorunları yansıtamaması", "klinik verilere karşı yeterli model doğrulamasının sağlanamaması" olarak belirtilmiştir. FDA, EMA (European Medicines Agency) ve diğer düzenleyici otoriteler sanal biyoeşdeğerliğin potansiyelini fark etmiş ve uygulanmasına yol göstermek için çeşitli girişimler ve rehberler oluşturmaya başlamıştır. EMA tarafından "Fizyolojik Temelli Farmakokinetik (PBPK) Modelleme ve Simülasyonun Raporlanmasına İlişkin Rehber" yayımlanırken, FDA tarafından ise endüstri için, "Fizyolojik Temelli Farmakokinetik Analizler-Format ve İçerik" ve "Fizyolojik Temelli Farmakokinetik Analizlerin Kullanımı-Ağız Yolu ile Uygulanan İlaç Ürünü Geliştirme İçin Biyofarmasötik Uygulamalar, Üretim Değişiklikleri ve Kontrolleri" rehberleri yayımlanmıştır [8,36,37]. Her bir otorite tarafından hazırlanan rehberler, modelleme ve simülasyon yaklaşımları ve bunların başarılı bir şekilde uygulanabileceği durumlar hakkında genel bir bakış sunmaktadır. Bu rehberlerde modeller geliştirilirken göz önünde bulundurulması gereken etkenler, geliştirme stratejileri, veri gereksinimleri, doğrulama ve geçerleme yaklaşımları ayrıntılı olarak verilmiştir. Bu rehberlerin en önemli özelliği, çözünme yönteminin geliştirilmesi, klinik olarak ilgili çözünme spesifikasyonlarının belirlenmesi, kalite risk değerlendirmesi, onay sonrası değişikliklerin desteklenmesi, klinik çalışma sayısının azaltılması, kalite spesifikasyonlarının ve biyomuafiyet oluşturulmasına yardımcı olan PBPK model geliştirme uygulaması ile bu modellerin dosyada sunulmasıdır [8,36-38]. FDA ilk kez, klinik *in vivo* farmakodinamik sonlanım noktalı çalışma yerine fizyolojik temelli farmakokinetik ile sanal biyoeşdeğerlik kullanan diklofenak sodyum içeren jel preparatını dermal kullanım için 2019 yılında, kısaltılmış yeni ilaç başvurusu olarak onaylamıştır. Bu onayda, tipik klinik biyoeşdeğerlik çalışmasına alternatif olarak deri yoluyla diklofenak emiliminin nicel açıklaması için Simcyp™ Simulator (Sürüm 17; Certara, Princeton, NJ, ABD) içinde uygulanan çok fazlı çok katmanlı (Multi-Phase Multi-Layer, MPML) MechDermA (Mechanistic Dermal Absorption) modeli kullanılmıştır.

Ayrıca Japonya'da yasal otorite, PBPK model analizi ve analiz raporları için çalışmalar yapmış, bu çalışmaları konuyla ilgili paydaşların görüşüne sunmuştur [35]. PBPK'nın yenilikçi ve jenerik ilaç geliştirmedeki uygulamalarına FDA'nın bakış açısı 2019 yılında jenerik ilaç çalıştayında tartışılmıştır. Yeni geliştirilen ilaçlar için klinik öncesi aşamada aday seçimi, hayvandan insana ekstrapolasyon çalışmaları, ön formülasyon seçimi, hastalıkların etkisinin değerlendirilmesi, özel popülasyon için doz ayarlaması (organ yetmezliği, çocuk popülasyonu) gibi uygulamalar, jenerik ilaçlar için ise ürüne özgül rehberler (ilaç salım mekanizmasındaki değişiklik için risk değerlendirmesi), biyoeşdeğerliğin kanıtlanması için alternatif yaklaşımlar (biyoeşdeğerlik yerine *in vitro* testler, lokal etkili ilaçlar), alt popülasyonlarda biyoeşdeğerlik değerlendirmesini ekstrapole etme (hastalık, yaş), ilaçların klinik özellikleri için "Safe Space (güvenli alan)" oluşturulması (*in vitro* çözünme özellikleri) gibi uygulamalar, PBPK'nın yenilikçi ve jenerik ilaç geliştirme sürecinde, FDA'nın yaklaşımını ortaya koymaktadır.

Türkiye'de sanal biyoeşdeğerlik çalışmaları ve PBPK için herhangi bir rehber henüz yayımlanmamıştır. Sanal biyoeşdeğerlik, modelleme ve simülasyon tekniklerini kullanarak ilaç eşdeğerliğinin verimli bir şekilde değerlendirilmesini sağlar, dolayısı ile kaynak gereksinimlerini en aza indirir. Kapsamlı ve yüksek maliyetli klinik çalışmalara olan gereksinimi azaltarak maliyetin düşmesine, insan deneylerine olan bağımlılığın azalmasına, bu üstünlüklerden dolayı ilaçların ruhsat başvuru süresinin kısalmasına olanak sağlar [39].

Sanal Biyoeşdeğerlik Uygulamaları

PBPK modellemenin biyofarmasötik uygulamalar için *in vivo* kestirim yeteneği, ilaç endüstrisinde formülasyon geliştirme de dahil olmak üzere geniş bir kullanım alanına sahiptir. Bu uygulamalar, biyokestirimsel çözünme yönteminin geliştirmesini, klinik olarak ilgili ürün

spesifikasyonlarının belirlenmesini, kalite risk değerlendirmesini ve ilacın yaşam döngüsü yönetimini içerir. FDA'nın ilgili rehberinde, biyofarmasötik uygulamalar için PBPK modellemesinin, ilk onay sürecinde formülasyon ve/veya üretim süreci değişikliklerine bağlı olarak yapılması gereken *in vivo* biyoyararlanım/biyoeşdeğerlik çalışmalarının sayısını azaltabileceği, ayrıca ürün ölçek büyütme ve onay sonrası değişiklikleri de destekleyebileceği belirtilmiştir [8]. Sanal biyoeşdeğerlik uygulamalarının yapıldığı çalışmalar aşağıdaki başlıklar altında incelenebilir.

İnsan Farmakokinetiğinin/Dozunun Kestirimi

Matematiksel modeller ve simülasyonlar, farmakokinetik ölçütlerin ve farklı dozlara yanıtların kestiriminde de kullanılır. Bu yaklaşım sayesinde, klinik çalışmalar başlamadan önce en uygun dozaj şekli, en güvenli ve etkili doz belirlenebilir. Az çözünen aripiprazol için, oral çözeltiye karşı tablet, enzalutamid için, sıvı kapsüle karşı amorf katı dispersiyon içeren tablet formülasyonlarında sanal biyoeşdeğerlik değerlendirmeleri yapılmıştır. Mide bağırsak kanalının fizyolojik parametrelerindeki değişkenlikler, *in vitro* biyouyumlu çözünme testlerinde, *in siliko* modelleme ve PBBM simülasyonlarında dikkate alınmıştır. Dozaj şekilleri arasındaki biyoeşdeğerliği kestirebilmek için, sanal insan gönüllülerin performanslarındaki bireylerarası değişkenlikler *in vitro* çalışmalardan kestirilmiş, mide boşalması ve midedeki sıvı hacmindeki değişkenlikler dikkate alınmıştır. Her iki model de ilacın çözelti ve tablet formülasyonlarının *in vivo* performansını yeterli düzeyde kestirebilmiş ve daha duyarlı kestirimler yapabilmek için, mide bağırsak kanalının fizyolojik parametrelerinin değişkenliklerinin, birey içi ve bireylerarası değişkenliklerin ve farklı zamanlardaki durum değişikliklerinin betimlenmesinin önemli olduğu sonucuna varılmıştır [40]. Diğer yandan, imatinib için uygulanan PBPK modeli, kronik faz ve akselere faz hastalarda tek başına ve altı modülatörle birlikte uygulanan, karaciğer yetmezliği olan kronik faz hastalarda, imatinibin minimum plazma konsantrasyonunu başarılı bir şekilde kestirmiş ve doz ayarlamalarına yönelik öneriler sunmuştur [41].

Formülasyon/Üretim Yöntemi Farklılıklarında Biyoeşdeğerliğin Kestirimi

İlaçla ilgili tüm süreçlerde oldukça katı yasal düzenlemeler söz konusudur, özellikle üretim yeri değişiklikleri gibi üretim sürecinde yapılan değişiklikler genellikle karmaşık ve maliyeti yüksek işlemlerdir. Sanal biyoeşdeğerlik, ilaç araştırma ve geliştirme çalışmaları sırasında formülasyonda ve/veya üretim yönteminde yapılan değişikliklerin ürünün biyoeşdeğerliği üzerindeki potansiyel etkilerini incelemek amacıyla uygulanabilir. Özellikle, sanal biyoeşdeğerlik değerlendirmesi, yeni üretim yöntemlerinin ve/veya yeni formülasyonların referans ilaçla aynı terapötik eşdeğerliğe sahip olup olmadığını belirlemek için yapılabilir. BCS sınıf 2 bir etkin madde olan piroksikam ile yapılan bir çalışmada PBPK modellemesi, köpeklerde farmakokinetiği kantitatif olarak kestirmek ve türler arası ekstrapolasyon yapmak için bir araç olarak kullanılmıştır. Öncelikle, köpeklerde açlık ve tokluk durumlarında PBPK modelleri oluşturulmuş, daha sonra, *in vitro* çözünme oranlarının *in vivo* farmakokinetik performans üzerindeki etkisini değerlendirmek için bir Z-faktörü modeli entegre edilmiş, piroksikamın hemen salım sağlayan dozaj şekillerinin biyoeşdeğerlik açısından beklenenden çok daha geniş bir çözünme alanına sahip olduğu gösterilmiştir. Parametre duyarlılık analizi (Parameter Sensitivity Analysis, PSA) testine göre, partikül büyüklüğü 150 μm'nin altında yüksek oral emilim elde edilmiştir. Son olarak, birleştirilmiş PBPK modelleri insanlara ekstrapole edilerek kalite kontrol stratejisi belirlenmiştir. Bu ekstrapolasyon, piroksikamın hemen salım sağlayan formülasyonları için biyomuafiyetin söz konusu olabileceğini, ayrıca geliştirilen yöntemle insan farmakokinetiğinin kantitatif olarak kestiriminin yapılabileceğini ve elde edilen bulguların ölçek büyütme veya onay sonrası değişiklikler için anlamlı olabileceğini göstermiştir [42].

Optimum Klinik Çalışmalar Tasarlamak

Modelleme ve simülasyon teknikleri kullanılarak klinik çalışma tasarımını optimize etmek amacıyla sanal biyoeşdeğerlik kullanılabilir. Bir başka deyişle, klinik çalışmaya başlamadan önce, sanal biyoeşdeğerlik, hangi dozaj şeklinin, hangi yitilikte, hangi popülasyona, hangi koşullarda uygulanacağı hakkında bilgi sağlayabilir.

Yeni İlaç Formülasyonlarını Değerlendirmek

Sanal biyoeşdeğerlik, yeni geliştirilen ilaç formülasyonlarının geliştirme sürecini hızlandırmak amacıyla kullanılan bir yöntemdir. Bu yöntem, daha az klinik çalışmayla ürünün etkililiği ve güvenliliğinin değerlendirilmesine dolayısı ile daha güvenli ve etkili formülasyonların kısa sürede piyasaya çıkmasına olanak sağlar. Bu bağlamda, tasarımda kalite (Quality by Design, QbD) paradigması altında bir ilacın akılcı tasarımında emilim modellemesinin potansiyel uygulamasını göstermek için bir dizi olgu çalışması yapılmıştır. Bu çalışmalarda ilk olarak yüksek mide pH'sına karşı en az duyarlılığa sahip bir formülasyon geliştirilmesi için emilim modellemesinin insan çalışmalarından önce uygulanması örneklenmiştir. Bu yaklaşımda öncelikle proton pompası inhbitörleri ve/veya H2 reseptör antagonistleri ile birlikte kullanıldığında etkileşimi azaltan bir formülasyonun geliştirilmesi planlanmıştır. İkinci olarak, minimum plazma konsantrasyonunu sağlamak ve günde bir dozlamayı mümkün kılmak için optimum salım hızına sahip kontrollü salım formülasyonunun tasarımı hakkında çalışmalar yapılmıştır. Üçüncü olarak etkin maddenin partikül büyüklüğü dağılımının tablet biyoyararlanımı üzerindeki etkisini anlamak ve geç faz formülasyon tasarımını yönlendirmek için emilim modellemesi kullanılmıştır. Dördüncü olarak etkin maddenin kolay çözünen tuz şekli veya katı dispersiyon halinde stabilize edilen amorf şekli ile daha güç çözünen serbest şeklinin (baz şekli) oranlarının değiştirilmesi sonucu, ürün performansı üzerindeki etkisinin değerlendirilmesi ve spesifikasyonlarının yönlendirilmesi amacıyla çalışma tasarlanmıştır. Son olarak, çözünme hızı değişikliklerinin formülasyonun biyoperformansı üzerindeki etkisini araştırmak ve uygun spesifikasyonları sağlamak için çalışma planlanmıştır. Bu olgu çalışmaları, hastalara optimum yarar sağlayacak stabil bir ilacın tasarımını yönlendirmek için, ürün performansını etkileyen kritik etkenlerin anlaşılmasında, fizyolojik temelli emilim modellemesi kullanımının önemini vurgulamaktadır [43].

İlaç-İlaç Etkileşimlerinin Kestirimi

Sanal biyoeşdeğerlik, kombine ilaçların daha güvenli ve etkili bir şekilde kullanılabilmesi için önemli bir araç olarak öne çıkmaktadır. Özellikle, farklı etkin maddelerin birbirleriyle etkileşimlerini kestirebilmek, bu etkileşimlerin farmakokinetik parametreler üzerindeki etkilerini incelemek amacıyla sanal biyoeşdeğerlik yöntemleri kullanılabilir. Sanal biyoeşdeğerlik sayesinde ilaç kombinasyonlarının olası etkileşimleri önceden tahmin edilebilir, bu da klinik çalışmalara geçmeden önce ilaç kombinasyonlarının güvenli ve etkili bir şekilde kullanılmasını sağlar. Ayrıca, sanal biyoeşdeğerlik, ilaç kombinasyonlarının dozlarının belirlenmesinde de kullanılabilir. Etkileşimlerin kestirimi sayesinde, her bir ilacın etkisini en üst düzeye çıkarmak ve yan etkileri en aza indirmek için uygun dozlar belirlenebilir. Bu da hastaların tedaviye daha iyi yanıt vermesini sağlayabilir [44].

İlaç-Besin Etkileşimlerinin Kestirimi

Sanal biyoeşdeğerlik, ilaçların besinlerle etkileşimini, bu etkileşimin biyoeşdeğerlik üzerine etkilerini önceden kestirebilir. Özellikle PBPK modellemesi kullanılarak besin etkilerinin kestirimi ile endüstri ve ilaçla ilgili yetkili otoriteler için destekleyici veriler sağlanır. Bu modellemeler tokluk biyoeşdeğerlik çalışmalarının temelini oluşturabilir [19,45]. Örnek bir uygulamada BCS sınıf 2 etkin madde içeren amorf ve kristal yapıda iki farklı formülasyon için tokluk koşullarında yapılan araştırmada, besin etkisi ve biyoeşdeğerlik riski değerlendirilmiştir. Mide bağırsak parametrelerinin değişkenliği göz önünde bulundurularak yiyecek etkisinin GastroPlus™'ta simülasyonu için tokluk durumu fizyolojisi seçilmiştir. Bu durumda mide hacmi, pH değeri ve gastrointestinal geçiş süresi de değişmektedir. Oluşturulan model, ilaçların besin alımıyla nasıl etkileşime gireceğini ve bu etkileşimlerin biyoeşdeğerlik üzerindeki potansiyel etkilerini kestirebilmiştir [46]. Başka bir çalışmada ise, BCS sınıf 1, 2 ve 3 etkin maddeleri içeren altı farklı hemen salım sağlayan ilacın açlık ve tokluk biyoeşdeğerliklerinin kestirimi yapılmıştır. Bu çalışmada, sanal biyoeşdeğerlik çalışmasına olan güveni artırabilecek ilaç özellikleri, modelleme ve doğrulama verileriyle ilgili bazı kısıtlamalar vurgulanmıştır. Örneğin, BCS sınıf 3 etkin madde içeren ilaç için biyoeşdeğerlik tahminlerinin karmaşık olabileceği ve bu durumun ilacın besin bileşenlerine bağlanması nedeniyle daha fazla araştırma gerektirdiği belirtilmiş ve bu etkin maddeyi içeren formülasyon için biyoeşdeğerlik sağlanamamıştır. Besin etki mekanizmaları bilinen BCS sınıf 1 ve 2 etkin maddelerde model doğrulaması için açlık koşullarında *in vivo* verilerin

elde edilmesi durumunda ve farmakokinetik parametrelerin güvenilir kestirimi sonucu sanal biyoeşdeğerlik çalışmaları kullanılarak tokluk biyoeşdeğerlik çalışmalarından vazgeçilebilir. Ancak, daha fazla örnek üzerinde çalışma yapılması ve daha geniş kapsamlı araştırmaların yapılması gerekmektedir [47].

Farklı Klinik Popülasyonlardaki Farmakokinetik Sonuçların Kestirimi

Sanal biyoeşdeğerlik, bir ilacın farklı klinik popülasyonlardaki (pediyatri, geriyatri, organ bozukluğu, hastalık durumları, etnik farklılıklar gibi) farmakokinetik sonuçlarını kestirmek için kullanılabilir. Bu yaklaşım, ilacın geniş bir hasta kitlesi üzerindeki etkililiğini ve güvenliliğini önceden kestirmeyi amaçlar. Dikkat eksikliği hiperaktivite bozukluğu (DEHB) olan çocuklar ve ergenlerde guaifenesin uzatılmış salım sağlayan tabletin doz ayarlaması için potansiyel gereklilikleri belirlemek amacıyla yapılan bir çalışmada, sanal biyoeşdeğerlik kullanılmıştır. Bu çalışmada, ilacın CYP3A4 inhibitörleri ve indükleyicileri ile birlikte kullanılması durumunda oluşabilecek ilaç etkileşimlerinin kestiriminin gerekliliği üzerinde durulmuştur. Simcyp™ Simulator PBPK modeli, klinik farmakokinetik veriler kullanılarak geliştirilmiş ve doğrulanmıştır. Bu model, ilacın farklı popülasyonlardaki farmakokinetik özelliklerini öngörmek, özellikle, CYP3A4 inhibitörleri ve indükleyicileri ile birlikte kullanıldığında ilaç etkileşimlerini önceden kestirebilmek ve bu etkileşimlerin doz ayarlaması gerektirip gerektirmediğini belirlemek için uygulanmıştır [48].

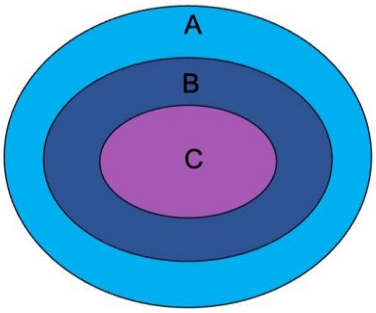
Formülasyonun İn Vivo Performans Üzerindeki Etkisinin İn Vitro Çözünme Davranışı İle Kestirimi

Formülasyonun *in vitro* çözünme hızının *in vivo* davranışı üzerindeki etkilerini anlamak ve formülasyonu bu doğrultuda optimize etmek için sanal biyoeşdeğerlik kullanılabilir. BCS sınıf 2 ilaç özelliklerinin incelendiği, emilim bölgesinde zayıf asidik ve iyonize olabilen ilaçlarla yapılan bir çalışmada, yapay sinir ağları kullanılmıştır. Mide bağırsak kanalının farklı bölgelerindeki çözünürlük ve çözünmeyi kestirmek için pH ve yüzey etkin madde varlığı gibi değişkenler dikkate alınmıştır. Kestirimi yapılan *in vitro* çözünme bulguları deneysel olarak test edilerek doğrulanmış, bu verilerin biyoeşdeğerlik kriterlerine uygunluğu değerlendirilmiş ve *in vivo* çözünmenin kestiriminin yapılabileceği belirtilmiştir [49].

Referans ve Jenerik İlaçlar İçin Güvenli Alanın Belirlenmesi

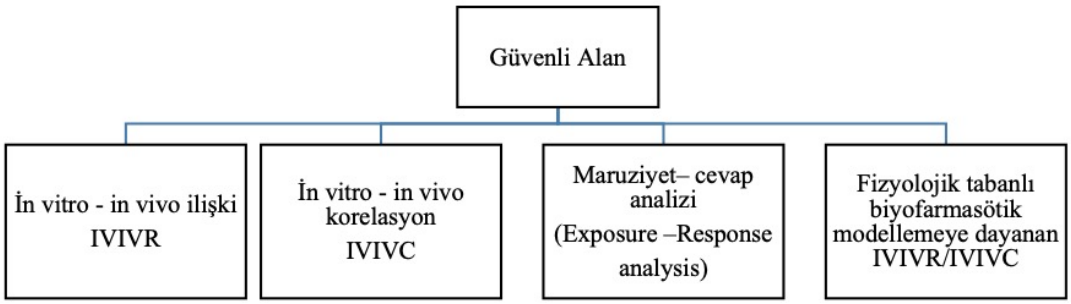
Başarılı bir ilaç geliştirme için ağız yolu ile uygulanacak ilaçların geliştirilmesinde kritik öneme sahip olan etkenlerden birisi de biyoeşdeğerlik güvenli alanının tanımlanmasıdır. Bu süreçte, yeni biyoeşdeğer formülasyonların belirlenmesi veya ilaç ürünü kalitesinin sağlanması için klinik olarak ilgili *in vitro* spesifikasyonların belirlenmesi gerekmektedir. Güvenli alan, ilaç ürünü uyarlamalarının biyoeşdeğer olmasının beklendiği çözünme profili sınırlarını veya ilaç ürünü uyarlamalarının diğer kalite özelliklerini tanımlamaktadır. PBBM ile bu güvenli alanın oluşturulması, emilim mekanizmasının ve kritik biyoyararlanım özelliklerinin (Critical Bioavailability Attributes, CBA) daha iyi anlaşılması için mekanistik *in vitro* ve *in vivo* ilişkilerin (*In Vitro-In Vivo* Relationship, IVIVR) kurulmasını sağlar. Yeni olgu çalışmalarında ve literatür örneklerinde, *in vitro* çözünme/partikül büyüklüğü dağılımı spesifikasyonlarının belirlenmesi, f_2 testlerinin yerini alacak şekilde, çözünme spesifikasyonunun genişletilmesi veya ölçek büyütme ve onay sonrası değişikliklere yönelik uygulama gibi, biyoeşdeğerlik güvenli alan uygulamalarına yer verilmiştir [50]. Biyoeşdeğerlik güvenli alanı "*in vitro* spesifikasyonların (çözünme veya diğer ilgili ilaç ürünü kalite özellikleri) sınırlarını tanımlar ve bu sınırlar içinde ilaç ürünü uyarlamalarının birbirlerine biyoeşdeğer olması beklenir" [8] (Şekil 1). Güvenli bir alanın oluşturulması, *in vitro* ilaç ürünü özelliklerinin, *in vivo* farmakokinetik performans üzerindeki etkisinin mekanik olarak anlaşılmasını sağlar ve beklenmedik veya standartın altındaki *in vivo* farmakokinetik sonuçların eksiltilmesine yardımcı olabilir [50]. Jenerik ve referans ürün geliştirmede güvenli alanın oluşturulması, ürün geliştirme kararlarından ruhsatlandırma gerekçelerine kadar uzanan bir dizi üstünlük ve uygulama sunmaktadır. Ürün geliştirmenin erken aşamalarındaki başarılı PBBM uygulamaları, kritik biyoyararlanım özelliklerinin belirlenmesine yardımcı olur. Kritik biyoyararlanım özellikleri, partikül büyüklüğü, çözünme hızı, tablet basım kuvveti gibi "bir ilaç ürününün biyoyararlanımını kritik ölçüde etkilemesi" beklenen formülasyon özellikleridir [50]. Kritik biyoyararlanım özellikleri; kritik işlem parametreleri, kritik madde özellikleri, kritik kalite özellikleri ve

kritik formülasyon değişkenleri ile birlikte kullanılan daha yeni bir terimdir. Kritik biyoyararlanım özellikleri bu özelliklerden herhangi biri olabilir ve jenerik ürün geliştirme sırasında tanımlanırsa, *in vivo* özelliklerin ürün geliştirmeye dahil edilmesine yardımcı olur. Çözünme ve kritik biyoyararlanım özellikleri için güvenli alanın tanımlanması, kalite kontrol ortamında çözünme spesifikasyonlarının doğrulanmasına, biyoeşdeğer olmayan serilerin reddedilmesine ve çözünme sınırlarının belirlenmesine yardımcı olur. Ayrıca güvenli alan, çözünme farklılığı veya IVIVC eksikliği nedeniyle daha düşük veya daha yüksek dozlarda biyoeşdeğerliğe ulaşılamadığı durumlarda, biyoeşdeğerliği göstermek için etkili bir araç olabilir. Bu uygulamaların yanı sıra güvenli alanın tanımlanması, üretim işlemindeki değişiklikler, içerik değişiklikleri ve üretim yeri değişikliği ile ilgili biyomuafiyet durumlarını desteklemeye de yardımcı olabilir [50-52].



Şekil 1. Güvenli Alan (Biyoeşdeğerlik alanı). A: Bilgi Alanı: Ürün geliştirme sürecinde elde edilen tüm bilgiler, B: Tasarım Alanı: Kaliteyi sağlamak için çok boyutlu kritik madde özellikleri ve kritik işlem özelliklerinin etkileşimi ve çok boyutlu kombinasyonu, C: Güvenli Alan: Güvenli alandaki tüm seriler biyoeşdeğerdir [52,53]

Güvenli alanı belirlemek için dört farklı yaklaşımdan söz etmek mümkündür (Şekil 2) [52,54].



Şekil 2. Güvenli Alan (Biyoeşdeğerlik alanı) belirlemede kullanılan yaklaşımlar [52]

- ***İn vitro* - *in vivo* ilişki (IVIVR):** Kritik madde özellikleri, kritik işlem özellikleri ve kritik fizyolojik değişkenler ile *in vitro* çözünme verileri arasındaki ilişkiyi belirlemeye yönelik işlemlerdir. Bu süreçte, ilgili bir *in vivo* yanıt ile *in vitro* salım profilleri arasında niteliksel bir sıralama ilişkisi kurulur.
- ***İn vitro* - *in vivo* korelasyon (IVIVC):** Geleneksel modelleme yaklaşımlarını kullanarak, uzatılmış dozaj şeklinin *in vitro* özelliği (genellikle ilacın çözünme veya salım hızı ve miktarı) ile ilgili *in vivo* yanıt (emilen ilacın plazma ilaç konsantrasyonu veya miktarı) arasındaki korelasyonu kestirime yönelik matematiksel işlemlerdir.
- **Maruziyet - cevap analizi:** Bu çalışmalar maruziyet-cevap verilerine dayanan, çözünmenin güvenli alanı dışındaki kestirimlerindeki risk analizi işlemleridir. Maruziyet-cevap analizleri, klinikte daha önce iyi çalışılmamış yeni dozaj şekilleri ve dozaj rejimleri için önceki klinik sonuçların interpolasyonu, yeni dozaj şekillerinin ve formülasyonların piyasaya sunulması, farklı uygulama yollarına izin verilmesi ve farmakokinetik farklılıklara yol açan bileşen ve

üretim yöntemi değişiklikleri olması durumunda kabul edilebilir ürün performansı sağlanması amacıyla uygulanmaktadır [55]. Güvenli alanın sınırları, etkililik ve güvenliliği sağlamak için maruziyet-cevap analizine dayanılarak daha fazla desteklenmelidir [56].

- **Fizyolojik tabanlı biyofarmasötik modellemeye dayanan IVIVR/IVIVC:** Kritik madde özellikleri, kritik işlem özellikleri ve kritik fizyolojik değişkenler ile *in vitro* çözünme verileri arasındaki ilişkiyi, fizyolojik tabanlı biyofarmasötik modellemeler kullanarak belirlemeye yönelik işlemlerdir. Bu süreçte, *in vivo* etki (*in vivo* çözünme veya *in vivo* salım) dikkate alınır.

BCS sınıf 1 bir ilaç olan zolpidem için, Simcyp® Simulator'de yapılan bir PBPK/PD (Pharmacodynamic) modeli çalışmasında, ticari hemen salım sağlayan formülasyondan zolpidemin çözünmesi için güvenli alan oluşturulması amaçlanmıştır. Bu çalışmada, zolpidemin olumsuz besin etkisine sahip olması nedeniyle, dozlama zamanının PK/PD sonuçları üzerindeki etkisi de araştırılmıştır. Simülasyonlara göre, ilacın %85'i 45 dakika veya daha kısa sürede çözündüğünden, zolpidemin farmakokinetik ve farmakodinamik profilleri üzerindeki etkisi minimum olacaktır. FDA'ya göre, BCS-biyomuafiyet işlemi uygulanarak biyoeşdeğerlikten vazgeçmek için ilacın, test ve referans ürünlerden 30 dakikada %85'den fazlasının çözünmesi gerekmektedir. Ancak, PBPK/PD modeli, mevcut BCS-biyomuafiyet kriterlerinin hemen salım sağlayan zolpidem tabletleri için gereğinden daha kısıtlayıcı olduğunu göstermektedir. Bu durumda, farmakokinetik ve farmakodinamik ilişki göz önünde bulundurulduğunda, zolpidem tabletlerinin ürün etiketinde yazdığı gibi, yemekle birlikte veya yemekten hemen sonra alınmasının uygun olamayacağı sonucuna varılmıştır [57]. Değiştirilmiş salım sağlayan formülasyon geliştirme, ağız yolu ile uygulanan dozaj şekillerinde besin etkisi değerlendirmesi, biyouyumlu ortamlardaki çözünme koşulları ve spesifikasyonlarının belirlenmesi, biyoeşdeğerlik ve biyomuafiyete partikül büyüklüğünün etkisi gibi konularda PBPK çalışmaları yoğun bir şekilde devam etmektedir [38].

İn Siliko Modellemede Yazılımlar

İn siliko modelleme ve yazılımların kullanımı, insanlarda ilaç maruziyetinin tahmin edilmesinde büyük öneme sahiptir. Bu *in siliko* platformlar arasında, sanal biyoeşdeğerlik çalışmalarında PBPK modelleme ve simülasyon yeteneklerini kullanan SimCyp® (Certara, Sheffield, İngiltere), doğrusal olmayan karışık etkiler modellemesi için NONMEM® (ICON Plc., Dublin, İrlanda) ve mekanistik oral emilim modelleme ve simülasyonu PK-Sim® (Bayer Technology Services GmbH, Leverkusen, Almanya), GastroPlus™ gibi özel yazılımlar öne çıkmaktadır. SimCyp®, NONMEM®, PK-Sim® ve GastroPlus™ platformlarının, *in vitro* verilerden, plazma konsantrasyonunu kestirim yetenekleri birçok çalışmada gösterilmiştir [58]. Bu platformlar, sanal biyoeşdeğerlik çalışmalarının yürütülmesinde, özellikle biyomuafiyet genişletme ve ilaç ürünlerinin geliştirilmesinde zaman ve maliyetten tasarruf sağlayabilir. *İn siliko* biyoeşdeğerlik çalışmalarında kullanılan bazı platformlar Tablo 1'de listelenmiştir [58,59].

İlaçla ilgili yetkili otoriteler, simülasyonların yer aldığı ruhsat dosyası başvurularında platformların güvenilirliğini sorgulamakta, süreci daha iyi anlama konusunda ise artan bir ilgi göstermektedir [60]. Avrupa Birliği (AB), formülasyon değişikliklerini esas alan, ağız yolu ile uygulanan ilaçlarda emilimle ilgili kestirim araçlarını geliştirmek için, oral biyofarmasötik araçlar (OrBiTo) projesi kapsamında yeni iş paketi uygulamalarını başlatmıştır [61]. FDA iç araştırma girişimleri ise, popülasyon farmakokinetik modelleme algoritmalarını PBPK modellerine entegre eden, sanal biyoeşdeğerlik deneme simülasyon platformunun geliştirilmesini desteklemektedir [58,62].

Tablo 1. Biyoeşdeğerlik çalışmaları için simülasyon ve modellemede kullanılan bilgisayar yazılımları [58,59]

BilgisayarYazılımı/*İn Vitro*	Uygulama Örnekleri
GastroPlus™	• Araştırma alanına bağlı olarak farklı modüller ile kullanılabilen çok yönlü bir uygulama yazılımı • Yerleşik modülleri kullanarak popülasyonun sanal gönüllüleri için plazma konsantrasyonunun taklit edilmesi, • İlaç veri tabanının girdilerinin dikkate alınmasıyla fizyolojik ve farmakokinetik değişkenliğin birleştirilmesi • Biyomuafiyetin değerlendirilmesi • İlaç çözünmesi ve partikül büyüklüğü spesifikasyonuna dayalı olarak hemen salım sağlayan tabletler için kontrol stratejisinin oluşturulması
SimCyp®	• Mekanik bir gastrointestinal simülasyonun geliştirilmesi ile emilim modelinin tahmin edilmesi • IVIVC'lerin kurulması için IVIVC modülünün iki aşamalı yaklaşımının kullanılması • İlacın çözünmesini ve emilimini modellemek için gelişmiş çözünme, emilim ve metabolizma (Advanced Dissolution, Absorption, and Metabolism, ADAM) modelinin kullanılması • Deri yoluyla ilaç emilimini ölçmek için MPML MechDermA modelinin kullanılması • Sanal biyoeşdeğerlik çalışmalarının simülasyonunun gerçekleştirilmesi
NONMEM®	• Biyoeşdeğerlik çalışmalarının Monte Carlo simülasyonlarının gerçekleştirilmesi • Popülasyon farmakokinetik/farmakodinamik analizinin gerçekleştirilmesi • Sanal biyoeşdeğerlik çalışmalarının gerçekleştirilmesi • Doğrusal olmayan karışık etkilerin modellenmesi
MATLAB®	• Biyoeşdeğerlik çalışmasının sonuçlarının kestirimi için *in vitro in vivo* simülasyon (*In Vitro-In Vivo* Simulation, IVIVS) yaklaşımının geliştirilmesi • Plazmadaki ilaç konsantrasyonlarını simüle eden modelin, kompartmantal emilim ve geçişin (Compartmental Absorption and Transport, CAT) oluşturulması • NONMEM® çıktılarının, model simülasyonlarının ve istatistiksel ve grafiksel analizlerin ek olarak incelenmesi
Stella® Professional	• *İn siliko* ilaç emilim modelinin geliştirilmesi • PBBM'nin oluşturulması
B^2O simulator	• Klinik ve klinik olmayan verilerin entegrasyonuyla PBPK modelinin oluşturulması • Farklı çözünme profilleri verileri kullanılarak biyoeşdeğerliğin tahmin edilmesi
PK-Sim®	• Ağız yolu ile uygulanan ilaçların biyoeşdeğerliğinin kestirimi için PBPK modellemesi ile, biyouyumlu ortamlardaki *in vitro* çözünme testinin (USP-4 aygıtı, akış hücresi) birlikte kullanılması • İnsanlar ve çeşitli laboratuvar hayvanları üzerinde PBPK modelinin gerçekleştirilmesi • Hemen salım ve uzatılmış salım sağlayan dozaj şekillerinin gastrointestinal geçiş, dağılma ve çözünme süreçlerinin dozaj şekline bağlı olarak dikkate alması

Zorluklar ve Gelecekteki Fırsatlar

Günümüzde sanal biyoeşdeğerlik çalışmalarının, ağız yolu ile uygulanan ilaçlar için uygulanması diğer uygulama yollarına göre daha yaygındır, ancak pratikte hala üstesinden gelinmesi gereken birçok zorluk bulunmaktadır. Sanal biyoeşdeğerlik uygulamalarında; uygun girdi parametrelerinin seçimi, çözünme profili modelinin seçimi, model kabul kriterlerinin belirlenmesi ve güvenli alanın genişletilmesi gibi zorluklar bulunmaktadır [50]. Başarılı sanal biyoeşdeğerlik uygulamaları genellikle BCS sınıf 1, 2 ve 3 ilaçlarda uygulanmış, BCS sınıf 4 ilaçlar için çok daha az uygulama yapılmıştır. BCS sınıf 4 ilaçlar, düşük çözünürlük ve düşük geçirgenlik nedeniyle mide bağırsak kanalı fizyolojisine daha duyarlı olduğundan, model simülasyonunun zorluğunu artırır. Sanal çalışmalar nadiren özel

popülasyonlara (örneğin, pediyatrik ve geriyatrik) ve hastalara uygulanmaktadır. Ayrıca, bazı koşullarda model simülasyonu, insanlarda yapılan klinik çalışma sonuçları ile desteklenememektedir [19]. İnsanlarda ibuprofenle yapılan bir çalışmada, test ürününün eğri altında kalan alan (Area Under the Curve, AUC) açısından referans ürüne biyoeşdeğer olmadığı sonucuna varılmış ancak model simülasyonu tarafından sonuçlar doğrulanamamıştır. AUC için taklit edilen ve gözlemlenen sonuçlar arasındaki uyumsuzluğun nedeni olarak, klerensle ilgili değişkenliğin simülasyona yansıtılamaması gösterilmiştir [63]. Sanal biyoeşdeğerlik sonuçlarına güven oluşturmak için, yalnızca formülasyona bağlı sistemik maruziyetin değil, aynı zamanda beklenen popülasyon değişkenliği derecesini kestirme yeteneğinin de gösterilmesi gerekir. Biyoeşdeğerliği etkileyen değişkenlik kavramı, bağıl biyoyararlanımda %90 güven aralığını dikkate alan kabul kriterleri ile daha belirgin hale gelir. Dolayısıyla, bir referans ürünü kendisiyle karşılaştıran klinik çalışmalar, bireyin aynı formülasyonu aldığı iki durumla ilişkili birey içi değişkenlikler nedeniyle başarısız olabilir. Bu çalışmalarda, farmakokinetik parametrelerdeki gerçekçi güven aralıklarını yakalamak için fizyolojik değişkenlikler PBPK modellemede yer almalıdır [64]. Sanal biyoeşdeğerlik çalışmaları için sınırlı *in vivo* bilgi, güvenilir biyokestirimsel *in vitro* araçlarını ve modelleme yaklaşımlarını sınırlandırır [19]. Ancak her geçen gün teknolojilerin gelişmesi ile ilaç-vücut etkileşim mekanizmalarını anlama konusunda çok sayıda çalışma yapılmaktadır. Ayrıca, yapay zekâ uygulamalarının artması ve model algoritmalarının iyileştirilmesi (Monte Carlo simülasyonu, Bootstrap, Bayesian gibi), *in vitro* biyokestirimsel araçlarının geliştirilmesine büyük ölçüde yardımcı olmaktadır [65].

PBPK/PBBM'ler sadece ağız yolu ile uygulamalar için değil, aynı zamanda diğer önemli ilaç uygulama yolları için de geliştirilmelidir. Pulmoner, oftalmik, topikal ve transdermal olarak uygulanan ilaçlar için PBPK modellerinin geliştirilmesine yönelik şu anda az sayıda çalışma bulunmaktadır. Öncelikle ağız yolu ile uygulanmayan dozaj şekilleri ile test ve referans formülasyonlar arasındaki tutarsızlıkları tespit edebilen biyokestirimsel *in vitro* tekniklerin oluşturulması, güvenilir verilerle daha mekanistik PBPK modellerinin modellenmesi, *in vivo* ilaç davranışı ile *in vitro* veriler arasında iyi bir ilişki kurulması gibi konular üzerinde çalışılmalıdır. Peptitler, antikor-ilaç konjugatları veya anti-sens oligonukleotitler gibi yeni ilaçlar için, deri altı ve inhaler ilaç uygulaması, son yıllarda yaygın olarak kullanılan uygulama yolları arasındadır. PBBM'ler gelecekte bu ürünlerin ve ilaç formülasyonlarının kalite yönlerini desteklemek amacıyla yardımcı maddelerin, ilaç konsantrasyonunun, uygulama aracının veya cihazın etkisini değerlendirmek için kullanılabilir [10].

Ayrıca, yapay zekâ algoritmalarının kullanımı, sanal biyoeşdeğerlik çalışmalarının verimliliğini artırabilir. Doğal dil işleme (Natural Language Processing, NLP) gibi yapay zekâ teknikleri, bilimsel literatürden, klinik araştırma verilerinden ve düzenleyici belgelerden bilgi çıkararak kapsamlı veri sentezine olanak sağlayabilir. Bu da sanal biyoeşdeğerlikte kullanılan hesaplama modellerinin doğruluğunu ve güvenliliğini artırmaya yardımcı olur. Makine öğrenimi ve derin öğrenme, ilaç davranışını, emilimini ve dağılımını taklit eden kestirim modelleri geliştirmek için kullanılabilir. Yapay zekâ, büyük veri kümelerini analiz ederek farmakokinetik ve farmakodinamik özelliklerin daha doğru kestirimini sağlar. Yapay zekâ ayrıca, yardımcı maddeler, dozaj şekilleri ve salım oranları gibi çeşitli formülasyon parametrelerini dikkate alarak biyoeşdeğerlik kriterlerini karşılayan optimize edilmiş formülasyonlar önerebilir. Bu durum da formülasyon geliştirme sürecini kolaylaştırır ve sanal eşdeğerlik değerlendirmelerinin verimliliğini artırır. Yapay zekâ, sanal biyoeşdeğerliğin gerçek zamanlı izlenmesini kolaylaştırabilir ve ilgili sorunların zamanında ele alınmasına yardımcı olabilir. Yapay zekâ algoritmaları, elektronik sağlık kayıtları, advers olay veri tabanları ve pazarlama sonrası gözetim dahil olmak üzere çeşitli kaynaklardan gelen verileri analiz ederek, jenerik ilaçlarla ilgili potansiyel sinyalleri ve güvenlik sorunlarını belirleyebilir, bu da zamanında müdahalelere, risk azaltmaya ve sanal biyoeşdeğerliğin sürekli değerlendirilmesine olanak sağlar [39,66]. Ribosislib ile ilgili bir çalışmada, permeasyon kontrollü emilime sahip ilaçlar için PBBM'nin formülasyon değişikliklerinden sonra benzer *in vivo* performansları göstermek için yeni fırsatlar sunulmuş, BCS veya IVIVC tabanlı biyomuafiyet kapsamında olmayan veya f_2 benzerliği açısından karşılaştırmalı çözünme çalışmasında başarısız olan ilaç ürünleri için bu konudaki eksiklik giderilmiştir. Bazı durumlarda PBBM ile kanıtlanmış biyoeşdeğerlik güvenli alanı, f_2 çözünme profili benzerliğini değersiz hale getirebilir. Ribosislib içeren ve çözünme profilleri benzerlik göstermeyen kapsül ve tablet formülasyonlarının PBBM ile biyoeşdeğer olduğu tahmin edilmiştir. Önemli olan nokta, insan midesindeki tahmin edilen hızlı çözünmenin,

çözünme hızından daha yavaş permeasyon hızıyla birlikte, her iki formülasyon için de benzer emilim kinetiği ve farmakokinetiğe yol açmasıdır. Bu tahminler, klinik geliştirme sırasında mevcut olan tablet serisini (Biyoeşdeğerlik serisi) kullanarak bir biyoeşdeğerlik çalışması yürütme ve tablet serilerinin daha fazla geliştirilmesine yatırım yapmama kararını destekleyerek zaman ve maliyet tasarrufu sağlayacaktır. Böyle bir durumda, formülasyon çalışmalarında çözünmenin ötesinde üretim yöntemine ve üretilebilirliğine odaklanılmalıdır, çünkü bir IVIVC olası değildir. Geçirgenlik kontrollü emilim için biyoeşdeğer güvenli alanı, bağırsak geçirgenliğinin formülasyonlar arasında değişmediği dikkate alınarak oluşturulur. Formülasyonların geçirgenliği genellikle formülasyon yardımcı maddeleri tarafından değiştirilmez. Burada önemli olan, formülasyonların geçirgenliği ve geçiş süresini etkilediği bilinen yardımcı maddeleri (örneğin yüksek düzeyde mannitol) içermemesini sağlamaktır. Klinik çalışmanın sonuçları, kapsül ile tablet (Biyoeşdeğerlik serisi) arasındaki biyoeşdeğerliği doğrulayarak PBBM'nin sağlamlığını ayrıca ortaya koymuştur. BCS biyomuafiyet kriterlerini karşılamayan ilaçlar için (yani BCS 2 ve 4): bazı pH değerlerinde çözünürlük düşüktür, çözünme benzerliği yoktur ve IVIVC söz konusu değildir, bu durumda PBBM destekli güvenli alanın f_2 çözünme benzerliğinin yerini aldığı uygun bir formülasyon stratejisi geliştirmek mümkündür. PBBM, bir biyoeşdeğerlik çalışmasının başarı oranını tahmin etmek ve bir biyoeşdeğerlik çalışmasına başlama veya yeni formülasyonu daha da geliştirme konusunda bilinçli bir karar vermek için uygundur. Genel olarak, PBBM'nin birçok zayıf baz ve asit için 'geçirgenlik kontrollü' emilimi göstermek için kullanılabileceği ve biyoeşdeğerlik güvenli alanını yalnızca karar vermeyi geliştirmek için değil, aynı zamanda model bazlı biyomuafiyet fırsatını sunabileceğine de dikkat çekilmektedir [67].

Ayrıca ürün-hasta etkileşimlerinin ve bireysel insan fizyolojisindeki değişkenlik düzeyinin mekanik olarak anlaşılması, PBBM'lerin insan fizyolojisinin ve proteomunun ilgili biyobelirteçlerine dayalı olarak her bir hasta için kişiselleştirilmiş dozu, uygulama programını veya salım hızı gereksinimlerini hesaplamak üzere sanal ikizler oluşturarak hassas dozlamayı bulmaya da olanak sağlayabilir [10].

SONUÇ VE TARTIŞMA

Son yıllarda, *in siliko* simülasyon programları, insanlarda ilaç maruziyetinin tahmini için mekanistik bir çerçeve sağlayan fizyolojik temelli farmakokinetik modellere dayalı olarak yeni ilaç ve jenerik ilaç ruhsat başvurularında önemli bir rol oynamaktadır. Tartışmalarda odak noktası genellikle ağız yolu ile uygulanan jenerik ilaçlar olmuştur, ancak kestirime dayalı bu modellemeler oküler, pulmoner, transdermal ve parenteral uygulama yolları için de geçerlidir, bu alandaki çalışmaların artması beklenmektedir.

Sanal biyoeşdeğerlik çalışmalarının önemli ölçüde gelişeceği, klinik çalışma tasarımlarının optimize edilmesinden, *in vivo* klinik çalışmalardan muafiyete kadar ilaç endüstrisi ve ilaçla ilgili yasal otoritelere birçok konuda destek olması beklenmektedir. PBPK/PBBM modellerinin giderek daha fazla ürün için kullanılacağı ve mevcut kısıtlamaların üstesinden gelmek için daha fazla gelişeceği öngörülmektedir. Bu bağlamda, ilaç araştırma merkezlerinin, referans ve jenerik ilaç endüstrilerinin, ilaçla ilgili yetkili otoritelerin ve akademi dünyasının, bilgiyi yaymak ve kısıtlamaları ele almak için iş birliği yapmaları, rehberlerin oluşturulması, konu ile ilgili tüm paydaşların görüşlerine sunulması oldukça önem taşımaktadır.

YAZAR KATKILARI

Kavram: T.G., H.D., L.Ö.; Tasarım: T.G., H.D., L.Ö.; Denetim: T.G., H.D., L.Ö.; Kaynaklar: T.G., H.D., L.Ö.; Malzemeler: - ; Veri Toplama ve/veya İşleme: - ; Analiz ve/veya Yorumlama: - ; Literatür Taraması: T.G., H.D.; Makalenin Yazılması: T.G., H.D.; Kritik İnceleme: L.Ö.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1180-1188, 2024
Doi: 10.33483/jfpau.1423707

DERLEME MAKALE / REVIEW ARTICLE

ORAL KOLAJEN TAKVİYELERİ VE OLASI ADVERS ETKİLERİNİN DEĞERLENDİRİLMESİ

ORAL COLLAGEN SUPPLEMENTS AND DETERMINATION OF THEIR POSSIBLE ADVERSE EFFECTS

Senanur ÖZSOY[1,2], Özge CEMİLOĞLU ÜLKER[3]*, Aylin ÜSTÜNDAĞ[3]

[1]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji Anabilim Dalı, 06110, Ankara, Türkiye
[2]Başkent Üniversitesi Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, 06790, Ankara, Türkiye
[3]Ankara Üniversitesi Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, 06560, Ankara, Türkiye

ÖZ

Amaç: *Bugüne kadar oral kolajen takviyeleri hakkında yapılan birçok çalışma kolajen takviyelerinin eklem ve cilt sağlığına üzerindeki etkileri üzerinedir. Kolajenlerin vücudun diğer fonksiyonları üzerine yararlı etkileri bilinmekte olup bunlar hakkında yapılan çalışmalar yetersiz kalmaktadır. Mevcut veriler birçok insan tarafından günlük diyetin önemli bir parçası haline gelen kolajen takviyelerinin herhangi bir toksik etkisi olmadığını, kullanımlarının güvenli olduğunu göstermektedir. Ancak yapılan çalışmalar sonucu birbirini destekleyen verilerin olmamasından dolayı kolajen takviyelerinin bu kadar sık kullanımlarına rağmen toksisiteleri üzerine ek çalışmalar yapılmasına ihtiyaç duyulmaktadır.*
Sonuç ve Tartışma: *Yapılan literatür taramalarının sonuçları incelendiğinde, olası advers etkilere ait çok az veriye rastlanmıştır. Spesifik kolajenin uzun süreli oral uygulanması vücutta zararlı hücresel hasarlara sebep olabilir, kilo kaybına sebep olabilir, organları hedef alarak işleyişini aksatabilir. Değişken kolajen kaynaklarının içerikleri hakkında bilgi sahibi olmak ve kullanım sürelerine, kullanım miktarlarına dikkat edilmesi gerekir.*
Anahtar Kelimeler: *Kolajen, oral kolajene bağlı advers etkiler, oral kolajen kullanım alanları, oral kolajen tipleri*

ABSTRACT

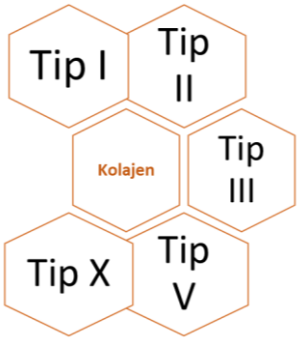
Objective: *Many studies on oral collagen supplements to date are on the effects of collagen supplements on joint and skin health. The beneficial effects of collagen on other functions of the body are known, but studies are insufficient. Available data show that collagen supplements, which have become an important part of the daily diet by many people, do not show any toxic effects and are considered to be safe to use. However, due to the lack of supporting data, it is necessary to draw attention to the need for additional studies on the toxicity of collagen supplements despite their frequent use.*
Result and Discussion: *When the results of the literature reviews were examined, very little data on possible adverse effects were found. Long-term oral administration of specific collagen can cause harmful cellular damage in the body, weight loss, and disrupt the functions of target organs. It is necessary to have information about the contents of variable collagen sources and pay attention to their usage durations and amounts.*
Keywords: *Collagen, oral collagen related adverse effects, oral collagen types, oral collagen uses*

* **Sorumlu Yazar / Corresponding Author:** Özge Cemiloğlu Ülker
e-posta / e-mail: oulker@pharmacy.ankara.edu.tr, **Tel. / Phone:** +905053952958

Gönderilme / Submitted : 22.01.2024
Kabul / Accepted : 14.06.2024
Yayınlanma / Published : 10.09.2024

GİRİŞ

Yunan kökenli kelimelerden türediği düşünülen ve doğal yapıştırıcıyı adlandırmak için kullanılan eski bir terim olan kolajen, hayvan kemiklerinin pişirilmesiyle elde edilir. Kolajen, hayvansal dokularda bulunan üçlü sarmal yapıya sahip bir veya daha fazla bölge içermesiyle ayırt edilen büyük bir yapısal protein ailesi olarak tanımlanır ve hayvan vücudundaki toplam protein içeriğinin yaklaşık %30'unu oluşturduğu düşünülmektedir [1,2]. Şu ana kadar elde edilen verilere göre bitkiler veya tek hücreli mikroorganizmalar gibi diğer canlılarda kolajenin varlığına dair bir kanıt yoktur. Bu canlılarda kolajenin yapısal rolünü, polisakkaritler veya protein-polisakkarit kompleksi gibi diğer bileşikler almıştır. Kolajenin üçlü sarmal yapısı ilk olarak 1950'lerde derideki kolajen liflerinin X-ışınları kullanılarak aydınlatılmıştır. Bu yapı, sağ yönlü sarmal bir yapıya sarılmış üç tane sol yönlü polipeptit zincirinden oluşur. Kolajen polipeptit zincirlerinin ayrımı, tekrarlanan spesifik bir birime (Gly-XY) sahip olmalarıyla yapılır; burada Gly doğadaki en küçük amino asit olan Glisin aminoasididir; X ve Y genellikle prolin (Pro) ve hidroksiprolindir (Hyp). Bu özgül yapıdan dolayı, kolajen üçlü sarmalında, merkez ekseni boyunca çeşitli moleküler etkileşimler meydana gelir. Moleküler bileşim ve moleküller arası organizasyondaki supramoleküler organizasyona göre 28 tip kolajen vardır; Supramoleküler organizasyonlarına göre kolajen tipleri, ek olarak fibril oluşturan kolajenler, bazal membran kolajenleri ve mikrofibriler kolajenler olmak üzere farklı sınıflara ayrılır [2]. Fibril oluşturan kolajenler doğada en çok bulunan kolajenlerdir. Tip I, tip II, tip III, tip V ve tip X fibril oluşturan kolajenler sınıfındadır (Şekil 1). Tüm bu kolajen türleri çeyrek fibril dizisi olarak bir araya gelme yeteneğine sahiptir. Bu grupta tip I ve tip II en sık görülenlerdendir [3]. Tip I kolajen, cildin hücre dışı matrisinde yer alan temel proteinlerden biridir ve granülasyon aşamasında cildin yenilenmesi ve olgunlaşmasına destek olur [4]. Gözler, kemikler, bağlar, tendonlar ve kaslarda bol miktarda bulunan tip I kolajen, önemli bir yapısal proteindir [4,5]. Tip I kolajen fibrozda önemli bir rol oynar. Tip II kolajen, üç özdeş polipeptit zinciri içerir. Kıkırdaktaki toplam kolajenin yaklaşık %80'ini oluşturmakla beraber vitröz gövdesi veya kornea gibi diğer dokularda da bulunurlar [6].



Şekil 1. Kolajen tipleri sınıflandırması

Kolajen Biyosentezi

Kolajen biyosentezi mezenkimal hücrelerde; fibroblastlar, kondroblastlar, osteoblastlar ve ayrıca epitelyal hücreler gibi diğer hücrelerde gerçekleşen ve kolajen genlerinin transkripsiyonuyla başlayan, çeşitli hücre içi reaksiyonlarla devam eden ve hücre dışı işlemlerle sonlanan kompleks bir süreci içerir [2]. Kolajen bazlı biyomateryaller; zayıf antijenik özellikleri, güçlü biyouyumlulukları ve hızlı biyobozunurluklarından dolayı uzun yıllar boyunca çeşitli endüstriyel alanlarda, ilaç endüstrisinde ve doku mühendisliğinde sık olarak kullanılmaktadır [7-9].

Kolajen Kaynakları

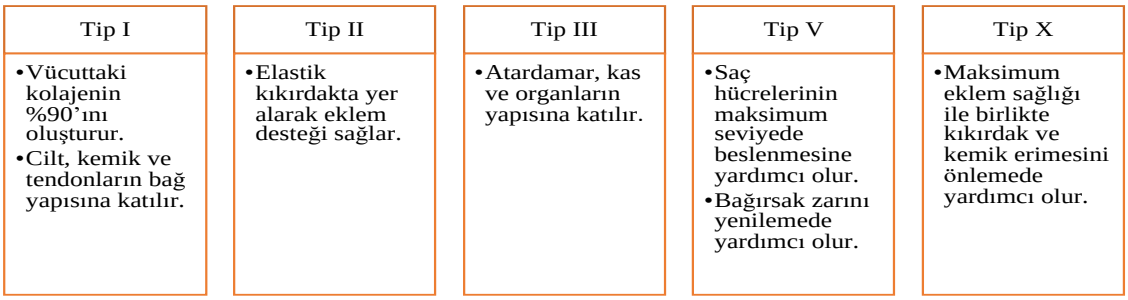
Kolajenler, kaynaklarına göre genel olarak doğal ve rekombinant kolajen olarak 2 sınıfa ayrılabilir. Doğal kolajen tipik olarak sıçan kuyruğu, domuz derisi veya sığır tendonu gibi memeli dokularından yapılır. Doğal kaynaklı kolajenler için; değişken kalite, düşük saflık, prion kontaminasyonu riski ve dini kısıtlamalar yoktur [10]. Rekombinant kolajenler ise laboratuvar

şartlarında üretilen sentetik ve özel teknoloji gerektiren ürünlerdir [11]. Günümüz piyasasında bulunan kolajenlerin hammaddesinin büyük çoğunluğu hayvansal kökenlidir. Temel olarak bağ dokusunu içeren ve bol miktarda tip I kolajen kaynağı olan deri, tendon ve kemikler en bilinenleridir [12]. Kıkırdaklar tip II kolajenin üretiminde kullanılır [13,14]. Takviyelerde bulunan Tip 2 kolajenin büyük çoğunluğu tavuktan elde edilir. Arjinin, glutamin, glisin ve prolin amino asitlerinin sarmal şeklinde birbirine bağlanarak güçlü bir yapı oluşturmasıyla oluşurlar. Yapılan çalışmalar sonucunda osteoartrit gibi yaygın olarak görülen kronik eklem rahatsızlıklarının tedavisinde tip 2 kolajen takviyeleri rol oynar. Osteoartrit patogenezinde eklemlerin yapısal bileşenlerinin kaybı, inflamasyon ve kondrositlerin yaşlanmasıyla sonuçlanan çoklu yollar arasındaki karmaşık etkileşimler rol oynar. Tip 2 kolajen ve kondroitin sülfat içeren gıda takviyelerinin osteoartrit üzerinde yararlı etkiler gösterdiği düşünülmektedir. Pek çok çalışma tavuk kıkırdağından elde edilen kondroitin sülfat ile ilişkili gıda takviyelerinin antiosteoartrit etkisini doğrulamıştır [15]. Tip I, V ve X kolajenin elde edilmesi için nadiren yumurta kabuğu zarları kullanılmaktadır [16]. Kolajen üretiminde genel olarak domuz ve sığır tercih edilir, ancak kümes hayvanları ve balık kolajeni [17], domuz kolajeninin kullanımının dini sakıncaları ve sığırlardaki zoonotik hastalıklara ilişkin kaygılar nedeniyle daha sık kullanılmaya başlanmıştır. Son zamanlarda balıklar ve yan ürünleri artan ilgi görmüştür [18,19]. Ek olarak literatürde, denizanasından (Catostylus mosaicus) elde edilen kolajenin hücre çoğalmasını desteklediği gözlenmiştir [20]. Balık kolajen eldesi, genel olarak balık pullarından, deriden veya kemiklerden olur [21]. Literatürlere kazandırılan birçok çalışma, balık mesanelerinden antioksidan ve antiaging aktif peptidlerin ekstraksiyonu uygulamalarına dikkat çekmektedir [22-25]. Sıcak su balıklarından elde edilen kolajenin, soğuk su balıklarından elde edilen kolajene göre termal stabilitesi daha yüksektir, çünkü protein peptitleri denatürasyona karşı daha dayanıklıdır [26]. Doğal kaynaklardan kolajen eldesi süreci genel olarak sonuç ürünün temel özelliklerini fizikokimyasal özellikler veya biyolojik aktiviteler olarak değiştiren farklı ekstraksiyon ve saflaştırma yöntemlerini içerir. Sonuçta; "çözünmeyen, denatüre doğal kolajenler", "çözünür doğal kolajenler", "denatüre kolajenler", "kolajen hidrolizatları" ve "kolajen peptidleri" şeklinde farklı kolajen ürünleri tanımlanmıştır. Çözünmeyen, doğası değiştirilmemiş doğal kolajenler, kullanılan ham maddeye bağlı olarak antijenik epitop göstermeleriyle karakterize edilir. Fibriler yapıyla ilişkili kolajen epitoplarının korunmasının immün etkilerde görev aldığı bilinmektedir [27,28]. Ham madde tavuk göğüs kemiği gibi kıkırdak dokudan oluşuyorsa çözünmeyen, denatüre olmayan tip II kolajen eldesi yapılmaktadır [29]. Çözünür doğal kolajenlerde, üçlü sarmal yapı sağlamdır, ancak daha az çapraz bağa sahiptir. Kimyasal açıdan molekül ağırlığı ortalama 300 kDa olan kolajenler üçlü sarmal yapısını korur. Üretim prosesi düşük sıcaklıklarda gerçekleştirilir [30]. Denatüre kolajen jelatin olarak bilinir. Yüksek sıcaklık gibi denatüre edici bir etkenin etkisiyle üçlü sarmal yapısını kaybederek farklılaşır [31]. Üçlü sarmal yapı denatüre olduğu için jelatin antijenik özellik göstermez. Kolajen hidrolizatların da üçlü sarmal yapısı denatüre olmuştur, ancak jelatinlerden farklı olarak polipeptit zincirleri kimyasal veya enzimatik bir hidroliz işlemine tabi tutularak parçalanır. Üçlü sarmal yapı bozulduğu için antijenik özellik göstermezler ancak farklı biyoaktif özelliklere sahip olabilirler. Kolajenin antioksidan aktivitesinin araştırıldığı çalışmalarda, ACE-I inhibitor aktivite veya DPP-4 inhibitör aktivite gibi farklı *in vitro* aktiviteler gösterdiği ileri sürülmüştür [32]. Kimyasal sentez veya biyoteknolojik mekanizmalarla "hayvansal olmayan kolajenlerin" üretimine yönelik bazı girişimlerde bulunulmuştur bunlar doğal hayvansal kaynaklardan elde edilen kolajenlere göre son derece basitleştirilmiş moleküllerdir [33].

Kolajen Kullanım Alanları

Kolajenin etki mekanizmaları molekül yapısına göre farklılık gösterebilmektedir bu nedenle gıda takviyelerinde kullanılan kolajenler, gıda takviyesinin etki amacına göre farklılık göstermektedir. Örneğin doğal kolajen, proteinaz enzimine karşı dirençli olduğundan ağızda ve gastrointestinal sistem boyunca sindirilmez [34]. Üçlü sarmal yapısını koruduğu için de eklem sağlığını korumak amacıyla kullanılır [35]. Jelatin ve hidrolize kolajen ise, üçlü sarmal yapısı bozulduğu için gastrointestinal sistem boyunca kolayca sindirime uğrar ve bu nedenle eklem sağlığına yönelik herhangi bir fonksiyon mekanizması tanımlanmamıştır [36]. Buna karşın piyasadaki birçok takviye gıdanın içerisinde düşük çözünürlüğünden dolayı jelatin bulunur [37]. İnsan vücudundaki en büyük organ olan deri dış çevreden gelen zararlı etkenlere karşı bir bariyer görevi görür. Cildin ana bileşenleri olan kolajen, elastin ve

hyaluronik asit, cilt yapısının ve neminin korunmasında önemli rol oynar. Cilt kolajeni, dermisin hücre dışı matris bileşenini üreten fibroblastlar tarafından üretilir [38]. Hücre dışı matris cilt bileşenlerinin yapısını oluşturur. Dermiste en çok bulunan bağ dokusu olan kolajen dermisin gücünden ve esnekliğinden sorumludur [39,40]. İç ve dış faktörlerin etkisinden dolayı, bireylerin cildi bazı faktörlerden hasar görür ve bu durum işlev kaybına sebep olur. Bu faktörlere güneş ve ultraviyole radyasyon, hava kirliliği, sigara dumanı, kötü beslenme örnek olarak verilebilir [41]. Ciltte en fazla bulunan protein olan tip I kolajenin yapısındaki değişikliklerin, yaşlanmış insan derisinin göstergesi olduğu ve direncin azalarak kırışıklık oluşumuna sebep olduğu gösterilmiştir. İlerleyen yaşla birlikte çevresel faktörlerin de etkisiyle kolajen üretimi azalır. Kolajen eksikliğinde kırışmış, sarkık bir cilt; kas ağrıları, kas zayıflığı, elastikiyeti azalmış tendonlar, eklem ağrıları, hareket problemleri ve gastrointestinal sistem rahatsızlıkları meydana gelebilir. Bundan dolayı günümüzde yan etkisi olmadığı düşünülen ve yaşlanma belirtilerini geciktirebilecek bir takviye alma fikri günden güne önem kazanmaktadır. Yapılan araştırmalara göre 2014'ten bu yana kolajenle ilgili araştırmalar artarak devam etmektedir [42]. Kolajen ilk olarak cilt kremlerinde ve serumlarda bir bileşen olarak ortaya çıkmıştır. Kolajen cildin daha alt katmanlarında bulunduğu ve kolajen lifleri cildin dış katmanlarına nüfuz edemeyecek kadar büyük olduğu için topikal kullanımı konusunda soru işaretleri giderek artmıştır. İlaçlar, tozlar ve bazı gıdalarla oral olarak alınan kolajenin vücut tarafından daha hızlı ve etkili bir şekilde emildiği ve bu sayede etkinliğinin daha çok arttığı düşüncesi son zamanlarda yaygınlaşmıştır. Kolajen takviyeleri, protein yapıtaşı olan aminoasitleri içerir. Buna ek olarak bazı kolajen takviyeleri, C vitamini, biotin, çinko gibi ek besinleri de içererek saç ve cilt için daha sağlıklı bir görünüm oluşmasını destekler [43]. Hidrolize kolajen, güvenli bir besin olarak kabul edilen düşük moleküler ağırlıklı peptitlerin karışımından oluşan nutrasötik bir takviyedir. İnsan vücudu tarafından emilimi diğerlerine göre daha fazla olduğu için takviye gıdalarda tercih edilen form olarak kabul edilir. Son yıllarda hidrolize kolajen oral alımının birçok yararı olduğu kabul edilmiştir. Bunlar arasında eklem ağrılarına iyi gelmesi, kardiyovasküler hasarı önlemesi, kan glikoz düzeyini dengelemesi, anemiye karşı kullanılması, cildin nem ve elastikiyetini destekleyerek cilde koruyucu bir bariyer oluşturması gibi fonksiyonlar vardır [44]. Bu kullanım alanları Şekil 2'de özetlenmiştir.



Şekil 2. Kolajen tiplerine göre kullanım alanları

Kolajen takviyeleri hakkında yapılan çoğu araştırma eklem ve cilt sağlığı üzerinedir. İnsanlar üzerinde yapılmış çalışmalar yetersiz kabul edilmektedir. Araştırmalar kolajen takviyelerinin eklem hareketliliğini arttırabildiğini ve cilt elastikiyetini sağlayabildiğini göstermektedir. Takviye amaçlı endüstriyel kullanımı olan kolajen peptitlerinin kullanımı genel olarak güvenli kabul edilir. Tarım ve Orman Bakanlığı onaylı ve sürekli denetimi gerçekleşen tesislerde, Türk Gıda Kodeksi Takviye Edici Gıdalar Tebliği'ne uygun olarak üretimi gerçekleştirilir ve piyasaya sunulur [43].

Kolajen Farmakokinetiği

Kolajen sindirim işlemi sonrasında kısmen gastrointestinal sistem tarafından emilen, çözünürlüğü değişken bir proteindir. Besin takviyesi olarak kullanım amaçları genel olarak gerekli aminoasitlerin sağlanmasıdır. Kolajende bulunan aminoasitlerin çoğunluğu değişken çözünürlükte oldukları için vücutta yeni protein sentezinde yapıtaşı olarak kullanılabilir. Balıklardan elde edilen kolajen hidrolizatların metabolizması ve itrahına ilişkin insan ve hayvan çalışmaları bulunamamıştır [47].

Advers Etkiler/Toksikolojik Veriler

Alia ve arkadaşlarının Irak'ta gebe tavşanlarda spesifik kollajen (kollajen-α®) kullanarak yaptığı çalışma bilinen ilk kronik toksisite çalışmasıdır. Bu çalışmaya göre 30 gün boyunca 1 ml oral kolajen takviyesi alan gebe tavşanlarda anti-obezite etkisi görülmüş kolajen peptidi alımının yağ miktarını azalttığı ve karaciğerde β-oksidasyonu arttırdığı görülmüştür. Belirgin bir zararlı etki gözlenmemiştir [45]. Kore'de Heung-Sik Seo ve arkadaşları tarafından sıçanlar üzerinde yapılan ve olası subkronik toksisiteyi belirlemek için yapılan bir başka çalışmaya göre de, oral kolajen takviyesinin test edilen canlılarda hiçbir olumsuz yan etkisinin gözlenmediği, hatta kilo kaybı, biyokimyasal parametreler, organ ağırlığı, histopatolojik bulguların belirli sınırlar dahilinde çıktığı tespit edilmiştir. Yapılan çalışmada, advers etki gözlenmeyen seviyesi (NOAEL) 2000 mg/kg/gün olarak tespit edilmiş ve hiçbir hedef organda toksisite gözlenmemiştir. Norveç Gıda Güvenliği Bilimsel Komitesi, Norveç Gıda Güvenliği Kurumunun isteği üzerine piyasada halihazırda bulunan gıda takviyeleri ve enerji içeceklerinde bulunan ve literatür taraması yapıldığında hakkında çok fazla bilgi bulunmayan maddelerin riskini değerlendirmiştir. Mevcut risk değerlendirmesini balık derisi üzerinde yaptığı önceki risk değerlendirmesine dayanarak yapılmıştır. Norveç piyasasında bulunan gıda takviyeleri kolajen formlarından birisi olan kolajen hidrolizat içerebilir. Norveç Gıda Güvenliği Kurumu tarafından yapılan kolajen risk değerlendirmesinde, balık derisinden yapılan gıda takviyeleri 750 mg/gün olarak tespit edilmiş ve bildirmiştir. Balık derisinden kolajen takviyesi çocuklar (10-14 yaş), ergenler (14-18 yaş) ve yetişkinler (> 18 yaş) yaş grupları için tahmin edilmiştir. İnsanlar üzerinde balık kolajeninde herhangi bir toksisite çalışması bulunmamıştır. Sıçanlar üzerinde somon balığı (Oncorhynchus keta) derisinden elde edilen deniz kolajen peptitleri etkileri üzerine 2 yıl boyunca yapılan oral toksisite çalışması kolajenin hiçbir olumsuz etkisi olmadığını göstermiştir. Aynı çalışma kromozom anormallikleri üzerine ve kobaylardaki alerjik duyarlılık üzerine de yapılmıştır ve bir olumsuz etkisi olmadığı rapor edilmiştir. Risk karakterizasyonunda karşılaştırma için kullanılan tahmini maruziyet değeri sıçanlar üzerinde yapılan kronik oral toksisite çalışmasından elde edilen günlük 8,6 g/kg vücut ağırlığı NOAEL değeridir. NOAEL değerinin maruziyete oranı olan MOE değeri hesaplanmıştır. Hayvan çalışmalarından elde edilen verilerin kabul edilebilir MOE değeri ≥ 100'dür. Balık derisinden günlük 750 mg/kg kolajen alımında tüm yaş grupları üzerinde MOE değeri 100'ün üzerinde bulunmuştur. Balıklardan elde edilen kolajenin bir balık alerjeni olduğu tespit edilmiştir, bundan dolayı balığa alerjisi olan kişilerin toleransı oldukça azdır ve balık kolajeninin olumsuz etkilerine daha çok maruz kalır. Balığa alerjisi olan kişilerin balık derisinden elde edilen jelatine de alerjik reaksiyon gösterebileceği bildirilmiştir. Yamamato ve arkadaşları (2014) tarafından yapılan genotoksisite çalışmasına göre, kromozomal anormalliklerin indüksiyonu 1.3, 2.5 ve 5 µl/konsantrasyonlarda balık kolajenine (çözünür forma getirilmiş Tilapya derisinden elde edilen) maruz bırakılan CHL/IU hücrelerinde (yenidoğan dişi çin hamster akciğer fibroblast hücresi) incelenmiştir. 24 saat sonrasında tedavi ve kontrol grupları arasında anlamsal ölçütte yapısal veya sayısal kromozom sapması olmadığı tespit edilmiştir. Aynı şekilde alerji testi çalışmalarına göre de kobaylara enjekte edilen %0,1 balık kolajeni ile muamele edilen bölgelerde cilt üzerinde bir reaksiyon gözlenmemiştir [46]. Kronik hastalıklardan olan romatoid artrit ve osteoartrit eklemleri etkileyen iki hastalıktır. Her iki hastalıkta da kolajene karşı oluşan immün cevap hastalığın başlamasında ve ilerlemesine sebep olabilir. Geçmiş yıllarda yapılan çalışmalarda tip II hidrolize kolajen takviyesi yapılan hastalarda yüksek yan etki ve etkinlik gözlenmiştir ancak nihai sonuç için daha çok çalışma yapılmasına ihtiyaç vardır [47]. Kore'de yapılan bir başka çalışmada oral kolajen peptitlerinin antiaging etkisi araştırılmış ve 84 kişi üzerinde 12 hafta boyunca yapılan çalışmada kaz ayağı, göz kırışıklıkları, cilt pürüzlükleri önemli ölçüde iyileşmiş olup çalışma süresi boyunca test materyaline karşı hiçbir alerjik reaksiyon gösterilmediği rapor edilmiştir [48]. Yapılan bir başka çalışmaya göre ise kolajen peptitleri uygulanmasının immün yanıt üzerindeki etkilerinin aydınlatılması amaçlanmış olup farelere kolajen peptit diyeti yaptırılmıştır. Farelere ovalbumin (OVA) ile immünizasyon yapılmış ve sonuçlar serum OVA'ya özgü IgE üretiminin baskılandığını ve anaflaksi yanıtlarını azalttığını göstermiştir [49].

Ancak, kolajen takviyesinin gereksiz yere alınmasının kardiyosit vakuolasyonu, hipertrofi, aritmi, miyokardit gibi olumsuz etkilere yol açabileceği bazı çalışmalarda belirtilmiştir [50]. Spesifik kolajenin uzun süreli oral uygulanması, vücutta zararlı hücresel hasarlara, kilo kaybına sebep olabilir, organları hedef alarak işleyişini aksatabilir. Değişken kolajen kaynaklarının içerikleri hakkında bilgi sahibi

olunması ve kullanım sürelerine ve miktarlarına dikkat edilmesi gerektiği bildirilmiştir [51].

SONUÇ VE TARTIŞMA

Kolajen takviyeleri ciltte kolajen üretimini desteklemek, saç ve tırnak yapısını güçlendirmek iddialarıyla piyasada satışa sunulmaktadır. Bu takviyeleri kullanan kişiler, aylar içerisinde cildin daha parlak, saçların ve tırnakların daha güçlü olduğu şeklinde yorumlar yapmaktadır. Bu konuyla ilgili yapılan bilimsel çalışmalar oldukça azdır, daha çok çalışma yapılmasına ihtiyaç vardır. Kolajen takviyelerinde bulunan kolajen peptitleri ciltteki kuruluğu ve kırışıklıkları azaltmaya yardımcı olduğu bildirilmiştir. Düzenli kullanımda deri kuruluğunda, çizgilerde azalma olduğu, deri kan akımında ve kolajen miktarında artış olduğu tespit edilmiştir. Kolajen takviyesi sonucunda, deri yaşlanmasının gecikmesi ve elastikiyetinin artması, su tutma kapasitesinin artması beklenir. Tablet, kapsül, toz veya sıvı olarak satışa sunulan kolajen takviyeleri vücuda alındığında önce sindirim sisteminde yapıtaşları olan aminoasitlere parçalanır ve kana karışır. Kolajen takviyelerinde biyoyararlanımı arttırmak için düşük molekül ağırlıklı kolajenler tercih edilebilir. Kolajen takviyesi almadan önce mutlaka bir doktora başvurulması gerekmektedir. Özellikle hamileler, emziren anneler, deniz ürünlerine, sığır-tavuk etine alerjisi olanlar ve diyabet hastalarının istenmeyen yan etkilerle karşılaşma olasılığı daha yüksektir. Kolajen takviyelerinin tam olarak ne içerdiği ve etiketlerinde gösterilen amaçları taşıyıp taşımadıkları hakkında bilgi eksikliğinden dolayı kullanımlarına dikkat edilmelidir. Mevcut verilere göre oral kolajen alımı genellikle hiçbir yan etkisi olmayan güvenli bir yöntem olarak kabul edilmektedir ancak yapılan çalışmaların belirli coğrafi bölgelerdeki insanları, yaş gruplarını ve cinsiyetleri kapsamasından dolayı detaylı değerlendirilmesine ihtiyaç vardır. Birbirini destekleyen verilerin olmaması oral alınan kolajenin vücudun diğer bölgeleri yerine genel olarak derinin dermis tabakasında lokal olarak birikmesi hastalarda oral kolajen alımının tartışmalı bir hale gelmesine sebep olmuştur. Oral alımın topikal alımla desteklendiği düşünülmektedir. Yapılacak olan ek çalışmalar, değerlendirme yöntemlerinin standartlaştırılarak, ideal dozlar belirlenerek ve en önemlisi hangi hastalarda kolajen takviyesinin gerekliliği göz önüne alınarak yapılmalıdır.

YAZAR KATKILARI

Kavram: S.Ö., Ö.C.Ü., A.Ü.; Tasarım: S.Ö., Ö.C.Ü., A.Ü.; Denetim: S.Ö., Ö.C.Ü., A.Ü.; Kaynaklar: S.Ö., Ö.C.Ü., A.Ü.; Malzemeler: S.Ö., Ö.C.Ü., A.Ü.; Veri Toplama ve/veya İşleme: S.Ö., Ö.C.Ü., A.Ü.; Analiz ve/veya Yorumlama: S.Ö., Ö.C.Ü., A.Ü.; Literatür Taraması: S.Ö., Ö.C.Ü., A.Ü.; Makalenin Yazılması: S.Ö., Ö.C.Ü., A.Ü.; Kritik İnceleme: S.Ö., Ö.C.Ü., A.Ü.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1189-1200, 2024
Doi: 10.33483/jfpau.1459437

DERLEME MAKALE / REVIEW ARTICLE

FUSARİUM TOKSİNLERİNİN EPİGENETİK MEKANİZMALAR ÜZERİNE ETKİLERİ: FUMONİSİN B1 VE ZEARALENON

THE EFFECTS OF FUSARIUM TOXINS ON EPIGENETIC MECHANISMS: FUMONISIN B1 AND ZEARALENONE

Elif PERÇİN[1], Ecem Fatma KARAMAN[2], Sibel ÖZDEN[3]*

[1]Medipol Üniversitesi, Sağlık Bilimleri Fakültesi, Moleküler Tıp ve Biyoteknoloji Anabilim Dalı, 34810, İstanbul, Türkiye
[2]Biruni Üniversitesi, Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, 34015, İstanbul, Türkiye
[3]İstanbul Üniversitesi, Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, 34116, İstanbul, Türkiye

ÖZ

Amaç: *Günümüzde en çok çalışılan Fusarium mikotoksin türleri arasında fumonisin B1 (FB1) ve zearalenon (ZEA) bulunmaktadır. FB1 ve ZEA farklı moleküler mekanizmaları etkilemekte olup birçok toksik etkiye sebep olmaktadır. Bu derlemede FB1 ve ZEA'nın DNA metilasyonu, histon modifikasyonları ve mikroRNA (miRNA) seviyeleri gibi epigenetik mekanizmalar üzerine etkileri ve moleküler düzeyde gözlenen toksik etkilerinin özetlenmesi amaçlanmıştır.*
Sonuç ve Tartışma: *FB1 ve ZEA'nın DNA metilasyonunu, histon modifikasyonunu ve miRNA seviyelerini uygulama süresi ve doza bağlı olarak değiştirdiği çeşitli çalışmalarda gösterilmiş olup bu mikotoksinlerin moleküler mekanizmalarında epigenetik çalışmaların önemi vurgulanmıştır.*
Anahtar Kelimeler: *Epigenetik mekanizmalar, fumonisin B1, fusarium toksinleri, zearalenon*

ABSTRACT

Objective: *Fumonisin B1 (FB1) and zearalenone (ZEA) have been the most widely studied Fusarium mycotoxins. It is demonstrated that FB1 and ZEA affect different molecular mechanisms and cause many toxic effects. In this review, it has been aimed to summarize the effects of FB1 and ZEA on epigenetic mechanisms such as DNA methylation, histone modifications, and microRNA (miRNA) levels, as well as their toxic effects at the molecular level.*
Result and Discussion: *It is shown in various studies that FB1 and ZEA change DNA methylation, histone modification and miRNA levels with dose and application time dependent. Additionally, it is identified that epigenetic studies are important in the molecular mechanisms of these mycotoxins.*
Keywords: *Epigenetic mechanisms, fumonisin B1, fusarium toxins, zearalenone*

GİRİŞ

Mikotoksinler, insan ve hayvanlar tarafından tüketimi fazla olan arpa, buğday, mısır ve pirinç gibi gıdaların *Aspergillus, Penicillium* ve *Fusairum* gibi küfleri tarafından üretilen sekonder metabolitlerdir. Kontamine gıdaların tüketimi sonucunda akut toksisite, teratojenik, mutajenik ve karsinojenik etkiler ortaya çıkabilmektedir. Ayrıca, insan ve hayvanlarda çeşitli organlarda hasara sebep olduğu yapılan çalışmalarla gösterilmiştir [1-3]. Mikotoksinlerin 300'den fazla farklı türü olduğu bildirilmektedir [4].

* **Sorumlu Yazar / Corresponding Author:** Sibel Özden
e-posta / e-mail: stopuz@istanbul.edu.tr, **Tel. / Phone:** +902124400000-13567

Gönderilme / Submitted : 26.03.2024
Kabul / Accepted : 29.06.2024
Yayınlanma / Published : 10.09.2024

Bunlar arasından okratoksinler, zearalenonlar, fumonisinler ve trikotesenler günümüzde en çok çalışılan mikotoksinler arasında yer almaktadır. Bu derleme kapsamında *Fusarium* toksinlerinden FB1 ve ZEA'nın toksik etkilerinde epigenetik mekanizmaların rolü incelenmiştir.

Fumonisin B1

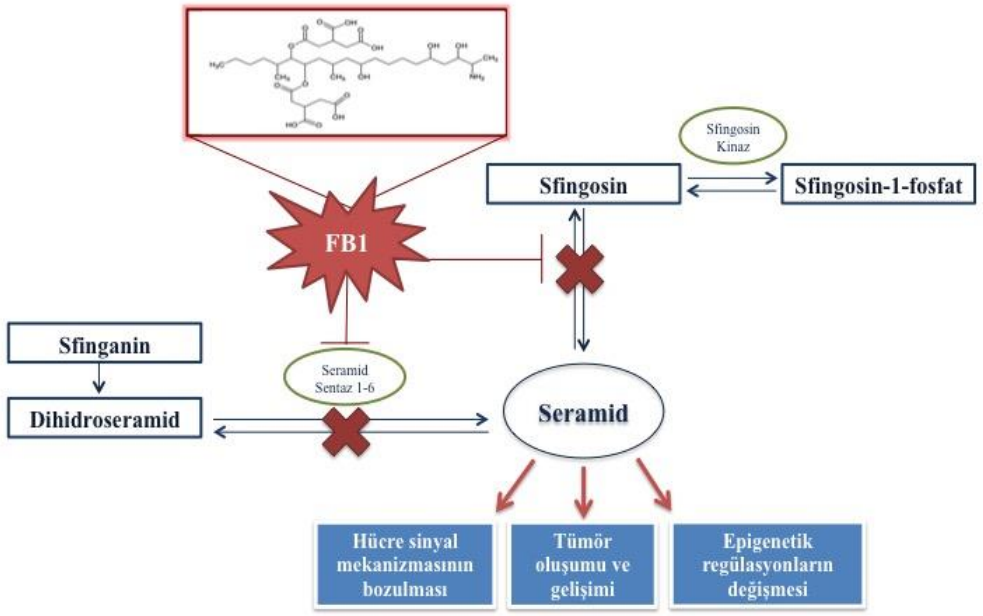
Fumonisinler, *Fusarium* küfleri tarafından üretilen önemli bir mikotoksin türüdür [5]. Genellikle, mısırın kontaminasyonu sonucu oluşan fumonisinlerin yedi türü vardır; fumonisin B1 (FB1), FB2, FB3, FB4, FA1, FA2 ve FC1 [6]. FB1 günümüzde en çok çalışılan ve toksisitesi en fazla olan fumonisin türüdür. Nörotoksisite, hepatotoksisite, nefrotoksisite ve immünotoksisite dahil olmak üzere hayvanlarda birçok toksik etkiye yol açtığı gösterilmiştir [7].

Yüksek oranda hidrofilik bir bileşik olan FB1, insan dışında tüm hayvanlarda benzer bir kinetiğe sahiptir. Hayvanlarda, gastrointestinal emiliminin zayıf olduğu görülmüş, sıçanlarda küçük bir kısmının safra ile atıldığı ve enterohepatik siklusa az miktarda geçişin olduğu gösterilmiştir [8-10]. Bunların yanı sıra, idrarda, karaciğerde, böbrekte ve kırmızı kan hücrelerinde FB1'e rastlanırken; plazma, plasenta, kalp ve beyinde ise rastlanmamıştır [11]. Kan beyin bariyerini geçebildiğine dair ise çok az kanıt bulunmaktadır [12]. İnsanlarda, safra ve az miktarda da idrar yoluyla vücut dışına atıldığı tespit edilen FB1'in özellikle karaciğerde ve böbrekte biriktiği bildirilmiştir [13]. Ayrıca, hayvanlar üzerinde yapılan çalışmalarla çeşitli organları olumsuz etkilediği gösterilmiştir. Karaciğer ve böbrek olumsuz etkilenen başlıca organlar arasındadır [14]. Ek olarak, FB1 içeren mısır ya da mısır içeren yemleri tüketen atlarda beyin zarı iltihabı [15], domuzlarda akciğer ödemi [16], farelerde böbrek hastalıkları ve erkek sıçanlarda sıçanın türüne göre karaciğer ve böbrek kanseri [14] geliştiği tespit edilmiştir. Fumonisin içeren besinlerin tüketildiği düşük sosyoekonomik toplumlarda ise özofagus kanserinin görüldüğü rapor edilmiştir [17].

Bunların yanı sıra, FB1 birçok hücresel mekanizmayı etkilemektedir. Sfingolipid sentezini inhibe etmesi de bu mekanizmalardan biridir. Sfingolipidler, hücre proliferasyonu, ölümü, göçü ve birçok sinyal yolağı olmak üzere önemli süreçleri kontrol eden önemli biyoaktif lipidlerdir [18]. Şekil 1'de gösterildiği gibi, FB1 kimyasal yapısı bakımından sfingozin ve sfinganine benzemekte olup seramid sentaz enzimini inhibe etmektedir. Bunun sonucunda, hücre içinde serbest sfingoid birikimine ve sfingolipid miktarının azalmasına sebep olduğu hem *in vitro* hem *in vivo* modellerle gösterilmiştir [19,20,23] Ayrıca, sfingolipidlerin yapısının bozulmasının hücre büyümesi, farklılaşması ve ölümü başta olmak üzere önemli hücresel mekanizmalarla ilişkisi olduğu rapor edilmiştir [19]. Domuz böbrek hücreleri (LLC-PK1) üzerine yapılan bir çalışmada, sfingolipid miktarında azalma gözlenmiş ve bunun sonucunda da hücre büyümesinin engellendiği ve hücre ölümünün meydana geldiği görülmüştür [21,23]. Maymun böbrek hücreleri (MARC-145) üzerine yapılan çalışmada ise otofajiye bağlı hücre ölümünün indüklendiği görülmüştür [24].

Yanlış katlanmış proteinlerin hücre içinde birikmesiyle endoplazmik retikulum (ER) fonksiyonunda bozukluklar meydana gelmekte ve bu bozukluklar ER stresini tetiklemektedir. ER stresi, otofaji mekanizmasının kontrolünde önemli bir yere sahiptir. Yapılan çalışmalarda FB1'in ER stresini indükleyerek karaciğer, kolon ve böbrek gibi organlarda otofaji üzerinden hücre ölümüne sebep olduğu gösterilmiştir [18]. Ayrıca, FB1'in hücre içi sinyal iletiminde önemli rol oynayan mitojenle aktive edilen protein kinaz (MAPK) yolağını aktive ettiği *in vivo* ve *in vitro* modellerle gösterilmiştir [25]. MARC-145 üzerinde yapılan çalışmada, FB1 toksisitesi MAPK yolağını aktive ederek otofajiyi indüklemiş ve bunun sonucunda hücre ölümü gerçekleşmiştir [24].

Oksidatif hasarın temel oluşum mekanizmalarından biri hücre içindeki reaktif oksijen türlerinin (ROS) artışıdır. Birçok mikotoksin türünde olduğu gibi, FB1 toksisitesi ile redoks dengesinin bozulduğu, bunun sonucunda da DNA, protein ve lipidlerde oksidatif hasarın meydana geldiği gözlenmiştir [18]. İnsan nöroblastoma hücre hattı (SH-SY5Y) ve sıçan primer astrositleri üzerinde yapılan çalışmada, FB1 maruziyetinin mitokondriyal solunum sisteminde rol alan kompleks I'in inhibisyonu üzerinden ROS miktarını arttırdığı gözlenmiştir. Oksidatif fosforilasyon miktarında azalma ve mitokondriyal homeostazide bozulma da elde edilen diğer sonuçlar arasındadır [26]. Ayrıca, insan fibroblast [27], glioblastoma (U-118MG) [28] ve karaciğer (HepG2) hücreleri [29] üzerinde yapılan çalışmalarda da ROS seviyesinin arttığı bildirilmiştir.



Şekil 1. FB1'in sfingolipid metabolizması üzerine etkisinin şematize edilmiş gösterimi

Bunun yanında, FB1 maruziyeti sonucunda çeşitli dokularda apoptoz artışı görülmüş olup tümör oluşumunda etkili olabileceği belirtilmiştir [30]. 2021 yılında, Yu ve arkadaşları tarafından yapılan çalışmada, FB1 maruziyetinin özofagus epitel hücrelerinde (HEEC) hücre proliferasyonunu ve göçünü desteklediği, histon deasetilaz enzimi (HDAC) ekpresyon seviyesini arttırdığı, fosfatidilinositol 3-kinaz PI3K/Akt sinyal yolağını aktive ettiği ve buna bağlı olarak karsinojeneze sebep olabileceği rapor edilmiştir [31].

Zearalenon

Zearalenon (ZEA), *Fusarium graminearum, F. culmorum, F. crookwellense, F. equiseti* ve *F. semitectum* küfleri tarafından üretilen ve mısır, çavdar, arpa ve yulaf gibi tahıllarda bulunan östrojen benzeri bir mikotoksindir [1].

Yapısı bakımından östrojen hormonuna benzerliği nedeniyle östrojenik etkilere sebep olarak endokrin sistem üzerine etkiler göstermektedir. ZEA'nın östrojen reseptörlerine (ER-α and ER-β) bağlanarak domuz gibi birçok memeli hayvanda üreme sisteminde toksik etkilere sebep olabileceği [32], ayrıca gebeliğin devamında sorunlar görülebileceği, luteal fonksiyonun bozulması ve fetüs büyümesinin gecikmesine neden olabileceği yapılan çalışmada belirtilmiştir [33]. Ayrıca, ZEA'nın östrojen reseptörlerine bağlanmasıyla hücre proliferasyonunu etkilediği belirtilmiştir [34]. Farklı hücre türleri üzerinde yapılan çalışmada, doza bağlı olarak ZEA maruziyetinin DNA fragmentasyonunu ve apoptozu indüklediği, hücre döngüsünün bozulmasına neden olduğu bildirilmiştir [35]. 18-21 günlük sıçanlardan alınan primer sertoli hücreleri üzerinde yapılan çalışmada, ZEA maruziyetinin mitokondrinin yapısını bozduğu ve mitokondriyal membran potansiyelinde doza bağlı azalmaya sebep olduğu gösterilmiştir [36]. Bununla beraber, oksidatif stresi arttırarak ROS miktarının artmasına neden olmaktadır [34].

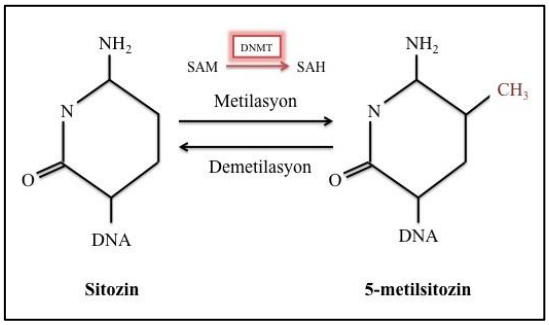
Epigenetik Mekanizmalar

Epigenetik; DNA dizisinde bir değişme olmadan fenotipte meydana gelen, kalıtımsal olarak aktarılabilen, geri dönüşümlü ve çevre ile modifiye edilebilen değişiklikleri inceleyen bilim dalıdır. Epigenetik terimi, ilk kez 1940'ların başında biyolog Conrad Waddington tarafından kullanılmıştır [37,38]. Epigenetik düzenlemeler, farklılaşma ve gelişim açısından önemli bir süreç olup bu düzenlemelerdeki herhangi bir hata sonucunda epigenetik hasarlar meydana gelmektedir. Bu hasarlar, DNA'nın farklı kromatin yapılarına paketlenmesiyle genlerin ifadesinin artmasına veya azalmasına neden olmaktadır. Böylece, bu durum önemli anahtar genlerin regülasyonunu etkileyerek karsinojeneze

yatkınlığı arttırmaktadır. Epigenetik düzenlemelerdeki değişikliklerin ayrıca, nörolojik bozukluklar, diyabet, astım ve kardiyovasküler hastalıklar ile ilişkili olduğu yapılan çalışmalar ile de gösterilmiştir [39,40]. Üç önemli epigenetik düzenleme vardır; DNA metilasyonu, histon modifikasyonu, mikroRNA'ların (miRNA) regülasyonu [41].

DNA Metilasyonu

DNA metilasyonu, kromatin yapısında meydana gelen başlıca modifikasyonlardan biridir. DNA metilasyonu, CpG dinükleotidindeki guanozin tarafından takip edilen C bazının 5. konumundaki karbonuna bir metil grubunun (-CH_3) bağlanmasıyla gerçekleşir. Bunun sonucunda, 5-metilsitozin (5-mC) oluşur [42]. %70'i metillenmiş durumda olan CpG dinükleotidleri, genom genelinde dağınık halde bulunmaktadır. CpG adaları ise özellikle dokularda devamlı ifade edilen bazı genlerin promoter bölgeleri ve ilk ekzonlarında ve metillenmemiş halde bulunurlar [43]. Promoter bölgelerinde 5-mC varlığı, bulunduğu kromozom bölgesinde lokalize olan genlerin sessizleşmesine yol açmaktadır. Şekil 2'de de gösterildiği gibi, S-adenozil metiyonin (SAM) tarafından sağlanan metil grubu, DNA metiltransferaz (DNMT) tarafından bağlanmaktadır. Bu bağlamda, SAM ve DNMT bu mekanizmada önemli bir yere sahiptir. DNMT1, DNMT2, DNMT3a, DNMT3b ve onun izoformu olan DNMT3L olmak üzere 5 türü olan DNMT enziminin ifadesi SAM miktarına bağlıdır [44]. SAM miktarındaki artış, DNMT'leri aktive ederek hipermetilasyonu tetikler ve ayrıca, global hipometilasyona karşı genomu korur [45,46]. DNA metilasyonu, hücre farklılaşması ve doku oluşumunda önemli bir yere sahiptir. Bu mekanizma dokuya özgü olup genin ifade derecesi ve metillenme derecesi arasında ters bir ilişki kurulabilir [47].



Şekil 2. DNA metilasyonunun şematik gösterimi. SAM: S-adenozil metiyonin; SAH: S-adenozil homosistein

DNA metilasyonundaki anormallikler, tümör gelişimi açısından kritik bir öneme sahiptir. DNA metilasyonu 3 farklı mekanizma üzerinden tümör gelişimine katkı sağlamaktadır. İlk olarak, 5-metilsitozinin deaminasyonu sonucu oluşan mutasyonun DNA onarım mekanizması tarafından fark edilemeyerek onarılamamasıdır. Diğer bir mekanizma ise global hipometilasyondur. Genom boyunca metile durumda olan CpG dinükleotidlerinin global hipometilasyonu ile kromozomda instabilite meydana gelmekte ve bunun sonucunda onkogenlerin ifadelerinde artış görülmektedir. Ayrıca, transpozonların aktivasyonu ve anoploidinin meydana gelmesine sebep olmaktadır. Son olarak, tümör baskılayıcı genlerin anormal hipermetilasyonu sonucunda, bu genlerin ifadeleri baskılanarak etkisiz hale getirilmekte, böylece tümör gelişimine katkı sağlanmaktadır. Bozulan önemli moleküler yolaklar arasında P16/Rb, p53/p14 ve APC/β-katenin yer almaktadır [47-50].

Histon Modifikasyonları

Histon modifikasyonları, kromatin yapısını ve gen ifadesinin değiştirilmesine aracılık eden post-translasyonel modifikasyonlardır. Histonlar, ökaryotik hücrelerin çekirdeğinde bulunan, DNA'nın paketlenmesinde görev alan bazik proteinlerdir. Paketlenme sonucunda nükleozomlar oluşur. Histon modifikasyonları, nükleozomlardaki histon proteinlerine enzimler yardımıyla farklı moleküllerin eklenmesi veya çıkarılmasıyla gerçekleşir. Fosforilasyon, metilasyon, ubiquitinizasyon, asetilasyon, ADP ribozilasyon ve sumozilasyon başlıca histon modifikasyonlarındandır [51]. Modifikasyonlar,

histon proteinlerinin elektriksel yükünü değiştirip kromatin yapısında değişikliklere sebep olur. Bu değişimlerle, DNA paketlenmesi, replikasyonu, tamiri ve gen ifadesi gibi önemli biyolojik mekanizmalar kontrol edilir [52-55].

MikroRNA (miRNA)'lar

miRNA'lar, 18-25 nükleotid uzunluğunda, kodlanmayan RNA'lardır. miRNA'lar, hücre bölünmesi, hücre farklılaşması ve apoptoz dahil olmak üzere birçok biyolojik mekanizmada önemli bir rol almaktadır. Ayrıca, birçok hastalıkta da miRNA'ların ekspresyon profilleri biyobelirteç olarak kullanılmaktadır [56].

FB1 ve ZEA'nın DNA Metilasyonu Üzerine Etkileri

Farklı modellerde, ZEA ve FB1 maruziyetinin DNA metilasyonu üzerine etkilerinin incelendiği çalışmalarda, doz ve uygulama süresine göre DNA metilasyonunda değişikliklerin gözlendiği rapor edilmiştir. Bu çalışmalar, Tablo 1 ve Tablo 2'de özetlenmiştir.

HepG2 hücrelerinde FB1 maruziyetinin, global DNA metilasyonunda ve DNMT1, DNMT3A, and DNMT3B metiltransferazların seviyelerinde azalmaya sebep olduğu belirtilmiştir [57]. İnsan kolorektal adenokarsinoma hücreleri (Caco-2) ile yapılan çalışmada, 72 saat 10 μM FB1 uygulamasıyla global DNA metilasyonunda artış tespit edilmiştir [58]. Sıçan karaciğer epitel hücreleri (Clone-9) ve sıçan böbrek epitel hücreleri (NRK-52E)'nde global DNA hipometilasyonunda değişiklikler gözlenmezken, tümör baskılayıcı genlerin promotor bölgelerinde hipermetilasyon görülmüş, ayrıca her iki hücre dizisinde *VHL* geninde metilasyon gözlenirken, clone-9 hücre dizisinde *c-myc* geninde metilasyon gözlenmiştir [59]. Bir diğer çalışmada ise, 24 saatlik 100 μmol/L FB1 maruziyeti ile insan embriyonik böbrek (HEK293) hücrelerinde global DNA metilasyonunun anlamlı bir şekilde arttığı bildirilmiştir [60]. İnsan böbrek hücreleri (HK-2) yapılan son çalışmada, 24 saatlik 100 μmol/L FB1 maruziyeti sonucunda DNMT3*a* ve DNMT3*b* seviyesinde artış görülürken; 50μmol/L ve 100 μmol/L maruziyet sonucunda DNMT1 seviyesinde azalma görülmüştür [61].

HepG2 ve insan meme kanseri hücreleri (MCF-7) ile yapılan çalışmalarda ZEA maruziyeti sonucunda DNMT seviyesindeki artışa bağlı olarak global DNA metilasyonunun arttığı tespit edilmiş, bununla beraber aynı çalışmada tümörijenik olmayan epitel hücre hattı MCF-10F'de bir değişiklik gözlenmemiştir [62,63]. ZEA maruziyeti ile bronşiyal epitel normal hücre hattı (BEAS-2B) üzerinde yapılan bir çalışma da ise, global DNA metilasyonunda azalma ve ROS seviyesinde ve apoptoza yönelimde artış gözlenmiştir [64]. Diğer bir çalışmada, fareden izole edilmiş germinal vezikül (GV) safhasında oositlerde 50 μM ZEA maruziyeti sonunda global DNA metilasyonunda artış olduğu belirtilmiştir [65].

FB1 ve ZEA'nın Histon Modifikasyonu Üzerine Etkileri

FB1 ve ZEA maruziyeti sonucunda histon modifikasyonlarında değişimler olduğu yapılan çalışmalarla gösterilmiştir. Bu çalışmalar, Tablo 1 ve Tablo 2'de özetlenmiştir.

Sıçan fetüsünden alınan karaciğer dokuları ile yapılan bir çalışmada; FB1 maruziyeti sonucunda histon H4 lizin 20 trimetilasyon (H4K20me3) seviyesi azalırken, H4K9me3 seviyesinde artış gözlenmiştir [65]. HepG2 ile yapılan bir çalışmada ise 200 μM FB1 maruziyeti sonucunda global histon modifikasyonlarında azalma görülmüştür [57]. NRK-52E hücrelerinde 24 saatlik 25 μM FB1 uygulaması sonucunda, H3K9me2 ve H3K9me3 seviyelerinde artış gözlenirken, H4K20me3 ve histon H3 lizin 9 asetilasyonu (H3K9ac) seviyelerinde azalma gözlenmiştir [67]. Fare embriyonik fibroblast hücre hattı (MEF) ile yapılan bir çalışmada ise 40 μM FB1 maruziyeti sonucu histon deasetilaz aktivitesinde düşüşe sebep olduğu gösterilmiştir [68]. İnsan özofagus epitel hücrelerinde (HEEC) yapılan bir çalışmada, FB1'in histon metiltransferazlardan *G9a, EZH2, SETD8, SETD1A, Suv39h1, PRDM2,* asetiltransferazlardan HAT1 ve deasetilazlardan *SIRT1* ifadelerinde önemli ölçüde azalmaya neden olduğu gösterilmiştir [31]. HK-2 ile yapılan çalışmada ise, FB1 maruziyeti sonucunda doza bağlı olarak kromatin-modifiye genlerin ifadelerinin azaldığı gözlenmiştir. 100 μmol/L FB1 maruziyeti sonucunda, *p16* geninde H3K9ac, H3K9me3 ve H3K27me3 modifikasyon seviyeleri anlamlı şekilde azalırken; 24 saatlik 100 μmol/L maruziyeti sonucu ise *p16* genindeki H3K27me3 modifikasyon

seviyesinin arttığı görülmüştür [61].

ZEA maruziyeti sonucunda ise, direkt ve dolaylı olarak etkilenen farelerde spermatogenezin bozulduğu ve fare testis dokularında H3K27, H3K9 ve H3K7 seviyelerinde artış gözlenmiştir [69,70]. Ayrıca, 12 saat boyunca ZEA maruziyeti sonucu fare GV safhasındaki oositlerde H3K4me2, H3K9me3, H4K20me1, H4K20me2 ve H4K20me3 seviyelerinde azalma gözlenmiştir [65]. HepG2 hücreleri üzerinde yapılan çalışmada ise; ZEA maruziyeti ile H3K9me3 ve H3K27me3 seviyelerinde artış ve kromatin yapısında değişikliğe sebep olan enzimleri kodlayan *EHMT2, ESCO1, HAT1, KAT2B, PRMT6* ve *SETD8* genlerinin de ifadelerinde artışa sebep olduğu belirtilmiştir [63].

Tablo 1. FB1 maruziyetinin epigenetik düzenlemeler üzerine etkisi

Model ve Maruziyet Koşulları		**Sonuçlar**	**Kaynak**
In Vitro			
İnsan Özofagus Epitel Hücre Hattı (HEEC)	0.325-5μM, 120 saat	Histon deasetilaz ifadesini arttırmış, histon asetilaz ifadesini azaltmıştır.	[31]
İnsan Karaciğer Kanseri Hücre Hattı (HepG2)	200 μM, 24 saat	Global DNA hypometilasyonu artmıştır. DNMT1, DNMT3A, ve DNMT3B metiltransferanslarının seviyeleri azalmıştır. KDM5B ve KDM5C seviyeleri artmıştır.	[57]
İnsan Kolorektal Adenokarsinoma Hücre Hattı (Caco-2)	10 μM, 72 saat	Global DNA metilasyonu artmıştır.	[58]
Sıçan Böbrek Epitel Hücre Hattı (NRK-52E) ve Sıçan Karaciğer Epitel Hücre Hattı (Clone-9)	10, 25 ve 50 μM, 24 saat	Her iki hücre hattında da *VHL* geninde metilasyon görülürken, Clone-9 hücrelerinde *C-myc* geninde metilasyon görülmüştür.	[59]
İnsan Embriyonik Böbrek Hücre Hattı (HEK293)	100 μmol/l, 24 saat	Global DNA metilasyonu ve DNMT aktivitesi artmıştır.	[60]
İnsan Böbrek Hücre Hattı (HK-2)	50 ve 100 μmol/l, 24 saat	100 μmol/l uygulamada DNMT3a ve DNMT3b seviyesi artmış, 50 ve 100 μmol/l uygulamada DNMT1 seviyesinde azalmıştır. *p16* geninde H3K9ac, H3K9me3 ve H3K27me3 modifikasyon seviyeleri azalmış, H3K27me3 modifikasyon seviyesi artmıştır.	[61]
Sıçan Böbrek Epitel Hücre Hattı (NRK-52E)	25 μM, 24 saat	Global H3K9me2 ve H3K9me3 seviyeleri artmış, H4K20me3 seviyesi azalmıştır.	[67]
İnsan Karaciğer Kanseri Hücre Hattı (HepG2)	200 μM, 24 saat	miR-135b, miR-181d, miR-27a/b ve miR-30c seviyelerinde azalma görülmüştür.	[71]
Fare Embriyonik Fibroblast Hücre Hattı (MEF)	40 μM, 24 saat	Histon deasetilaz aktivitesi düşmüştür.	[68]
İnsan Karaciğer Kanseri Hücre Hattı (HepG2)	200 μM, 24 saat	miR-30c seviyesi artmıştır.	[72]
In Vivo			
Sıçan Fetüsünden Alınan Karaciğer Dokuları	2 μg/kg/gün, 20 gün	H4K20me3 seviyesi azalmış, H4K9me3 seviyesi artmıştır.	[66]

Tablo 2. ZEA maruziyetinin epigenetik düzenlemeler üzerine etkisi

Model ve Maruziyet Koşulları		Sonuçlar	Kaynak
In Vitro			
İnsan Meme Kanseri (MCF7) ve Tümörijenik Olmayan Epitel Hücre Hattı (MCF10F)	50 μM, 24 saat	Global DNA metilasyonunun artmıştır. DNMT seviyesi ve 5mC miktarı artmıştır. MCF-10F'de bir değişim görülmemiştir.	[62]
İnsan Karaciğer Kanseri Hücre Hattı (HepG2)	10 ve 50 μM, 24 saat	Global DNA metilasyonu artmıştır. H3K9me3 ve H3K27me3 seviyeleri atmıştır.	[63]
Bronşiyal Epitel Normal Hücre Hattı (BEAS-2B)	40 μM, 24 saat	Global DNA metilasyonu azalmıştır.	[64]
Fare Leydig Hücre Hattı (TM3)	50 μmol/l, 24 saat	miR-96-5p, miR467e-3p, miR-19a-3p ve miR-221-5p ifadelerinde artış gözlenirken miR-96-3p, miR-146b-3p, miR-185-5p, miR-326-3p ifadeleri azalmıştır.	[77]
Fare Leydig Hücre Hattı (TM3)	0.01 μmol/l, 2, 4, 6 ve 18 saat	Let-7c-5p, miR21a-5p, miR-10b-5p ve miR10a-5p ifadeleri 18 saat sonunda artmış, miR7a-5p ise azalmıştır.	[78]
In Vivo			
Germinal Vezikül (GV) Safhasında Fare Oositleri	50 μM, 12 saat	Global DNA metilasyonu ve 5mC seviyesi artmıştır. H3K9me3, H3K4me2, H4K20me1, H4K20me2 ve H4K20me3 seviyeleri azalmıştır.	[65]
CD-1 Erkek Fare Testis Dokuları	20 ve 40 μg/kg, 5 hafta	5mC ve 5hmC seviyesinde azalış görülürken H3K27 seviyesinde artış görülmüştür.	[69]
Dolaylı Olarak Etkilenmiş Fare Testis Dokuları	20 ve 40 μg/kg, 7 gün	H3K9 ve H3K7 seviyeleri artmıştır.	[70]
Domuz Hipofiz Bezi Dokuları	7.5 mg/kg, 24 saat	miR-7 ifadesi artmıştır.	[75]
Domuz Uterus Dokuları	0.17mg/kg, 1.46 mg/kg ve 4.58 mg/kg, 28 gün	miR-424-5p, miR-450c-5p, miR-450b-5p, miR450a, miR-503 ve miR-542-3p ve miR-181c ifadeleri artmıştır.	[76]
Domuz Karaciğer ve Kolon Dokuları	40 μM/kg, 35 gün	miR-15a, miR-21, miR-192 ve miR-7 ifadeleri artmıştır.	[79]
Domuz Granüloza Hücreleri (pGC)	10 veya 30 μM, 48 saat	miR-744, miR-1343 ve miR-331-3p ifadeleri artmıştır.	[80]

FB1 ve ZEA'nın miRNA'lar Üzerine Etkileri

FB1 maruziyetinin miRNA'lar üzerinde etkisi üzerine literatürde çok az çalışma bulunmaktadır. HepG2 hücre dizisi üzerine yapılan çalışmada, 200 μM FB1 uygulamasıyla miR-135b, miR-181d, miR-27a/b ve miR-30c'de azalma görülmüştür [71]. HepG2 hücreleri ile yapılan bir diğer çalışmada ise, 24

saat 200 μM FB1 maruziyetinin miR-30c seviyesini arttırarak PTEN translasyonunu bloke ettiği, PI3K/Akt sinyal yolağı üzerinden kontrol noktalarının düzenlenmesinin engellendiği ve buna bağlı olarak DNA hasarı oluşumuna sebep olduğu bildirilmiştir [72].

Diğer yandan, ZEA'nın miRNA'lar üzerine etkisi farklı hücre dizileri ve hayvan modelleriyle çalışılmıştır. miRNA'ların hipofiz hormonlarının sentezini etkilediği ve üreme bozukluklarına yol açtığı hayvanlar üzerinde yapılan çalışmalarla gösterilmiştir [73,74]. ZEA'nın *in vivo* ve *in vitro* uygulamaları sonucunda, protein kinaz C (PKC) ve p38 sinyal yolu üzerinden miR-7 ifadesinde artış görülmüştür. 7,5 mg/kg/gün ZEA maruziyeti sonucu domuz hipofizinin incelendiği çalışmada; miR-7 ifadesindeki artışın, *FOS* geninin sentezini aktive ederek folikül uyarıcı hormon (FSH) sentezini ve salgılanmasını engellediği görülmüştür [75]. Domuzlar üzerinde yapılan başka bir çalışmada ise, 28 günlük 1,46 mg/kg ve 4,58 mg/kg olmak üzere iki farklı ZEA maruziyeti sonucu uterusta miRNA profilinin incelendiği çalışmada; gen ekspresyonu ve sinyal yolaklarının düzenlenmesinde etkili genleri hedef alan 14 miRNA'da doza bağlı olarak önemli değişiklikler görülmüştür [76]. Fare leydig hücreleri (TM3) üzerinde yapılan çalışmalarda ise, 50 μmol/L ZEA maruziyetinin 24. saatinde, 86 miRNA'da artma ve 111 miRNA'da azalma olmak üzere 197 miRNA'da değişiklikler gözlenmiş olup Rap1, PI3K-Akt, FOXO, AMPK ve RAS olmak üzere 5 önemli sinyal yolağının etkilendiği görülmüştür [77]. Aynı hücrelerde farklı zaman aralıklarında (0, 2, 6 ve 18 saat) 0,01 μmol/L ZEA maruziyeti sonucunda ise; Let-7a, miR-10b ve miR-21a ifadelerinde artış, buna bağlı olarak MAPK ve RAS-RAF-MEK-ERK gibi hücre proliferasyonu üzerinde etkili yolakların etkilendiği görülmüştür [78]. Başka bir çalışmada, 40 μM/kg/gün ZEA maruziyeti sonunda domuzlardan alınan karaciğerde ve kolonda; 35.gün sonunda karaciğerde miR-15a, miR-21 ve miR-192 seviyelerinde önemli bir artış gözlenirken, kolonda sadece miR-15a seviyesinde artış görülmüştür [79]. Domuzdan izole edilen granüloza hücreleri (GC) ile yapılan çalışmada ise, ZEA maruziyeti ile miR-744, miR-1343 ve miR-331-3p ifadelerinde artış gözlenmiş olup buna bağlı olarak apoptozla bağlantılı sinyal yolaklarının aktifleştiği ve hücre büyümesinin engellendiği görülmüştür [80].

SONUÇ VE TARTIŞMA

Mikotoksinler, çeşitli yollar ile tüketimleri sonucunda vücutta çeşitli hasara neden olabilen önemli toksinlerdendir. Son zamanlarda, yapılan çalışmalar artmış olup tüketimleriyle ilgili çeşitli önlemler alınmaktadır. FB1 ve ZEA en çok çalışılan mikotoksin türlerinden olup çeşitli hayvan ve hücre modelleri üzerinde çalışmalar yapılmıştır. Yapılan çalışmalar sonucunda, iki mikotoksin türünün vücutta farklı hasarlara sebep olabileceği gösterilmiştir. FB1'den farklı olarak ZEA, toksik etkilerin yanında östrojenik etkileri dolayısıyla endokrin ve üreme sistemi problemlerine sebep olabileceği de gözlenmiştir. FB1 ve ZEA'nın toksik etkilerinin ortaya çıkmasında epigenetik mekanizmaların da rolünün olabileceği gerçekleştirilen araştırmalarda gösterilmiştir. FB1 ve ZEA'nın DNA metilasyonunu, histon modifikasyonunu ve miRNA seviyelerini değiştirdiği çeşitli çalışmalarla belirtilmiştir. Doza ve uygulama süresine bağlı olarak global DNA metilasyonunu arttırdığı veya azalttığı, farklı miRNA seviyelerini ve histon modifikasyonları aktivitesini değiştirdiği farklı modellerde tespit edilmiştir. Ayrıca, epigenetik düzenlemelere bağlı olarak DNA proliferasyonunu değiştirdiği ve önemli sinyal yolaklarını etkilediği yapılan çalışmalarla gösterilmiştir. FB1 ve ZEA'nın toksik etkilerinin değerlendirilmesinde *in vivo* hayvan çalışmaların sayısının arttırılması ile elde edilecek sonuçlar, var olan araştırmaları desteklemesi açısından son derece önemlidir. Bu derleme ile FB1 ve ZEA'nın toksik etkilerinde epigenetik mekanizmaların rolü ile ilgili araştırmalar incelenmiş olup bu toksinlerin moleküler toksik etki mekanizmalarında epigenetik çalışmaların önemi vurgulanmıştır.

YAZAR KATKILARI

Kavram: E.P., S.Ö.; Tasarım: E.P., S.Ö.; Denetim: - ; Kaynaklar: - ; Malzemeler: - ; Veri Toplama ve/veya İşleme: - ; Analiz ve/veya Yorumlama: E.P., S.Ö.; Literatür Taraması: E.P., S.Ö.; Makalenin Yazılması: E.P., E.K., S.Ö.; Kritik İnceleme E.P., E.K., S.Ö.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1201-1218, 2024
Doi: 10.33483/jfpau.1459098

DERLEME MAKALE / REVIEW ARTICLE

OTOİMMÜN HASTALIKLARDA LUTEOLİN BİLEŞİĞİNİN ROLÜ

ROLE OF LUTEOLIN IN AUTOIMMUNE DISEASES

Ali Eren IŞKIN[1], Muhammed Ali KIZMAZ[1], Ferah BUDAK[1]*

[1]Bursa Uludağ Üniversitesi, Tıp Fakültesi, İmmünoloji Anabilim Dalı, 16059, Bursa, Türkiye

ÖZ

Amaç: *Bitkisel gıdalarda yaygın olarak bulunan ve kristalik bileşikler olarak da bilinen flavonoidler, optik aktivite özelliği gösteren bileşiklerdir. İskelet yapılarının farklılıklarına dayalı olarak alt tipleri bulunmaktadır. Luteolin (LUT), boyacı katırtırnağı (Genista tinctoria) ve muhabbet çiçeği (Reseda luteola) bitkilerinden elde edilen önemli bir flavondur. Yapılan çalışmalarda, anti-oksidan, anti-enflamatuvar, immünomodülatör, anti-kanser ve nöroprotektif gibi birçok özellik sergilediği bildirilmiştir. LUT gibi doğal ürünlerden elde edilen bileşiklerin, kanser, otoimmün, nörodejeneratif ve kronik hastalıkların gelişiminde önemli rolleri nedeniyle çok sayıda araştırmanın odak noktası haline gelmiştir. Bu derlemedeki amacımız, otoimmün hastalıklarda LUT bileşiğinin terapötik rolünü değerlendirmektir.*

Sonuç ve Tartışma: *Çeşitli sebzelerde, meyvelerde bulunan ve değişken bir fenolik madde yapısına sahip olan LUT bileşiğinin; sistemik lupus eritematozus, romatoid artrit, astım, multipl skleroz, otoimmün ensefalit, ülseratif kolit, otoimmün diyabet, psoriasis, otoimmün tiroidit ve üveit gibi bazı otoimmün hastalıklarda anti-enflamatuvar, anti-oksidan, immünomodülatör ve nöroprotektif aktiviteler sergilediği bildirilmiştir. Ancak, LUT bileşiğinin toksisitesi ile ilgili endişe verici bazı bulgular da raporlanmaştır. Gelecekte yapılacak olan farmakokinetik, toksisite, kombine tedavi ve moleküler biyoloji çalışmaları LUT bileşiğinin sağlık üzerindeki etkilerini daha iyi anlamamıza yardımcı olabilir.*

Anahtar Kelimeler: *Anti-oksidan, flavonoid, luteolin, otoimmün hastalıklar, otoimmünite*

ABSTRACT

Objective: *Flavonoids, also known as crystalline compounds commonly found in plant foods, are compounds that exhibit optical activity. There are subtypes based on differences in their skeletal structure. Luteolin (LUT) is an important flavone obtained from dyer's catnip (Genista tinctoria) and lovebell (Reseda luteola) plants. In studies, it has been reported to exhibit many properties such as antioxidant, anti-inflammatory, immunomodulatory, anticancer and neuroprotective. Compounds derived from natural products such as LUTs have become the focus of numerous studies due to their important roles in the development of cancer, autoimmune, neurodegenerative and chronic diseases. Our aim in this review is to evaluate the therapeutic role of LUT compound in autoimmune diseases.*

Result and Discussion: *The compound LUT, characterized by a variable phenolic structure and found in various vegetables and fruits, has been reported to exhibit anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective activities in several autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, asthma, multiple sclerosis, autoimmune encephalitis, ulcerative colitis, autoimmune diabetes, psoriasis, autoimmune thyroiditis, and uveitis. However, there have also been some concerning findings regarding the toxicity of the LUT*

* **Sorumlu Yazar / Corresponding Author:** Ferah Budak
e-posta / e-mail: fbudak@uludag.edu.tr, **Tel. / Phone:** +905326227485

Gönderilme / Submitted : 26.03.2024
Kabul / Accepted : 12.07.2024
Yayınlanma / Published : 10.09.2024

compound. Future pharmacokinetic, toxicity, combination therapy, and molecular biology studies could help us better understand the health effects of the LUT compound.
Keywords: *Antioxidant, autoimmune diseases, autoimmunity, flavonoid, luteolin*

GİRİŞ

Otoimmün hastalıklar, heterojen bir hastalık kümesidir. Genetik risk, çevresel faktörler gibi olası durumların otoimmün hastalıklarda rol aldığı ve immün sistemin kontrol mekanizmalarındaki bozulmalar ile otoimmünite gelişimine yol açabileceği düşünülmektedir [1]. Doğal bileşikler, yeni ilaç moleküllerinin kaynağıdır. Doğal bileşiklerin, düşük toksisite ve daha az yan etki gibi önemli biyolojik etkileri bulunmaktadır. Bilim insanlarının hastalıkları incelemesi ve bu hastalıklara karşı terapötik ilaç arayışları doğal bileşiklere olan ilgiyi arttırmıştır [2]. Luteolin (LUT) bileşiği; anti-oksidan, anti-enflamatuvar ve nöroprotektif gibi önemli farmakolojik etkilere sahiptir. Bu bileşik, birçok bitki türünde bulunan bir etken madde olarak da işlev göstermektedir [3]. Bu derlemedeki amacımız, doğal bir flavon olan LUT bileşiğinin immünojenik özelliklerini ve otoimmün hastalıklardaki olası terapötik etkilerini değerlendirmektir.

Flavonoidler

Polifenolik fitokimyasal özellik gösteren flavonoid sınıfı bileşikler, özellikle sebze ve meyvelerde olmak üzere bitkiler aleminde yaygın olarak bulunan bileşiklerdir. Bu bileşikler, bitkilerde polifenolik iskelete sahip bir grup sekonder metabolitlerle ilişkilidirler [4]. Şimdiye kadar 10.000'den fazla flavonoid bileşiği izole edilmiştir [5]. Flavonoidler; doymamışlık derecelerine, kimyasal yapılarına ve karbon halkasının oksidasyonuna göre sınıflandırılmaktadır. Flavonoidlerin her biri doğada doğal bulunur ve farklı alt gruplara ayrılırlar [6]. Bunlar; flavanonlar, flavanoller, flavanonoller, flavonoller, antosiyaninler, kalkonlar ve flavonlardır (Tablo 1) [7].

Tablo 1. Flavonoidlerin sınıflandırılması [7]

FLAVONOİDLER	Flavanonlar
	Flavanoller
	Flavanonoller
	Flavonoller
	Antosiyaninler
	Kalkonlar
	Flavonlar

Flavonoid bakımından zengin gıdalar, kanser, gastrointestinal, metabolizma ve nörodejeneratif hastalıkları engelleyebilir. Flavonoidlerin antikanser, anti-malaryal, anti-mikrobiyal, anti-viral, analjezik, anti-oksidan, anti-anjiyogenik, anti-proliferatif ve anti-enflamatuvar gibi birçok etkisi gösterilmiştir (Tablo 2) [8]. Flavonoidlerin en önemli alt gruplarından biri de flavonlardır. LUT, sinensetin, nobiletin, krisin, apigenin, izosinensetin ve tangeretin gibi önemli bileşikler flavonlar içerisinde yer almaktadır [9].

Luteolin ve Aktiviteleri

LUT; dört hidroksil grubunun 3, 4, 5 ve 7 pozisyonlarında yer alan, tetrahidroksiflavon olarak tanımlanan ve farklı bitki türlerinde bulunan doğal bir bileşiktir. $C_{15}H_{10}O_6$, bileşiğin moleküler formülüdür [10-12]. Polifenolik yapısı sayesinde bitki hücrelerini, mikroorganizma ve radyasyon gibi stresli uyaranlara karşı korur [10,11]. Lahana, enginar, kereviz, maydanoz, fesleğen, kekik, havuç, elma, nane ve brokoli gibi birçok meyve ve sebzenin LUT açısından zengin olduğu gösterilmiştir (Tablo 3) [12]. LUT bakımından zengin bitkiler, Çin tıbbında da kullanılmaktadır. Genellikle bu bitkilerin kanser, enflamasyon ve kardiyovasküler hastalıkların tedavisinde kullanıldığı bilinmektedir [13].

Tablo 2. Flavonoidlerin aktiviteleri [8]

FLAVONOİDLER	Anti-kanser
	Anti-malaryal
	Anti-mikrobiyal
	Anti-viral
	Analjezik
	Anti-oksidan
	Anti-anjiyogenik
	Anti-proliferatif
	Anti-enflamatuvar

Tablo 3. LUT içeren doğal ürünler [12]

LUTEOLİN	Elma
	Nane
	Brokoli
	Havuç
	Kekik
	Fesleğen
	Maydanoz
	Kereviz
	Enginar
	Lahana

Deneysel veriler LUT bileşiğinin anti-oksidan özelliklere sahip olduğunu ve bu etkileri çoklu mekanizmalarla sürdürebildiğini ortaya koymuştur. LUT, kendini okside ederek reaktif oksijen türleri (ROT) temizleyicisi olarak fonksiyon gösterebilir [14], ROT'u meydana getiren oksidazları inhibe edebilir [15], glutatyon redüktaz (Glutathione reductase; GR), glutatyon S-transferaz (Glutathione S-transferase; GST), katalaz (catalase; CAT) ve süperoksit dismutaz (Superoxide dismutases; SOD) gibi endojen anti-oksidanları koruyabilir ya da düzeylerini arttırabilir [16-18]. Bununla birlikte, doğrudan hücre bileşenlerinin oksidasyonunu katalize eden enzimleri engelleyerek anti-oksidan etkinlik gösterebilir.

LUT bileşiğinin nöron koruyucu ve anti-oksidan etkileri nükleer faktör eritroid 2-ilişkili faktör 2 (nuclear factor erythroid 2–related factor 2; Nrf2) yolağı ile ilişkilendirilmiştir [19]. Harris ve arkadaşları, LUT ve krisin bileşiklerinin anti-oksidan özelliklerini lipopolisakkarit (LPS) ile endüklenen bir makrofaj hücre hattında (RAW 264.7) değerlendirmişler ve bu iki bileşiğin prostaglandin E2 (PGE2) oluşumunu inhibe ettiğini gözlemlemişlerdir. Sonuç olarak araştırmacılar, yapısal olarak birbirine benzer flavonların (LUT ve krisin) farklı mekanizmalar ile benzer anti-enflamatuvar özellikler sergileyebildiğini belirtmişlerdir [20]. Bir diğer çalışmada, Reudhabibadh ve arkadaşları, LUT bileşiğinin anti-oksidan özelliğini, 1-metil-4-fenilpiridinyum iyodür ile endüklenen nöroblastoma (SH-SY5Y) hücrelerinde değerlendirmişler ve bulgularında LUT bileşiğinin apoptozu ve mitokondriyal ROT'a bağlı oksidatif stresi engellediğini bildirmişlerdir [21]. Proenflamatuvar faktörlerin neden olduğu enflamatuvar süreçte, LUT bileşiğinin enflamatuvar süreçleri önleyebileceği pek çok *in vitro* ve *in vivo* çalışmalarla gösterilmiştir. Bu etkiler arasında enzimlerin ve enzim aktivitesinin bloke edilmesi, nükleer faktör kappa B (Nuclear factor kappa B; NF-κB) yolağının baskılanması ve sitokin üretiminin engellenmesi bulunmaktadır [22].

Hücresel Homeostaz ve İmmün Yanıtta Luteolinin Rolü

İmmün sistemin patojenler, toksik bileşikler, ultraviyole (UV) ve hasarlı hücreler gibi yararlı olmayan uyaranlara verdiği cevaba enflamasyon adı verilir [23]. Makrofajlar enflamasyon sırasında çeşitli moleküller tarafından aktifleşir. Bu moleküller, patojenlerden gelen toksinler ve konakçıdan gelen sitokinlerdir. Aktifleşen makrofajlar, serbest radikaller (ROT ve reaktif nitrojen türleri), interlökin (IL)'ler ve tümör nekroz faktörü-α (TNF-α) gibi enflamatuvar molekülleri üretirler. Akut enflamasyon sırasında aktifleşen makrofajlar, lenfositler ve nötrofiller gibi enflamatuvar hücrelerin iltihaplı bölgeye toplanmasına patojenlerin ve zararlı etkenlerinin ortadan kaldırılmasına aracılık eder [24, 25]. Enflamasyonun bir türü de kronik enflamasyondur. Otoimmün hastalıklar, artrit, alerji, kanser ve ateroskleroz gibi hastalıklar kronik enflamasyonla ilişkilidir [26].

Enflamasyon belirteçleri, çeşitli hastalıkların araştırılmasında önemli hale gelmektedir. Bu enflamasyon belirteçleri arasında sitokin olarak IL-1, IL-6, IL-8, TNF-α; enzim ve protein olarak siklooksijenaz-2 (cyclooxygenase-2; COX-2) ve matriks metalloproteinazlar (MMP'ler) bulunmaktadır [26]. LUT bileşiği, pleiotropik etkiler gösterir. Enflamatuvar mediyatörleri *in vivo* ve *in vitro* araştırmalarda regüle ederek, anti-enflamatuvar etkiler gösterdiği bildirilmiştir [12]. Aktivatör protein 1 (Activator protein 1; AP-1), Janus kinaz (JAK) sinyal transdüseri ve transkripsiyon aktivatörü (STAT) [(Janus kinase (JAK)-signal transducer and activator of transcription (STAT); JAK STAT)], NF-κB gibi çeşitli sinyal yollarının regülasyonunda ve nitrik oksit (NO), IL-6, IL-1β, TNF-α gibi proenflamatuvar faktörlerin baskılanmasında da LUT bileşiğinin rol oynadığı gözlemlenmiştir [27]. LUT bileşiği, çeşitli enflamatuvar faktörleri ve enflamasyona neden olan biyokimyasal yolakları inhibe ederek birçok kronik hastalıkları önleyebilir [3].

Flavonoidler, organizmaları ve hücreleri stres faktörlerinden koruyabilirler. Bu koruma işlemini yapabilmelerinin en aktif yolu, hücre döngüsünün regülasyonunda görev alan enzimlerle etkileşime girmeleridir [12]. LUT bileşiğinin minimum üç sinyal yolağı ile etkileşime girdiği bilinmektedir. Bu sinyal yolakları arasında; NF-κB, sinyal dönüştürücü ve transkripsiyon 3 aktivatörü (signal transducer and activator of transcription-3; STAT-3) yolağı ve AP-1 yolağı bulunmaktadır [12]. Proenflamatuvar genlerin ekspresyonunda NF-κB transkripsiyon faktörü kilit bir rol üstlenir. LUT gibi bitkilerde bulunan doğal bileşikler, NF-κB transkripsiyon faktörünü inhibe ederek anti-enflamatuvar özellik sergilerler [28].

LUT bileşiğinin fotoyaşlanma ile ilişkili fibroblastlar ve keratinositler üzerinde de önemli yararlı etkileri bulunmaktadır. Weng ve arkadaşlarının yapmış olduğu bir araştırmada LUT bileşiğinin, NF-κB sinyalini inhibe ederek insan epidermal keratinositleri ve HaCaT hücreleri üzerinde IL-6 ve IL-8 gibi proenflamatuvar sitokinlerin üretimini azalttığı gösterilmiştir [29]. Bir başka çalışmada ise, keratinositlerde solar radyasyon aracılı proenflamatuvar TNF-α, IL-6 ve IL-20 gibi sitokinlerin LUT tarafından module edilebildiği ve dermal fibroblastlarda fotoyaşlanmayı hafifletebileceğine dair kanıtlar sunulmuştur [30].

Nörogelişimsel ve nörodejeneratif bazı hastalıklarda LUT bileşiğine dair ilgi çekici bulgular sunulmuştur. Taliou ve arkadaşlarının yapmış olduğu Otizm Spektrum Bozukluğu (OSB) tanılı 50 çocuğun yer aldığı bir klinik çalışmada, 26 hafta boyunca hastaların diyet takviyesinde kersetin (70 mg/kapsül) ve LUT (100 mg/kapsül) kombinasyonu uygulanmış ve bu kombinasyonun, herhangi bir önemli yan etki olmadan OSB semptomlarını azaltmada etkili olduğu bildirilmiştir [31]. LUT bileşiğinin Alzheimer hastalığının bir *in vitro* modelinde SH-SY5Y hücrelerinde doza bağlı bir şekilde, Ser262/356'daki çinko kaynaklı tau fosforilasyonunu azalttığını Zhou ve arkadaşları bildirmiştir [32]. Kou ve arkadaşları LUT bileşiği tedavisinin Alzheimer hastalığı modellenmiş üçlü transgenik fare modelinde IL-1β, IL-6, COX-2, NO, endüklenebilir nitrik oksit sentaz (inducible nitric oxide synthase; iNOS) ve TNF-α gibi nöroenflamasyonla ilişkili olan belirteçlerin seviyelerini inhibe ettiğini gözlemlemişlerdir [33].

LUT bileşiğinin anti-tümör rollerine dair sınırlı sayıda çalışmada önemli bulgular bildirilmiştir. LUT bileşiğinin A431 hücre hattında (invazif epidermoid karsinom hücre hattı), epitelyal-mezenkimal geçişi (EMT) engelleyerek metastazı önlediği gösterilmiştir. Aynı zamanda bu bileşiğin, vimentin ve N-kadherin gibi önemli mezenkimal belirteçlerin ekspresyonunu da baskıladığı gösterilmiştir [34]. Erdoğan ve arkadaşlarının yapmış oldukları bir çalışmada insan kolorektal kanser hücre hattı olan HT-

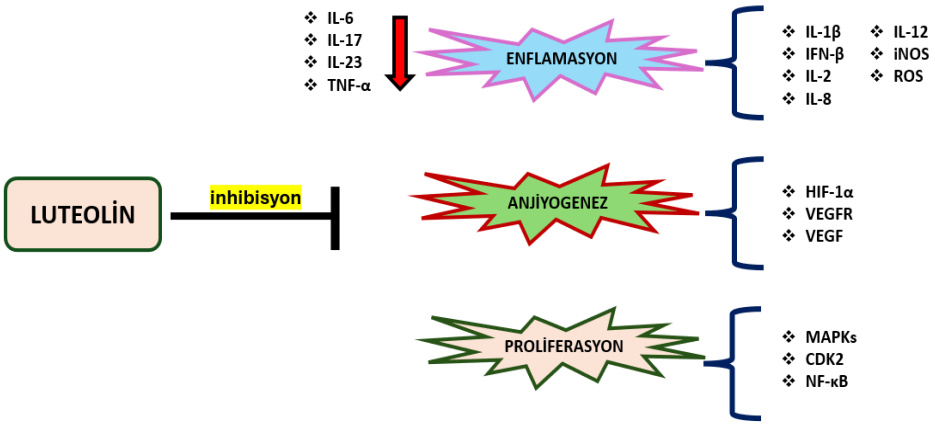
29'da, LUT ve kersetin bileşikleri ile 5-Florourasil (5-FU) ilaç kombinasyonunun anti-tümör özelliklerini incelemişlerdir. Bulgularında, bu iki bileşiğin HT-29 hücrelerinde 5-FU'nun anti-tümör ilaç özelliğini sinerjistik olarak arttırdığını belirtmişlerdir [35]. Zhao ve arkadaşları, *in vitro* deneylerde LUT tedavisinden sonra endometrial kanser hücrelerinde Vasküler Endotelyal Büyüme Faktörü (Vascular endothelial growth factor; VEGF), MMP1 düzeylerinin ve göç yeteneğinin azaldığını; IL-17 seviyesinin de baskılandığını göstermişlerdir. Sonuç olarak araştırmacılar, LUT bileşiğinin endometrial karsinomalı hastaların prognozunu düzeltebileceğini belirtmişlerdir [36].

LUT bileşiğinin demir metabolizması ile ilişkili etkileri olduğunu öne süren bazı araştırmacılar ilginç bulgular elde edilmiştir. Ferroptoz, reaktif oksijen türlerine ve fenton reaksiyonu aracılığıyla Fe^{+2} demir artışının meydana gelmesine bağlı olarak ortaya çıkan bir hücre ölüm tipidir. Yoğunlaştırılmış mitokondriyal membran, azalan veya kaybolan mitokondri kristası gibi önemli karakteristik özelliklere sahip olan ferroptoz, enfeksiyon hastalıkları başta olmak üzere birçok kanser ve nörodejeneratif hastalıklarla da ilişkisi bulunmaktadır [37]. Han ve arkadaşları, LUT bileşiğinin ferroptoza etki ederek terapötik etkiler gösterdiğini bildirmiştir [38]. Bu çalışmada, LUT bileşiğinin, heme oksijenaz 1 (heme oxygenase 1; HO-1) ekspresyonunu regüle ettiğini ve aynı zamanda kararsız demir havuzunu (labile iron pool; LIP) aktifleştirerek berrak hücreli renal hücreli karsinom (clear cell renal cell carcinoma; ccRCC)'da ferroptozu tetiklediğini gözlemlemişlerdir. Sonuç olarak araştırmacılar LUT bileşiğinin, potansiyel bir ferroptoz endükleyicisi olduğunu ve ccRCC'de anti-tümör etkiler sergilediğinin kanısına varmışlardır [38].

LUT bileşiğinin enflamasyon, anjiyogenez ve proliferasyon gibi birçok immünolojik süreci inhibe ettiği gösterilmiştir. Xie ve arkadaşları, akciğer hasarı üzerinde LUT bileşiğinin terapötik etkisini incelemişler ve bulgularında LUT bileşiğinin, $CD4^{+}CD25^{+}Foxp3^{+}$ regülatör T hücrelerinin (Treg) farklılaşmasını endüklediğini ve IL-10 sitokininin ekspresyonunu arttırarak akciğerdeki hasarı ve kontrolsüz enflamasyonu hafiflettiği görülmüştür [39]. Altı haftalık erkek C57BL/6 farelerde serulein ile endüklenen akut pankreatit modeli üzerine LUT bileşiğinin terapötik etkilerini Huang ve arkadaşları incelemiş; sonucunda LUT bileşiği tedavisinin, tübüler kompleks oluşumunu inhibe ettiğini; aynı zamanda fosforile epidermal büyüme faktör reseptörü (phosphorylated-epidermal growth factor receptor; p-EGFR), fosforile sinyal dönüştürücü ve transkripsiyon 3 aktivatörü (phosphorylated-signal transducer and activator of transcription-3; p-STAT-3) ve SRY-box transkripsiyon faktörü 9 (SRY-box transcription factor 9; SOX9) gibi önemli protein düzeylerini de azalttığını bildirmişlerdir [40]. Ly ve arkadaşları yapmış oldukları bir çalışmada, LPS ile endüklenen yenidoğan sıçan primer kardiyomiyositlerinde LUT bileşiğinin, TNF-α ekspresyonunu inhibe ettiğini göstermişlerdir [41]. LUT bileşiğinin, Sprague Dawley sıçanlarından elde edilen kemik iliği türevli makrofajlarda IL-6 ve TNF-α üretimini doza bağlı bir şekilde inhibe ettiği gösterilmiştir [42]. Benzer şekilde, LUT bileşiğinin, IL-6, IL-17, IL-23, granülosit makrofaj koloni uyarıcı faktör (Granulocyte-macrophage colony-stimulating factor; GM-CSF), TNF-α, IFN-β, IL-1β, IL-2, IL-8 ve IL-12 gibi önemli proenflamatuvar sitokinlerin seviyelerini azalttığı ve IL-10 sitokin seviyesini de arttırdığına dair faklı çalışmalar kanıtlar sunmuştur. (Şekil 1) [43, 44, 45]. Bir başka çalışmada ise, LUT bileşiğinin AP-1 aktivasyonunu ve CD40L ekspresyonunu inhibe edebildiği yapılan çalışmalarda belirtilmiştir. İnsan kökenli mast hücrelerinde LUT bileşiğinin, GM-CSF, immunglobulin E (IgE) aracılı histamin, prostaglandin D2 ve lökotrienler gibi önemli faktörlerin salınımını inhibe edebileceği gösterilmiştir [46].

LUT bileşiğinin, hücresel homeostazda etkin roller oynayabileceğine dair birçok kanıt sunulmuştur. Forkhead box O (FOXO), p53, Notch gibi nörotrofin yolaklarını LUT bileşiği tarafından düzenlenebileceği gösterilmiştir [43,44]. LUT bileşiğinin lökotrien, prostaglandin gibi önemli eikosanoidleri ve C-X-C Motif Kemokin Ligand 2 (C-X-C Motif Chemokine Ligand 2; CXCL2), CXCL8, CXCL9, C-C Motif Kemokin Ligand 2 (C-C Motif Chemokine Ligand 2; CCL2) gibi kemokinlerin düzeylerini de inhibe edebileceği belirtilmiştir [44]. Yang ve arkadaşlarının yapmış oldukları cıva klorür ($HgCl_2$) kaynaklı kunming fare hepatotoksisite modelinde LUT bileşiğinin, apoptoz ve enflamatuvar ilişkili proteinlerin Bcl-2 ile ilişkili X proteini (Bcl-2-associated X protein; Bax), B hücre lenfoma geni-2 (B-cell lymphoma gene-2; Bcl-2), Rapamisinin memeli hedefi (Mammalian target of rapamycin; mTOR), sirtuin1 (Sirt1), p53, TNF-α, ve NF-κB) düzeylerini tersine çevirdiği bildirilmiştir [47]. Zhang ve arkadaşları, farelerde çekal bağlama ve delme (cecal ligasyon ve puncture; CLP) yöntemi ile akciğer hasarı oluşturmuşlar ve fareleri LUT bileşiği ile tedavi etmişlerdir.

Bulgularında LUT bileşiğinin kontrol grubuna kıyasla; gasdermin D (GSDMD), kaspaz-1, kaspaz-11, IL-1α ve IL-1β protein seviyelerini önemli ölçüde azalttığını gözlemlemişlerdir [48].



Şekil 1. LUT'un bazı inhibisyon etkilerinin gösterimi [43-45]

Topluca ele alındığında, mevcut *in vitro* deneysel yaklaşımlar ve hayvan modeli çalışmaları ile LUT bileşiğinin anti-kanser, anti-oksidan, nöroprotektif ve anti-enflamatuvar özellikler sergileyebileceği birçok çalışmada bildirilmiştir. Apoptozu ve mitokondriyal ROT'a bağlı oksidatif stresi engelleyerek anti-oksidan aktivite gösterebileceği gibi AP-1, JAK STAT, NF-κB gibi önemli çeşitli sinyal yolaklarını regüle ettiği, hatta proenflamatuvar özellik gösteren belirteçleri inhibe edebileceği bildirilmiştir. Bununla birlikte, LUT gibi doğal bileşiklerin, NF-κB transkripsiyon faktörünü inhibe ederek anti-enflamatuvar aktivite sergileyebileceği gözlemlenmiştir. Destekler şekilde, LUT bileşiğinin, Treg hücre farklılaşmasını desteklediğini, alternatif makrofaj polarizasyonunu endükleyebildiği ve antienflamatuvar IL-10 sitokininin üretimini arttırdığına dair kanıtlar sunulmuştur. Aynı zamanda LUT bileşiğinin, nörogelişimsel ve nörodejeneratif bazı hastlarıklarda protektif roller oynayabileceği bildirilmiştir. Benzer şekilde, LUT bileşiğinin anti-tümör etkinliği ile ilgili umut verici bulgular bildirilmiştir. Sonuç olarak LUT bileşiğinin, önemli farmakolojik aktiviteler sergileyerek çeşitli hastalıklarda protektif bir rol oynayabileceği bildirilmiş ve literatüre önemli bilgileri kazandırılmıştır.

Otoimmün Hastalıklarda Luteolinin Rolü

Organizmanın self-antijenlerine karşı oluşturduğu immün cevaba otoimmünite adı verilmektedir [49,50]. İmmün sistem normal koşullarda, self-antijenlerine karşı sessiz kalır ve bir cevap oluşturmaz. Bu self-antijenlere karşı yanıtsızlık haline immün tolerans; immün sistemin self olanı tanıma, tepki vermeme veya saldırmama yeteneğine ise self-tolerans adı verilmektedir [51,52]. Self-tolerans özelliği kaybedilirse, vücut kendine özgü dokularına ve hücrelerine karşın otoimmünite durumu geliştirir ve bu süreç otoimmün hastalıklara neden olur [52].

Konağın bileşenlerine karşı normal olmayan T hücresi ve B hücresi aktivasyonuna yol açan, immün sistemin bozukluğuyla karakterize olan, herhangi bir organ sistemine tutulum sağlayan ve her yaştan bireyi etkileyip klinik belirtilerinin çeşitlilik gösterdiği hastalık grubuna otoimmün hastalıklar adı verilmektedir [53]. Otoimmün hastalık tanısını alan bireylerin dokularında genellikle makrofajların ve monositlerin infiltrasyonu görülmektedir. Bu hücreler, proenflamatuvar kemokin ve sitokinlerin salınmasını sağlayarak diğer immün hücrelerini enflamasyonun bulunduğu bölgeye toplar. Otoimmün hastalıklarda kronik enflamasyona; edinsel T ve B hücreleri, monositler ve makrofajlar birlikte katkıda bulunur [54]. Çoğu otoimmün hastalıklarda, kadınlar erkeklerden daha fazla etkilenir. Kadınların erkeklere göre otoimmün hastalıklardan etkilenme oranları, Sjögren sendromu ve Sistemik Lupus Eritematozus hastalığında dokuz kat iken Multipl skleroz ve Romatoid artrit hastalıklarında da bu oran iki ila üç kat arasındadır [55]. Bazı otoimmün hastalıkların tedavi süreçleri immünosupresif ilaç kullanımına dayanmaktadır. Bu tedavi seçeneği, hastalığın türüne veya ciddiyetine bağlı olarak

değişiklik gösterebilir. Siklofosfamid, mikofenolat mofetil, siklosporin gibi ilaçlar immünosupresifler arasında yer almaktadır [56].

Bilim insanları doğal ürünlerin kullanımını çeşitli kronik ve otoimmün hastalıkların tedavisinde, geniş çapta araştırmakta ve literatüre yeni bilgiler kazandırmaktadır. Doğal bir bileşik olan LUT etken maddesi; çok sayıda otoimmün, kronik, nörodejeneratif, gastrointestinal ve kanser hastalıkları üzerinde çalışılmış ve biyolojik özelliği bakımından çok sayıda terapötik faydasının olduğu düşünülen bir flavondur. Bu derlemede LUT bileşiğinin, otoimmün hastalıklardaki (Sistemik Lupus Eritematozus (SLE), Romatoid Artrit (RA), Astım, Multipl Skleroz (MS), Otoimmün Ensefalit, Ülseratif Kolit, Otoimmün Diyabet, Psoriasis, Otoimmün Tiroidit ve Üveit) terapötik etkileri özetlenmiştir.

Sistemik Lupus Eritematozus

Halk arasında kelebek hastalığı olarak tanımlanan ve otoimmün hastalık sınıfında yer alan Sistemik Lupus Eritematozus (SLE); dermatolojik, kardiyovasküler, renal ve nöropsikiyatrik semptomlarla karakterize bir hastalıktır. SLE, immün sistemin normal olmayan aktivitesi sonucu meydana gelir [57]. Bu hastalıkta en fazla görülen komplikasyonlardan biri lupus nefriti (LN)'dir. LN, glomerülonefrit türü olarak bilinmektedir. SLE hastalarında, mortalite ve morbiditeye yol açabilir [58]. Hipoksi ile endüklenen faktör-1 alfa (Hypoxia-inducible factor-1 alpha; HIF-1α) ve NF-κB gibi transkripsiyon faktörleri, LN gelişimine neden olabilir. Ding ve arkadaşları, LUT bileşiğinin HIF-1α'nın aracılık ettiği makrofaj oksidatif stresi üzerindeki etkisini değerlendirmiş ve LUT bileşiğinin LN fare modeli üzerinde koruyucu etkisini incelemişlerdir. LUT bileşiği tedavisinin, farelerin makrofajlarında ve böbrek dokularında HIF-1α ve NF-κB seviyelerini inhibe ettiğini gözlemlemişlerdir. Böylelikle, LN fare modelinde LUT bileşiğinin terapötik bir rol oynayabileceğini bildirmişlerdir [59]. Jieduquyuziyin karışımı (JQZP), Çin'in Zhejiang Eyaleti Geleneksel Çin Tıbbı Hastanesi'nde on yılı aşkın bir süredir SLE tedavisinde uygulanan ve bu hastalıkta meydana gelen enflamasyon durumunu azaltıcı bir etki gösteren karışımdır. Bu karışımın; LUT, apigenin ve salidrosid gibi bileşikleri içerdiği gösterilmiştir. Birçok moleküler biyolojik araştırmalar sonucunda bu karışımın; Nek7-NLRP3 sinyal yolunu regüle ederek enflamasyonu azalttığı, bağışıklığı düzenleyebildiği ve enfeksiyon oluşumunu azaltabildiği gösterilmiştir [60].

Liu ve arkadaşları *in vivo* ve *in vitro* metotlar oluşturarak JQZP'in SLE hastalığındaki terapötik rolüne odaklanmışlardır. JQZP'in *in vivo* ve *in vitro* deneyler sonucunda pro-enflamatuvar özellik taşıyan IL-1β ve IL-18 sitokin seviyelerini down regüle ettiğini göstermişlerdir. Aynı zamanda, yüksek performanslı sıvı kromatografi (High liquid pressure chromatography; HPLC) ve network farmakolojisi analizleri kullanarak JQZP ilaçlı serumda LUT, apigenin ve salidrosid gibi etken maddelerin olduğunu; docking analizi ile bu üç etken maddenin bir CARD içeren apoptozla ilişkili benek benzeri protein (Apoptosis-associated speck-like protein containing a CARD; ASC), Nek7 ve NLR ailesi pyrin alanı içeren 3 protein (NLR family pyrin domain containing 3 protein; NLRP3) proteinlerine bağlanabildiğini belirtmişlerdir [60].

Romatoid Artrit

Romatoid Artrit (RA); hiperplazi, romatoid faktör (RF), sinovyal enflamasyon ve anti-sitrulinlenmiş protein antikoru (ACPA) gibi otoantikorların üretimi ile karakterize olan otoimmün bir hastalıktır. Başta eklem ve bölgeleri olmak üzere göz, akciğer, böbrek, kalp, sinir ve sindirim sistemi gibi eklem dışı organları da etkileyebilir [61, 62]. Sekonder metabolitlerin terapötik etkileri, RA gibi birçok otoimmün hastalıkta bilimsel çalışmaya konu olmuştur. Shi ve arkadaşları, sıçanlar üzerinde bir artrit modeli oluşturmuş ve LUT bileşiğinin sıçan artrit modeli üzerindeki etkinliğini ve P2X4'ün RA'daki fonksiyonel mekanizmasını değerlendirmişlerdir. LUT bileşiğinin; IL-1β, TNF-α, IL-6 ve IL-17 sitokinlerini; P2X4, ASC, Caspase-1p10 ve NLR ailesi pyrin alanı içeren 1 protein (NLR family pyrin domain containing 1 protein; NLRP1) protein seviyelerini inhibe ettiğini gözlemlemişlerdir. Araştırmacılar buldukları sonuçlara göre, artrit tedavisinde LUT bileşiğinin terapötik bir etken madde olabileceği kanısına varmışlardır [63]. Bazı bilim insanları, LUT+farklı bir doğal bileşik ile kombinasyon tedavileri üzerinde araştırmalar yapmışlardır. Bu çalışmalardan birini Lou ve arkadaşları, IL-1β ile endüklenen RA sinovyal dokularında, fibroblast benzeri sinoviyositler (FLS) üzerinde yapmışlardır. LUT, klorojenik asit ve LUT+klorojenik asit kombinasyonu ile FLS hücrelerini tedavi

etmişlerdir. Bulgularında, LUT+klorojenik asit kombinasyonunun apoptozu endükleyerek FLS'lerin proliferasyonunu güçlü bir şekilde inhibe ettiğini bildirmişlerdir. Aynı zamanda bu kombine tedavinin FLS hücrelerinde, p-STAT3 seviyesini de düşürdüğünü göstermişlerdir [64]. Yang ve arkadaşları yapmış oldukları bir çalışmada LUT bileşiğinin, Freund'un tam adjuvanı ile endüklenen fare artrit modelinde pençe ödemini ve ROT oluşumunu engellediğini gözlemlemişlerdir. Aynı zamanda bu bileşiğin; elastaz salınımını, nötrofil hücre dışı tuzakları (Neutrophil extracellular trap; NET) oluşumunu, süperoksit anyon üretimini insan nötrofillerinde baskıladığını ve Raf1- mitojenle aktifleşen protein kinaz (mitogen-activated protein kinase kinase-1; MEK-1)-hücre dışı sinyalle düzenlenen kinaz (extracellular signal-regulated kinase; Erk) sinyal yolaklarını da inhibe ederek insan nötrofil enflamatuvar cevaplarını azalttığı kanısına varmışlardır [65]. Bir diğer çalışmada Hou ve arkadaşları sıçanlarda kollajen ile uyarılan deneysel artrit modelinde LUT bileşiğinin artriti önleyici etkisini incelemişler ve sonucunda bu bileşiğin, sıçan sinoviyal fibroblastlarının proliferasyon kabiliyeti üzerinde inhibe edici bir rol üstlendiğini gözlemlemişlerdir [66]. Xiao ve arkadaşları; *Siegesbeckia orientalis* L. (SOL) bitkisinin olası anti-RA etkisini değerlendirmiş ve SOL bitkisinde aktif bir bileşik olan LUT'un *in vivo* ve *in vitro* deneylerde anti-RA etki gösterdiğini ve toll benzeri reseptör 4 (toll like receptor 4; TLR4) sinyalini de inhibe ettiğini bildirmişlerdir [67]. Son olarak Impellizzeri ve arkadaşları, kollajen ile endüklenmiş fare artrit modelinde N-palmitoiletanolamin (PEA)+LUT (PEA-LUT) kombine tedavisinin oksidatif hasar belirteçlerini, proenflamatuvar sitokin ve kemokinlerin seviyelerini azalttığını bildirmişlerdir [68].

Astım

Astım, akciğer ve solunum yolları başta olmak üzere hem çocukları hem de yetişkinleri etkileyen, patolojik mekanizmaları tam olarak bilinmeyen kronik ve enflamatuvar bir solunum yolu hastalığıdır [69]. Jang ve arkadaşları, LUT bileşik tedavisinin alerjik astım ve rinitli fareler üzerindeki terapötik etkisini incelemişlerdir. LUT bileşik tedavisinin; T helper 2 (Th2)'de görev alan IL-4, IL-5 ve IL-13 gibi sitokin seviyelerinin fare akciğer parankiminde azalttığını tespit etmişler; rinit fare ve alerjik astım modelinde anti-alerjik özelliğe sahip olabileceği kanısına varmışlardır [70]. Kim ve arkadaşları, ovalbümin antijeniyle duyarlı hale getirilen fareler üzerinde LUT bileşiğinin; farelerin akciğer dokusunda $CD4^+$ T hücreleri, $CD3^-CCR3^+$ hücreleri, $CD19^+$ B hücreleri, $CD3e^+Gr\text{-}1^+$ hücre seviyelerini ve bronkoalveolar lavaj (BAL) sıvısında IL-13, IL-4, IL-5 ve IgE düzeylerini azalttığını bildirmişlerdir [71]. Wang ve arkadaşlarının fareler üzerinde alerjik astım modeli oluşturduğu bir çalışmada, LUT bileşik tedavisinin farelerin akciğer dokularında beclin1-fosfatidilinositol 3-kinaz (phosphatidylinositol 3-kinase; PI3KC3) protein kompleksi seviyesini azalttığını ve fosfatidilinositol 3-kinaz (Phosphatidylinositol 3-kinase; PI3K)/Akt/mTOR sinyal yolağını da aktive ederek otofajiyi inhibe ettiğini gözlemlemişlerdir. Böylelikle araştırmacılar alerjik astımda, LUT'un otofaji üzerinde düzenleyici bir mekanizmaya sahip olduğu kanısına varmışlardır [72]. THP-1 (insan monositik lösemi hücre hattı), astım hastalığının potansiyel mekanizmasını değerlendirmek amacıyla kullanılan bir hücre hattıdır. Yapılan bir çalışmada, Gong ve arkadaşları LUT bileşiğinin THP-1 ile endüklenen makrofajlarda hsa_circ_0001326 ekspresyonunu arttırarak M1 makrofaj polarizasyonunu inhibe ettiğini ve M2 makrofaj polarizasyonunu desteklediğini gözlemlemişlerdir [73]. Qiao ve arkadaşları, fareleri ovalbümin/LPS (OVA/LPS) ile endükleyerek oluşturdukları nötrofilik astım modelinde LUT bileşiğinin IL-36γ sekresyonu aracılı mitojenle aktifleşen protein kinaz (Mitogen-activated protein kinase; MAPK) yolağı üzerindeki rolünü incelemişlerdir. Araştırmacılar, LUT bileşiğinin farelerde oluşturulan nötrofilik astımı MAPK yolağını inhibe ederek hafiflettiğini bildirmişlerdir [74]. Shen ve arkadaşları OVA ile endüklenen fare astım modelinde, LUT bileşik tedavisinin $GABA_A$ reseptörlerinin (Gamma aminobutyric acid type A receptors; $GABA_AR$) inhibisyonunu sağlayarak goblet hücre hiperplazisini ve aşırı mukus üretimini azaltıcı bir etkiye sahip olduğunu gözlemlemişlerdir [75].

Multipl Skleroz

Multipl Skleroz (MS), merkezi sinir sisteminde enflamasyon, aksonal dejenerasyon ve demiyalinizasyon ile karakterize bir hastalıktır. Ataklarla belirti verir ve otoimmün bir hastalık özelliği taşır [76]. Yapılan araştırmalar sonucunda sekonder metabolitlerin; MS, Parkinson, Alzheimer hastalığı, Amyotrofik Lateral Skleroz (ALS) gibi nörodejeneratif hastalıklarda nöron koruyucu özelliklerinin

olduğu belirtilmiştir. Bu metabolitlerden biri de LUT bileşiğidir. Yapılan çalışmalarda bu bileşiğin, nöroprotektif özelliğinin olduğu gösterilmiştir [77]. Kempuraj ve arkadaşları 10 ve 100 μm konsantrasyonlarındaki LUT bileşiğinin, temel MS antijeni miyelin temel proteini (myelin basic protein; MBP) baskıladığını göstermişlerdir. Araştırmacılar, LUT bileşik tedavisinin MS gibi nörodejeneratif otoimmün hastalıklara karşı terapötik bir etki gösterebileceğini düşünmüşlerdir [78]. MS hastalarından izole edilmiş periferik kan mononükleer hücresi (peripheral blood mononuclear cell; PBMC) üzerinde Sternberg ve arkadaşlarının yapmış olduğu bir çalışmada LUT bileşiğinin, proenflamatuvar belirteçlerin (IL-1β, TNF-α ve MMP-9) seviyelerini ve MMP-9/Metalloproteinaz Doku İnhibitörü-1 (Tissue Inhibitor of Metalloproteinase-1; TIMP-1) oranını düşürdüğünü bildirmişlerdir. Böylelikle araştırmacılar, MS hastalarından elde edilen PBMC'ler üzerinde LUT bileşiğinin immünomodülatör özellik gösterdiği kanısına varmışlardır [79]. El-Deeb ve arkadaşları deneysel otoimmün ensefalomiyelit (DOE) sıçanlarında LUT bileşiğinin, MS tedavisinde anti-apoptotik, anti-enflamatuvar ve nörotrofik bir bileşik olarak kullanılıp kullanılmayacağını araştırmışlardır. Araştırmacılar LUT bileşiği ile tedavi edilen DOE farelerinde, total anti-oksidan kapasite (total antioxidant capacity; TAC) ve siklik AMP (cyclic AMP; cAMP) seviyelerinde artışın olduğunu; NF-κB, makrofaj enflamatuvar protein 1 alfa (macrophage inflammatory protein 1 alpha; MIP-1α) ve bölünmüş kaspaz-3 seviyelerinde ise azalmanın meydana geldiğini gözlemlemişlerdir. Böylelikle araştırmacılar LUT bileşiğinin, MS tedavisinde yeni bir umut kaynağı olabileceğini düşünmüşlerdir [80].

Otoimmün Ensefalit

Otoimmün ensefalit, beyin iltihabına yol açan otoimmün bir hastalık olup mekanizması tam olarak bilinmemektedir. Bu hastalıkta, otoimmün antikorların sinaptik proteinleri hedef alarak enflamasyona neden olduğu düşünülmektedir [81]. Contarini ve arkadaşlarının yapmış oldukları bir çalışmada kompozit PEALut [(palmitoylethanolamide, (PEA)+LUT] tedavisinin, MOG35-55/Freund'un tam adjuvan emülsiyonu uygulanarak bağışıklanan DOE farelerinde; IFN-γ, TNF-α, NLRP3, serum amiloid A1 gibi proenflamatuvar protein seviyelerini azalttığını bildirmişlerdir [82].

Ülseratif Kolit

Enflamatuvar bağırsak hastalıkları (İBH), intestinal mukozanın kronik enflamasyonu sonucu ortaya çıkar. Crohn hastalığı (CH) ve ülseratif kolit (ÜK) olmak üzere iki ayrı patolojide değerlendirilir [83]. ÜK, ülserler ve açık yaralar ile seyreden ve kronik bir hastalıktır. Kolon mukozasının yaygın enflamasyonu ile karakterizedir [84]. Li ve arkadaşları, dekstran sülfat sodyum (DSS) ile endüklenen ÜK sıçanlarında LUT bileşiğinin terapötik etkilerini incelediklerinde, LUT bileşik tedavisinin enflamatuvar cevabın inhibisyonunu sağladığını aynı zamanda kolon hasarını da hafiflettiğini bildirmişlerdir [85]. Li ve arkadaşlarının yapmış olduğu bir diğer çalışmada, DSS ile endüklenen ÜK sıçanlarında LUT bileşik tedavisinin metabolit fonksiyon, immün cevap ve oksidatif stres üzerindeki etkilerini incelemişlerdir. Araştırmacılar, LUT bileşik tedavisinin IL-33, C-reaktif protein (CRP), IL-13 ve TNF-α gibi belirteçlerin seviyelerini azalttığını gözlemişlerdir. Aynı zamanda bu tedavinin sıçanlarda, oksidatif stres belirteçlerini de azalttığını belirtmişlerdir. Böylelikle araştırmacılar LUT bileşik tedavisinin, ÜK sıçan modelinde oksidatif stresi ve immün cevabı iyileştirdiği; kolon hasarını da azalttığı kanısına varmışlardır [86]. GLM [2-(3,4-dihydroxydihydro)-5,7-dihydroxy-3-(hydroxymethy) chroman-4-one], çeşitli sinyal moleküllerinin regülasyonu ile anti-melanojenik özellik gösteren bir LUT türevidir. Kim ve arkadaşları GLM bileşiğinin, DSS kaynaklı fare kolit modelinde; Th1, Th2 ve Th17 hücrelerinin seviyelerini düşürerek *in vivo* koruyucu bir etki gösterdiğini bildirmişlerdir [87]. Xie ve arkadaşları LUT bileşik tedavisinin, DSS ile endüklenmiş ÜK farelerinde bağırsak bariyer fonksiyonunun iyileşmesine katkıda bulunduğunu bildirmişlerdir. Araştırmacılar, LUT bileşik tedavisinin NCR^-ILC3/NCR^+ILC3 dengesini onararak bu sürece katkıda bulunduğunu ifade etmişlerdir [88]. Li ve arkadaşları LUT bileşik tedavisinin, Nrf2 sinyal yolunun aktivasyonuyla DSS ile endüklenen fareleri kolitten koruyabileceğini bildirmişlerdir [89]. Farelerde DSS ile uyarılan kolit modellemesi üzerinde yapılan bir diğer çalışmada Xue ve arkadaşları, kolitli farelere uygulanan LUT bileşiğinin anti-enflamatuvar etkisini IKKα/β-NF-κB sinyal yolağını inhibe ederek gösterdiğini belirtmişlerdir [90]. Vukelić ve arkadaşları LUT bileşik tedavisinin, DSS ile endüklenmiş kolitli farelerde meydana gelen kolon doku hasarını iyileştirdiğini aynı zamanda otofajiyi, apoptozu ve enflamasyonu da azalttığını

bildirmişlerdir [91]. Tan ve arkadaşları ROT'a duyarlı bir sistem olan LUT@ d-α-tokoferil polietilen glikol süksinat (d-α-tocopheryl polyethylene glycol succinate; TPGS)-poli(β-tiyoester (poly(β-thioester); PBTE) nanopartikül (nanoparticle; NP) [LUT@TPGS-PBTE NPs]'lerin DSS ile endüklenen fareler üzerinde anti-ÜK etkinliğini araştırmışlardır. Araştırmacılar kolitli fareler üzerinde bu sistemin, Th17/Treg ve Th1/Th2 dengesini regüle ettiğini; IFN-γ, TNF-α ve IL-6 gibi proenflamatuvar sitokinlerin de seviyelerini inhibe ettiğini gözlemlemişlerdir. Böylelikle araştırmacılar bu sistemin, DSS kaynaklı kolite karşı koruyucu bir özellik sergilediğini bildirmişlerdir [92].

Otoimmün Diyabet

Halk arasında Tip 1 *Diabetes Mellitus* (T1DM) olarak isimlendirilen otoimmün diyabet hastalığı, insülin üreten pankreas beta hücrelerini etkileyen otoimmün bir hastalıktır. Bu hastalık, genetik olarak duyarlı kişilerde insülin yetmezliğine neden olur [93]. Zhang ve arkadaşlarının yapmış oldukları bir çalışmada LUT bileşiğinin, diyabetik kardiyopati modelli sıçanlarda nitrik oksit sentaz (NOS) yolunu aktifleştirerek kardiyoprotektif bir etki gösterdiğini bildirmişlerdir. Aynı zamanda araştırmacılar, LUT bileşik tedavisinin iskemi/reperfüzyon hasarını da düzelttiğini ifade etmişlerdir [94]. Wang ve arkadaşları LUT bileşik tedavisinin, Streptozotosin (STZ) ile endüklenmiş diyabetik sıçanlarda antioksidan özellikler sergilediğini ve diyabetin yol açtığı kalp fonksiyon bozukluğuna karşı da kardiyoprotektif bir etki gösterdiğini bildirmişlerdir [97]. Wang ve arkadaşları STZ ile endüklenmiş diyabetik sıçanlara uygulanan LUT bileşiği tedavisinin, protein kinaz B fosforilasyonunu ve HO-1 ekspresyon seviyelerini arttırarak sıçan böbrek dokularının yıkımını; kan üre nitrojen (Blood Urea Nitrogen; BUN) ve kreatinin seviyelerini azaltarak diyabetik nefropatiyi önleyebileceği kanısına varmışlardır [95]. Farklı bir çalışmada Lu ve arkadaşları, STZ ile endüklenen diyabetik sıçanlarda LUT bileşik tedavisinin VEGF, NF-κB ve IL-1β gibi proenflamatuvar belirteçlerin seviyelerini inhibe ettiğini, malondialdehit (Malondialdehyde; MDA), nitrotirozin gibi oksidatif stres belirteçlerinin de artışını önlediğini gözlemlemişlerdir. Böylelikle araştırmacılar LUT bileşik tedavisinin, retinal nörodejenerasyona karşı koruyucu bir etkisi olabileceği kanısına varmışlardır [96]. Li ve arkadaşlarının STZ ile endüklenmiş diyabetik kardiyomiyopati fare modeli üzerinde yaptıkları bir çalışmada LUT bileşik tedavisinin, AMP ile etkinleşen protein kinaz (AMP-activated protein kinase; AMPK) ve AKT/GSK-3 sinyal yolaklarını aktive ederek diyabetik kardiyomiyopatinin tedavisinde yeni bir umut kaynağı olabileceğinin kanısına varmışlardır [98]. Li ve arkadaşları, farelerde oluşturulan diyabetik kardiyomiyosit modelinde LUT bileşik tedavisinin, *in vivo* ve *in vitro* şartlarda apoptoz, fibrozis ve hipertrofiyi hafiflettiğini gözlemlemişlerdir. Aynı zamanda araştırmacılar, LUT bileşik tedavisinin diyabetik kardiyomiyosit modeli üzerinde oksidatif stres ve enflamasyonu inhibe edici bir özellik sergilediğini belirtmişlerdir [99].

Psoriasis

Psoriasis, halk arasında sedef hastalığı olarak isimlendirilen otoimmün bir deri hastalığıdır. Bu hastalığın tanısını alan bireylerde, eritematöz pullu lezyonlar ortaya çıkmaktadır. *Psoriasis vulgaris,* psoriasis hastalığının en yaygın türlerinden biridir. Derinin enflamasyonu sonucu ortaya çıkar ve etiyolojisi tam olarak netlik kazanmamış kronik bir hastalıktır [100]. Vijayalakshmi ve arkadaşları sıçanlarda oluşturulan psöriasis modelinde, *Givotia rottleriformis* kabuğunun anti-psoriasis aktivitesini incelemişlerdir. Yaptıkları sıçan modellemesi ve HPLC analizine göre rutin, LUT, kemferol ve kersetin gibi bileşiklerin sıçanlarda, nötrofillerin hareketini inhibe ederek keratinosit ve epidermal tabakalarının proliferasyonunu azalttığı sonucuna ulaşmışlardır [101]. Ly ve arkadaşları imikimod ile uyarılmış fare psöriazis modelinde LUT bileşik tedavisinin, eksozom sekrete edilmesini ve ısı şok protein 90'ın (HSP90) ekspresyon düzeyini baskılayarak psoriasis hastalığındaki semptomları hafiflettiğini gözlemlemişlerdir [102]. Wang ve arkadaşları LPS ile endüklenen keratinositlerde LUT bileşiğinin; IL-6, p65 ve NF-κB gibi proenflamatuvar belirteçlerin seviyelerini azalttığını gözlemlemişlerdir. Araştırmacılar aynı zamanda bu bileşiğin, keratinositlerin proliferasyonunu da inhibe ettiğini bildirmişlerdir [103]. Xu ve arkadaşları, imikimod ilacıyla fare psöriasis modeli oluşturmuşlar ve LUT-nanoyapılı bir lipit taşıyıcı (nanostructured lipid carrier; NLC) jel sisteminin anti-psoriatik etkisini incelemişlerdir. Araştırmacılar bu sistemin, psöriasisli farelerin kan örneklerinde ve deri lezyonlarında IL-23, IL-17, IL-6 ve TNF-α gibi proenflamatuvar sitokinlerin seviyelerini azalttığını gözlemlemişlerdir

[104]. Başka bir çalışmada ise imikimod ile oluşturulan fare psöriasis modelinde LUT bileşiği tedavisinin, farelerde meydana gelen cilt lezyonlarını iyileştirdiği; aynı zamanda IL-1β, IL-23, IL-17, IL-6 ve TNF-α gibi sitokinlerin seviyelerini de inhibe ettiği gösterilmiştir [105].

Otoimmün Tiroidit

Otoimmün tiroidit (autoimmune thyroiditis; AIT); tiroid dokusunun diffüz guatr, parankim dokuda fibrozis ve atrofi ile ilerlediği otoimmün bir hastalıktır. AIT hastalığı, geniş bir hastalık yelpazesine sahiptir. T hücresi aracılı organa özgü kronik enflamasyonun bir türü olan Hashimoto tiroiditi (HT), AIT'nin klasik formudur [106,107]. Xia ve arkadaşlarının deneysel AIT fare modeli üzerinde yaptıkları bir çalışmada LUT bileşik tedavisinin, farelerde tiroid folikül dejenerasyonunu hafiflettiği ve STAT3 fosforilasyonunu azalttığını bildirmişlerdir [108]. Gan ve arkadaşları, Çin tıbbında enflamasyonu inhibe edici ve baş ağrısı tedavisinde kullanılan *Prunella vulgaris* (PV) bitkisi ile yaptıkları bir çalışmada network farmakolojisi ve moleküler docking analizleri ile kemferol ve LUT bileşiklerinin PV'de ana aktif bileşenler olabileceğini ve HT tedavisinde yeni bir umut kaynağı olabileceğini bildirmişlerdir [109].

Üveit

Üveit; glokom, katarakt ve makula ödemi gibi önemli göz hastalıklara neden olan; uvea tabakasının enflamasyonu sonucu ortaya çıkan göz hastalığıdır [110,111]. Retinal otoimmün reaksiyonların, çeşitli uvea-retinal ve retinal bozuklukların patogenezinde önemli bir rol oynadığı düşünülmektedir. Üveit hastalığına sahip çeşitli hasta gruplarında, çoklu anti-retinal antikor (ARA)'ların gözlemlendiği bildirilmiştir. Ten Berge ve arkadaşlarının yapmış oldukları bir çalışmanın sonucuna göre, üveitli hastalarda sağlıklı kontrollere kıyasla serum ARA'ların daha yaygın olduğu bildirilmiştir [112]. Zhang ve arkadaşları üveit tedavisinde Si-Ni-San (SNS) ilacının potansiyel rolünü, moleküler docking ve network farmakolojisi analizleri kullanarak değerlendirdiklerinde; naringin, kemferol, LUT ve kersetin biyoaktif bileşenlerinin MAPK, JUN ve RELA genlerine karşı iyi bir bağlanma yeteneği sergilediğini gözlemlemişlerdir. Böylelikle araştırmacılar, SNS ilacının üveit tedavisinde çok yönlü sinerjistik bir etki gösterebileceğini ve mekanizmasının immün regülasyonla korele olabileceği kanısına varmışlardır [113]. Kanai ve arkadaşları sıçanlarda endotoksin kaynaklı üveit (EKÜ) modelinde LUT bileşiğinin terapötik etkisini değerlendirmişlerdir. Araştırmacılar LUT bileşiği tedavisinin EKÜ sıçanlarının aköz hümör örneklerinde; enflamatuvar hücre sayısını, PGE2, TNF-α ve NO düzeylerini baskıladığını bildirmişlerdir. Aynı zamanda bu araştırmacılar histolojik olarak LUT bileşiği tedavisinin, EKÜ sıçanlarının göz dokularında doza bağlı bir şekilde iyileşme sağladığını da gözlemlemişlerdir [114]. Kanai ve arkadaşlarının yapmış olduğu bir diğer çalışmada, sıçanlarda EKÜ modelinde LUT bileşiğinin anti-enflamatuvar etkisini Prednizolon (Pred) ilacı ile karşılaştırmışlardır. Araştırmacılar 10 mg/kg LUT bileşiğinin anti-enflamatuvar etkisinin, 1 mg/kg Pred ile karşılaştırılabilir düzeyde olduğu kanısına varmışlardır [115].

Luteolinin Güvenilirliği Ve Toksisitesi

Genellikle bitkilerde bulunan ve bir flavon türü olan LUT bileşiğinin toksisitesi ve koruyuculuğu net değildir. LUT bileşiği üzerinde yapılan bazı araştırmalarda, endokrin sistem üzerine olan etkilerinden dolayı LUT bileşiğinin toksik olabileceği bildirilmiştir. Nordeen ve arkadaşları yapmış oldukları bir çalışmada, LUT bileşiğinin güçlü ve çok işlevli hormon bozucu aktiviteye sahip olduğunu gözlemlemişlerdir. LUT, östrojene bağımlı dokularda hücre büyümesini yönlendirebilen östrojen agonisti aktivitesi gösterdiği; aynı zamanda glukokortikoid ve progesteron sinyalini antagonize edebileceği bildirilmiştir [116]. Yapılan bazı araştırmalarda ise LUT bileşik tedavisinin hayvanlar üzerinde güvenli olduğu belirtilmiştir. Xiong ve arkadaşlarının yapmış oldukları bir çalışmada 100 mg/kg dozajındaki LUT bileşiğinin farelerin böbrek ve karaciğer dokularında herhangi bir toksisiteye neden olmadığını gözlemlemişlerdir [117]. Zebra balığı larvalarında yapılan bir çalışmada da LUT bileşiğinin güvenilir olduğu, De Leo ve arkadaşları tarafından bildirilmiştir [118]. Son olarak LUT bileşiğinin, hayvanlarda oluşturulan sisplatin kaynaklı nefrotoksisite veya doksorubisin kaynaklı kardiyotoksisitede de korucuyu etkiler gösterdiği bildirilmiştir [119].

SONUÇ VE TARTIŞMA

Doğal ürünler; çeşitli kronik, enflamatuvar ve otoimmün hastalıkların tedavisinde kullanılan ve bilimsel çalışmalara konu olan umut verici bir kaynaktır. Flavonoidlerin; anti-oksidan, anti-enflamatuvar, anti-kanser ve anti-alerji gibi birçok fonksiyonel özelliklere veya etkilere sahip olduğu bilinmektedir. Bu bileşikler başlıca tıpta, ilaç ve kozmetik sanayisinde kullanılmaktadır. LUT, en yaygın flavonlardan biridir. Elma, nane, lahana, havuç, fesleğen gibi meyve ve sebzelerde tanımlanmış bir bileşiktir. Yapılan çalışmalarda LUT bileşiğinin; IL-6 ve TNF-α gibi pro-enflamatuvar sitokinlerin seviyelerini azalttığını; IL-10 gibi anti-enflamatuvar özellik gösteren sitokinin ise üretimini artırarak enflamasyonu regüle edebileceği tespit edilmiştir. Ayrıca bu bileşiğin, lenfositlerin ve makrofajların fonksiyonlarını etkileyerek immün yanıtlarını da optimize edebileceği bildirilmiştir. LUT bileşiği, pro-enflamatuvar özelliği ile bilinen NF-κB aktivasyonunu endükleyerek anti-oksidan özellik gösterebilir. Böylece, hücrelerin oksidatif stresten korunmasına yardımcı olabilir. Ayrıca LUT bileşiğinin; anti-kanser, anti-diyabetik, anti-psoriasis ve nöroprotektif gibi birçok özelliklere sahip olduğu yapılan çalışmalar sonucunda bildirilmiş, birden fazla molekül ve hedefle etkileşime girme kapasitesine de sahip olduğu gösterilmiştir.

Otoimmün hastalıklarda, LUT bileşiği tedavisi üzerinde birçok *in vivo* ve *in vitro* çalışma yapılmış ve literatüre bu çalışmalar sunulmuştur. Özellikle SLE ve RA otoimmün hastalıklarında LUT tedavisinin proenflamatuvar özellik gösteren önemli mediyatörlerin seviyelerini inhibe ederek anti-enflamatuvar aktivite sergilediği gözlemlenmiştir. Bu durum, SLE ve RA'nın yönetiminde LUT'un önemli bir rol oynayabileceğini göstermektedir. Ayrıca, alerjik astım hastalığında LUT bileşiğinin anti-alerjik aktivite ve immünomodülatör etkiler gösterdiği bildirilmiştir. Alerjik astım hastalığında LUT'un bu etkileri, hastalığa bağlı enflamatuvar süreçleri baskılayarak alerjik semptomların şiddetini azaltmasına katkı sağlayabilir. Bunun yanı sıra LUT'un, MS ve otoimmün ensefalit gibi nörodejeneratif hastalıklarda nöroprotektif etkiler gösterdiği ve bu hastalığa bağlı enflamatuvar süreçleri baskıladığı tespit edilmiştir. MS ve otoimmün ensefalit hastalıklarında LUT'un etkileri, beyin fonksiyonlarının korunmasına ve nörodejeneratif süreçlerin yavaşlatılmasına katkı sağlayabilir. Ek olarak LUT'un, ÜK hastalığına neden olan proenflamatuvar mediyatörlerin ve oksidatif stres belirteçlerinin seviyelerini azaltarak kolon hasarını ve bağırsak bariyer bütünlüğünü geri kazandırdığı gösterilmiştir. ÜK hastalığında LUT'un bu etkileri, bağırsak dokusunun korunmasına yardımcı olabileceğini ve hastalığın semptomları ile şiddetini hafifletme potansiyelini ortaya koymaktadır. Bununla birlikte, LUT'un diyabet üzerine etkisi olmadığı raporlanmasına rağmen, diyabet kaynaklı oluşan kardiyopati, nefropati ve retinal nörodejenerasyona karşı protektif özellikler gösterebildiği bildirilmiştir. LUT'un metabolik düzenleyici, antioksidan ve antienflamatuvar etkileri, birçok hastalığa ve bu hastalıklardan kaynaklanan patolojilere karşı koruyucu bir rol oynayabileceğini düşündürmektedir. Bunun yanı sıra LUT'un, AIT hastalığında tiroid epitelinin tahribatını ve tiroid bezlerinde lenfositik infiltrasyonu hafiflettiği; aynı zamanda psöriasis ve üveit hastalıklarında enflamatuvar özellik gösteren mediyatörlerin seviyelerini azaltarak hastalıkların semptomlarını azalttığı gösterilmiştir. Bu bulgular, AIT, psöriasis ve üveit gibi hastalıklara karşı LUT ve benzeri doğal bileşiklerin terapötik yaklaşımlarda değerlendirilme olasılığını gündeme getirmektedir.

LUT bileşiği genel olarak birçok hastalık ve patolojide koruyucu rolleri gösterebilse de bazı araştırmacılar toksisite açısından endişe verici bulgular rapor etmiştir. LUT'un etkin şekilde kullanım potansiyeli, dozaj, süre ve uygulama yöntemine bağlı olarak değişiklik gösterebilir. Bu nedenle, LUT'un tedavi amaçlı kullanımı dikkatli bir şekilde ele alınmalıdır. Bazı hastaların özellikle kronik hastalıklar veya metabolik bozukluklar nedeniyle farklı tepkiler gösterebileceği bilinmektedir. Bu durum, LUT'un potansiyel faydaları ile birlikte her hasta için bireysel olarak değerlendirilmesi gereken riskler ve olası yan etkilerin göz önünde bulundurulması gerektiğini vurgular.

Gelecekte yapılacak bilimsel çalışmalar ile LUT'un klinik kullanılabilirliği hakkında daha fazla bilgi edinmemize katkı sağlayacak ve yeni ilaçların geliştirilmesine sağlam bir temel oluşturacaktır. Bu çalışmalar ile LUT'un farklı otoimmün hastalıklar üzerindeki etkilerini daha kapsamlı bir şekilde inceleyerek, bu bileşiğin potansiyel tedavi edici özelliklerini daha iyi anlamamızı sağlayacaktır. LUT bileşiğinin güvenirliliği tam olarak netlik kazanamamıştır. Bazı yapılan çalışmalarda güvenilir olduğu; bazı çalışmalarda da güvenilir olmadığı tespit edilmiştir. Bu nedenle LUT bileşiğinin güvenilirliğini

daha iyi ispatlamak için tavşan, köpek veya farklı model organizmalar üzerinde deneysel toksikolojik, biyoyararlanım ve stabilite çalışmalarının yapılması gerekmektedir. Bu derlemede, LUT bileşiğinin otoimmün hastalıklardaki olası terapötik etkileri değerlendirilmiş ve elde edilen bilimsel veriler derlenmiştir. Bu veriler otoimmün hastalıklar başta olmak üzere immünsüpresif etkileri hedefleyen hastalıklara karşı yeni tedavi stratejilerinin geliştirilmesinde gözleri LUT bileşiğine çekmektedir.

YAZAR KATKILARI

Kavram: F.B.; Tasarım: A.E.I., M.A.K.; Denetim: F.B.; Kaynaklar: - ; Malzemeler: - ; Veri Toplama ve/veya İşleme: - ; Analiz ve/veya Yorumlama: - ; Literatür Taraması: A.E.I., M.A.K; Makalenin Yazılması: A.E.I., M.A.K.; Kritik İnceleme: F.B.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1219-1235, 2024
Doi: 10.33483/jfpau.1430569

DERLEME MAKALE / REVIEW ARTICLE

LAMIACEAE FAMİLYASININ TÜY MORFOLOJİSİ

TRICHOME MORPHOLOGY OF LAMIACEAE FAMILY

Ayla KAYA[1]*

[1]Anadolu Üniversitesi, Eczacılık Fakültesi, Farmasötik Botanik Anabilim Dalı, 26470, Eskişehir, Türkiye

ÖZ

Amaç: *Lamiaceae familyası hem dünya da hem de ülkemizde zengin bir tür çeşitliliğine sahiptir. Familya üyeleri taşıdıkları uçucu yağlardan dolayı tıbbi ve ekonomik açıdan son derece önemli olup, bitkilerin gövde, yaprak, kaliks gibi çeşitli organlarında farklı tüy tiplerine rastlanmaktadır. Bitkilerin taksonomik olarak ayrımında da kullanılan tüy yapılarının derlenmesi bu çalışmanın amacını oluşturmuştur.*

Sonuç ve Tartışma: *Lamiaceae familyasında yer alan bitkilerin tüy morfolojileri çok sayıda kaynaktan yararlanılarak incelenmiştir. Elde ettiğimiz veriler ışığında familyanın tüyleri; örtü tüyleri, salgı tüyleri ve karışık tüyler olmak üzere 3 ana gruba ayrılmaktadır. Bunlarda kendi aralarında basit, dallanmış, kısa ve uzun örtü tüyleri, kapitat ve peltat salgı tüyleri ile dallanmış ve yıldız tipi karışık tüyler olmak üzere alt gruplara ayrılabilirler. Çalışmamızın sonucunda Lamiaceae familyası bitkilerinin 36 farklı tüy tipi belirlenmiş ve tüy tipleri kaynaklardan elde edilen çizim, ışık mikroskobu ve taramalı elektron mikroskobu (SEM) görüntüleriyle desteklenmiştir. Familyadaki tüy morfolojilerinin özellikle bitkilerin taksonomisinde kullanılan önemli bir karakter olduğu görülmüştür.*

Anahtar Kelimeler: *Lamiaceae, morfoloji, tüy*

ABSTRACT

Objective: The *Lamiaceae family has a rich species diversity both in the world and in our country. Family members are extremely important medically and economically due to the essential oils they carry, and different trichome types are found in their morphology. This study aims to compile the trichome structures, which are also used in the taxonomic differentiation of plants.*

Result and Discussion: *The trichome morphologies of plants in the Lamiaceae family have been examined using many sources. According to the data we observed from this compilation, trichomes of the family are separated into three groups non-glandular, glandular and mixed trichomes. And these trichomes can be separated into subgroups as non-glandular trichomes; simple and branched, glandular trichomes; capitate and peltate and mixed trichomes; branched and star. Our research shows that Lamiaceae family plants have 36 different types of hair structures and are supported by scanning electron microscopy (SEM), light microscopy and drawing pictures obtained from sources. It has been observed that trichome morphologies in the family are an important character used especially in the taxonomy of plants.*

Keywords: *Lamiaceae, morphology, trichome*

* **Sorumlu Yazar / Corresponding Author:** Ayla Kaya
e-posta / e-mail: aykaya@anadolu.edu.tr, **Tel. / Phone:** +902223350580

Gönderilme / Submitted : 02.02.2024
Kabul / Accepted : 23.07.2024
Yayınlanma / Published : 10.09.2024

GİRİŞ

Türkiye'de yetişen 11466 civarında tür ve tür altı bitki taksonuyla ülkemiz, dünya üzerinde zengin bir floraya sahiptir. Yaklaşık 3600 kadarı endemik olan bu bitkiler arasında [1,2], Lamiaceae familyasının hem endemik hem de tıbbi ve aromatik bitkiler açısından önemi büyüktür. Yeryüzünün bütün bölgelerine yayılmış olan familya, özellikle Akdeniz bölgesi vejetasyonunun önemli bir kısmını oluşturan tek veya çok yıllık, otsu veya çalı şeklinde bitkilerden oluşur. Lamiaceae familyası içinde barındırdığı tıbbi bitkileriyle gıda ve endüstri sanayi, tıp, ecza, parfümeri ve kozmetoloji gibi çeşitli alanlarda kullanılışı nedeniyle ekonomik ve tıbbi öneme sahiptir. Ülkemizde kullanılışı olan, yerli ve kayıtlı 120 aromatik bitkinin % 40'nın da Lamiaceae familyası içinde yer aldığı belirtilmektedir [3].

Lamiaceae familyasının, Boissier'in ''Flora Orientalis'' adlı eserinde 66 cins yaklaşık 1100 kadar türü bulunmaktadır. Bu sayı tahminen Dünya'daki Lamiaceae türlerinin 1/3'ü kadardır. Değişik araştırıcılara göre Lamiaceae familyası yeryüzünde 230'dan fazla cins (245 cins) ve 7000'nin üzerinde (7886 tür) tür ile yayılış göstermektedir [4,5].

Türkiye florası'nda ise Lamiaceae familyası 48 cins ve 782 takson (603 tür 179 alttür ve varyete) ile temsil edilmektedir ki bunlardan 346 takson (271 tür, 75 alttür ve varyete) endemik olup, endemizm oranı yaklaşık % 44'dür [5].

Lamiaceae üyeleri başta gövde, yaprak ve kaliks olmak üzere genelde yoğun örtü ve salgı tüyüne sahiptirler. Salgı tüyleri de kapitat ve peltat olmak üzere iki tipten meydana gelmişitir [6]. Kapitat salgı tüyleri genelde sap ve baş hücresinden oluşur ve salgılama şekline göre 3'e ayrılırlar [6,7].

I. tipte salgı, kutikula parçalanmadan kutikuladaki gözeneklerden damlacıklar halinde dışarıya verilir.

II. tipte salgı, kutikula ile hücre çeperi arasındaki boşlukta birikir, kutikulanın parçalanmasıyla salgı dışarıya verilir.

III. tipte ise salgı, kutikula altındaki boşlukta salgılanır ve bu boşluk, baş hücre çeperinin aşağı doğru çökmesiyle genişler ve salgı tüyü kadeh şeklini alır. Salgılama sonunda baş kısım volkan kraterine benzer bir görünümdedir [7].

Peltat salgı tüyleri geniş ve kısa bir sap hücresi ile büyük ve yuvarlak bir baş hücresinden oluşur. Baş salgı hücreleri tek ya da iki daire üzerinde dizilim gösterirler. Bu tüylerde salgı, kutikulanın altındaki boşlukta birikir ve kutikulanın mekanik bir darbeyle parçalanmasıyla dışarıya verilir [6,7].

Kapitat salgı tüylerinde salgılama süreci bitki yapraklarının erken evrelerinde başlar ve biter. Bu nedenle kısa ömürlüdürler. Peltat tüyler ise yaprak gelişiminin geç evrelerinde salgılama aktivitesine başlar ve yapraklar olgunlaşıncaya kadar devam eder. Bu nedenle peltat tüyler uzun ömürlüdür ve bitki yapılarının korunmasında rol oynarlar.

Örtü ve salgı tüyleri bitkilerde önemli rol ve fonksiyonlara sahiptir. Salgı tüyleri yapılarında toksik terpenik bileşikleri içermelerinden dolayı kimyasal savunmada; yapışkan polisakkaritleri içermelerinden dolayı mekanik savunmada ve güçlü alelopatik maddeleri içermelerinden dolayı diğer bitkilerle rekabette rol aldıkları düşünülmektedir. Bazı kseromorfik bitkilerde ise, hem örtü tüyleri hem de salgı tüyleri bitki yüzeyinde oluşturdukları tabaka ile ışık yansımasını arttırarak yaprak yüzey sıcaklığını azaltmaya yardımcı olmaktadır [7].

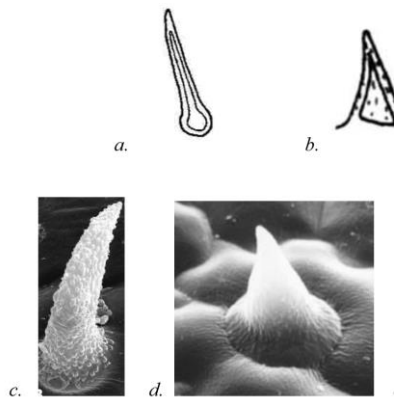
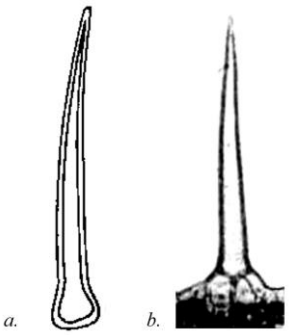
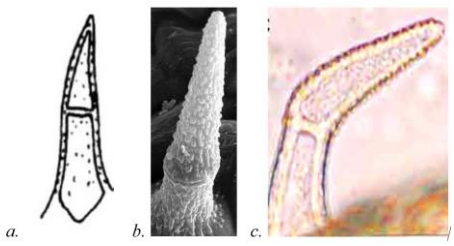
Bu derlemede Türkiye'de yetişen familya üyeleri öncelikli olmakla birlikte, ülkemizde yayılışı olmayan familya bitkileri de araştırılarak Lamiaceae familyası üyelerinin tüy morfolojik özelliklerinin ayrıntılı olarak belirlenmesi amaçlanmıştır.

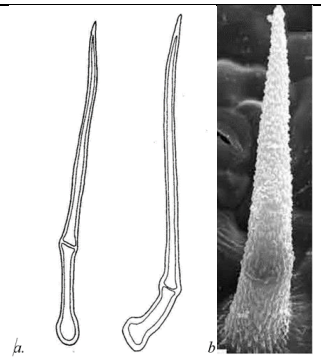
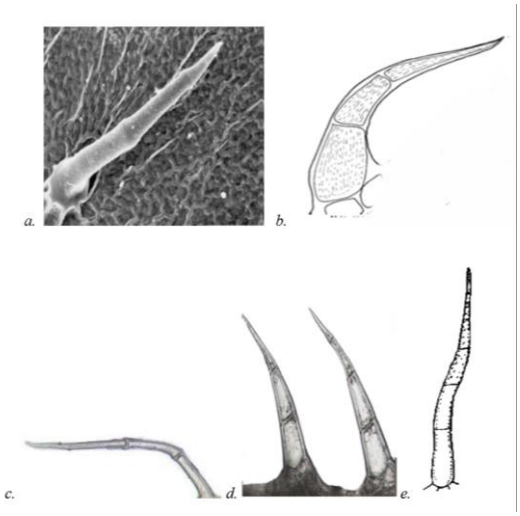
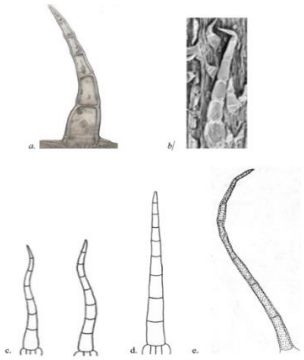
SONUÇ VE TARTIŞMA

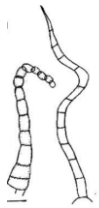
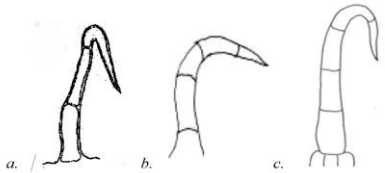
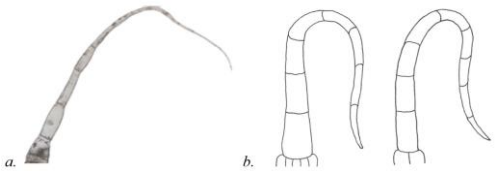
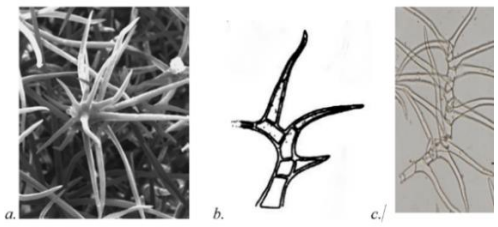
Örtü Tüyleri

Familya üyeleri basit ve dallanmış olmak üzere genelde iki tip örtü tüyüne sahiptir. Basit olanlar tek ve çok hücreli (2-14) olarak 2'ye ayrılırlar. Bunlarda kendi aralarında düz, eğik, kıvrık, kamçı ve kısa-uzun olmak üzere alt tiplere ayrılabilirler ve genellikle birkaç epidermal taban hücresinin üzerinde yer alırlar. Dallanmış örtü tüyleri ise yapısal olarak üstte ve tabanda dallanma (yıldız tip) göstermektedir [6,7]. Örtü tüyleri aşağıdaki Tablo 1'de listelenmiştir.

Tablo 1. Lamiaceae familyasındaki örtü tüyleri

1.Tek hücreli düz veya eğik kısa örtü tüyleri Tek, kısa terminal hücreli düz veya eğik örtü tüyleri bazen diş tüy şeklinde bazen ince konik şekildedir. Yüzeyde kutikula kabarcığı içerebilirler. *Acinos* L. *Ballota* L. *Cyclotrichium* (Boiss.) Manden. Et Scheng. *Dracocephalum* L. *Glechoma* L. *Hymenocrater* L. *Melissa* L. *Micromeria* Benth. *Nepeta* L. *Orthosiphon* Benth. *Otostegia* Benth. *Salvia* L. *Satureja* Tourn. ex Mill. *Syachys* L. *Sideritis* L. *Teucrium* L. *Thmbra* L. *Thymus* L *Ziziphora* L.	 a. *Ballota nigra* subsp. *nigra* [8] b. *Acinos* sp. [9] c. *Dracocephalum moldavicum* [10] d. *Salvia blepharophylla* [11]
2.Tek hücreli düz veya eğik uzun örtü tüyleri Tek, uzun terminal hücreli düz veya eğik örtü tüyler bazen sivri bazen uçları daha küt şekildedir. Yüzeyde kutikula kabarcığı içerebilirler. *Acinos* L. *Ajuga* L. *Ballota* L. *Elsholtzia* *Galeopsis* L. *Lamium* L. *Lycopus* L. *Micromeria* Benth. *Otostegia* Benth. *Salvia* L. *Sideritis* L. *Stachys* L. *Teucrium* L.	 a. *Otostegia fruticosa* var. *fruticosa* [12] b. *Stachys neurocalycina* [13]
3. İki hücreli düz veya eğik kısa örtü tüyleri İki hücreli örtü tüyleri genel olarak kısa taban ve kısa terminal hücreye sahiptirler, bazen yüzeyde kutikula kabarcığı içerebilirler. Uçları sivri veya daha küt olabilirler. *Acinos* L. *Cyclotrichium* (Boiss.) Manden. Et Scheng. *Dracocephalum* L. *Hymenocrater* Fisch. & C.A.Mey. *Hyptis* Jacq. *Micromeria* Benth. *Nepeta* L. *Orthosiphon* Benth. *Otostegia* Benth. *Salvia* L. *Satureja* Tourn. ex Mill. *Stachys* L. *Teucrium* L. *Thymus* L. *Thymbra* L. *Ziziphora* L.	 a. *Cyclotrichium* [14] b-c. *Dracocephalum moldavicum* [10]

4. İki hücreli düz veya eğik uzun örtü tüyleri İki hücreli düz veya eğik örtü tüyleri genel olarak kısa taban hücresi ve uzun terminal hücresine sahiptirler ve bazen yüzeyde kutikula kabarcığı içerebilirler. *Ballota* L. *Dracocephalum* L. *Galeopsis* L. *Lamium* L. *Nepeta* L. *Otostegia* Benth. *Sideritis* L. *Stachys* L. *Teucrium* L. *Thymbra* L. *Thymus* L.	 a. *Ballota nigra* subsp. *nigra* [8] b. *Dracocephalum moldavicum* [10]
5. Üç-beş hücreli düz veya eğik örtü tüyleri Üç, dört veya beş hücreli örtü tüyleri bazen eğik bazen düz şekilde uzundurlar. Yüzeyde kutikula kabarcığı içerebilirler. *Acinos* L. *Ajuga* L. *Ballota* L. *Calamintha* Mill. *Chelonopsis* Miq. *Clinopodium* L. *Coleus* Benth. *Glechoma* L. *Hymenocrater* Fisch. & C.A.Mey *Hyptis* Jacq *Lamium* L. *Leonitis* L. *Mellitis* L. *Mentha* L. *Micromeria* Benth. *Nepeta* L. *Ocimum* L. *Orthosiphon* Benth. *Otostegia* Benth. *Plectranthus* L. Hér. *Phlomis* L. *Salvia* L. *Satureja* Tourn. ex Mill. *Sideritis* L. *Teucrium* L. *Thymus* L. *Ziziphora* L.	 a. *Chelonopsis moschata* [15] b. *Mentha aquatica* [16] c. *Lamium truncatum* [17] d. *Salvia chrysophylla* [18] e. *Ajuga reptans* [19]
6. Altı-on hücreli düz veya eğik örtü tüyleri Altı, yedi, sekiz, dokuz ve on hücreli örtü tüyleri bazen düz bazen eğik şekilde uzundurlar. Yüzeyde kutikula kabarcığı içerebilirler. *Acinos* L. *Glechoma* L. *Hyptis* Jacq. *Nepeta* L. *Origanum* L. *Otostegia* Benth. *Plectranthus* L. *Salvia* L. *Teucrium* L. *Thymus* L. *Thymbra* L.	 *a. Salvia chrysophylla* [18] *b. Glechoma hederaceae* [20] *c. Salvia viridis* [21] *d. Salvia argentea* [7] *e. Thymus longicaulis* subsp. *Longicaulis* [22]

7. Onbir ve daha fazla hücreli düz veya eğik örtü tüyleri Onbir ve daha fazla hücreli örtü tüyleri bazen düz bazen eğik oldukça uzun tüylerdir. *Ajuga* L.	 *Ajuga chamaepitys* subsp. *chia* var. *chia* [19]
8. Tek hücreli kamçı örtü tüyleri Tek hücreli örtü tüyleri bazen kamçı şeklinde olabilir. *Ballota* L.	 *Ballota nigra* subsp. *nigra* [8]
9. İki-beş hücreli kıvrık veya kamçı örtü tüyleri İki, üç, dört veya beş hücreli kıvrık veya kamçı şeklindedir. *Salvia* L. *Sideritis* L.	 a. *Salvia aethiopis* [23] b. *Salvia hypargeia* [23] c. *Salvia argentea* [7]
10. Altı-on hücreli kıvrık veya kamçı örtü tüyleri Altı, yedi, sekiz, dokuz, on hücreli örtü tüyleri bazen kıvrık veya kamçı şeklindedir. *Salvia* L.	 a. *Salvia chrysophylla* [18] b. *Salvia argentea* [7]
11. Dallanmış (branched) örtü tüyleri ***a- Üst kısımda dallanmış örtü tüyleri*** Bazen örtü tüyleri tabandan çıkan bir hücrenin üst kısmından dalların çıkmasıyla dallanmış şekilde bulunabilirler. *Ballota* L. *Cyclotrichium* (Boiss.) Manden. Et Scheng. *Hyptis* Jacq. *Lavandula* L. *Perovskia* Kar. *Phlomis* L. *Rosmarinus* L. *Otostegia* Benth. *Origanum* L. *Stachys* L. *Teucrium* L.	 a. *Phlomis fruticosa* [24] b. *Cyclotrichium niveum* [14] c. *Stachys cydni* [25]
b- Tabanda dallanmış örtü tüyleri (yıldız tip) Örtü tüyleri bazen birçok tüyün tabandan çıkmasıyla yıldız şeklinde bulunabilirler. Bu şekilde tüy tipi içeren tek cinse rastlanmıştır: *Hyptis* Jacq.	 *Hyptis pectinat* [26]

Salgı Tüyleri

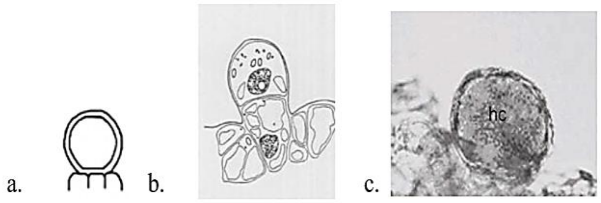
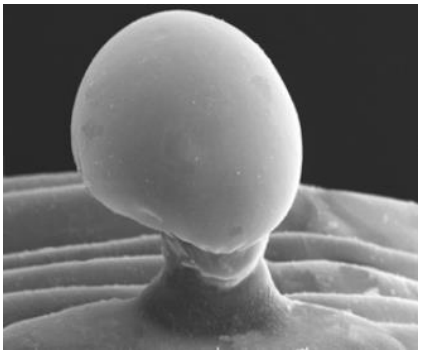
Familyayı karakterize eden uçucu yağların kaynağı salgı tüyleridir. Lamiaceae bitkilerinde, epidermis hücrelerinden gelişen salgı tüyleri, genellikle uçucu yağların sentezlendiği ve biriktirildiği yerler olarak bilinir. Olgun bir salgı tüyü bir sap ve bir baş kısmından meydana gelir. Hücre çeperleri ince ve selülozdur. Kutikula da ince ve düzdür. Salgı maddesi tüyün baş kısmındaki hücreler ile kutikula arasında toplanır. Bu bakımdan kutikula oldukça şişkindir. Bazen küçük bir darbe ile kutikula parçalanır ve salgı dışarı atılır [6,7,27].

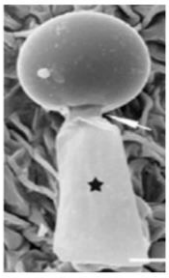
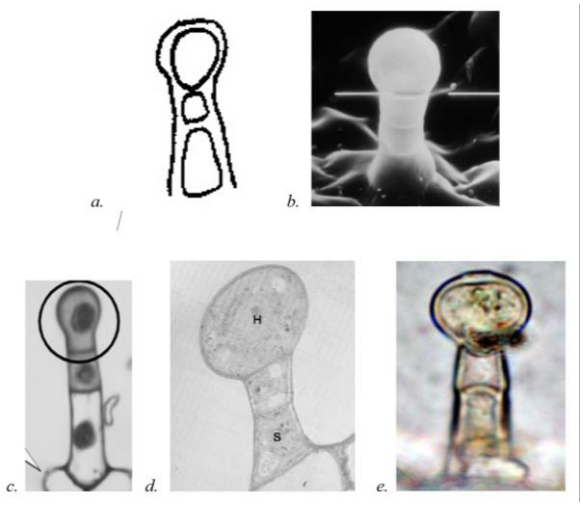
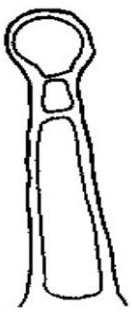
Lamiaceae familyasında salgı tüyleri kapitat ve peltat olarak iki kısma ayrılır.

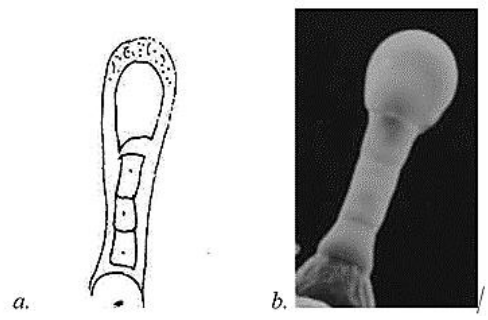
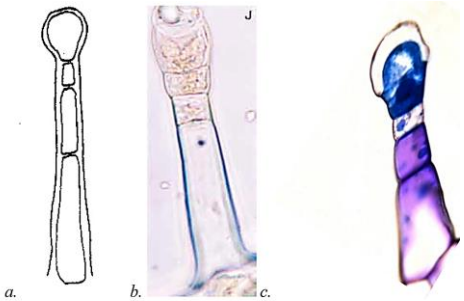
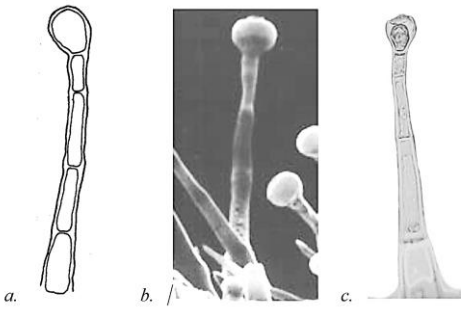
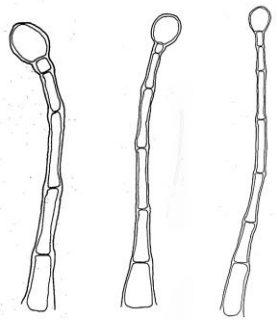
Kapitat Salgı Tüyleri

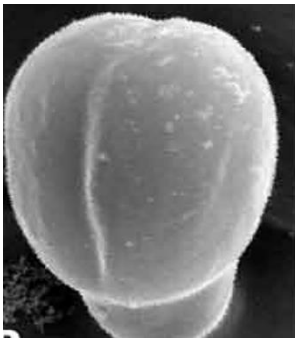
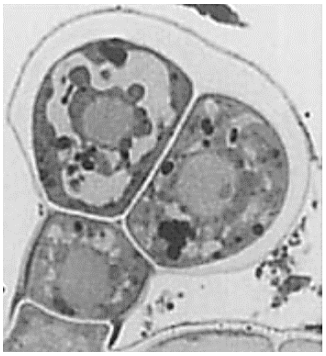
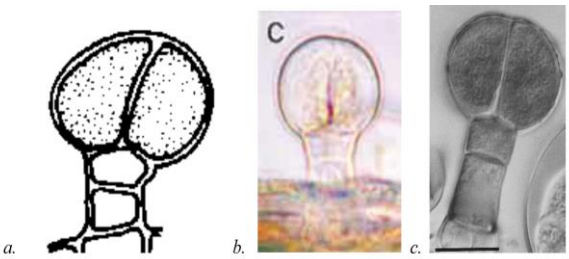
Kapitat salgı tüyleri sap ve baş kısmından oluşur. Ayrıca familyada nadir olarak da yıldız ve çatallı kapitat salgı tüyleri de bulunmaktadır. Kapitat salgı tüyleri aşağıdaki Tablo 2 de listelenmiştir;

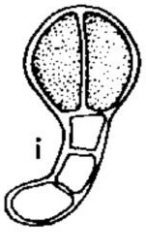
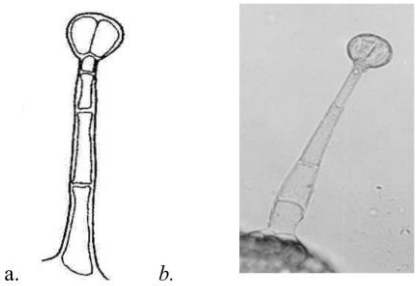
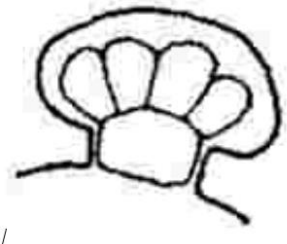
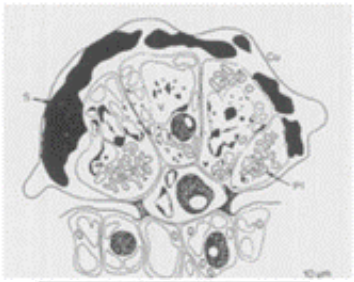
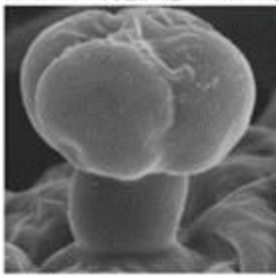
Tablo 2. Lamiaceae familyasındaki kapitat salgı tüyleri

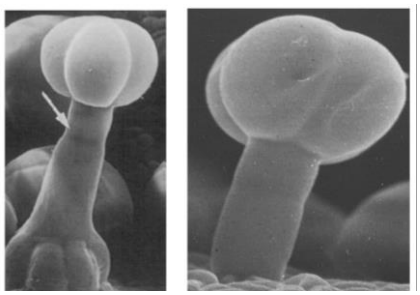
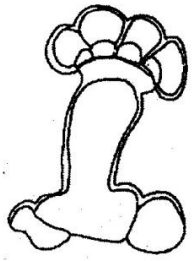
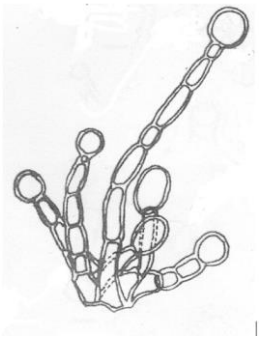
1. Başı bir hücreli, sapı olmayan salgı tüyleri Sapı olmayan bu tüylerde salgı oluşturan baş hücresi bazen oval bazen daha yuvarlak olabilirler. *Acinos* L. *Ajuga* L. *Glechoma* L. *Hyssopus* L. *Lallemantia* L. *Lavandula* L. *Leonitis* Benth. *Micromeria* Benth. *Salvia* L. *Scutellaria* L. *Stachys* L.	 a. *Salvia viridis* [7] b. *Leonitis leonurus* [27] c. *Salvia recognita* [27]
2. Başı bir hücreli, sapı bir hücreli olan kısa salgı tüyleri Başı ve sapı bir hücreden oluşan bu salgı tüylerinde baş hücresi yuvarlak, oval veya armut şekline sahip olabilirler. Bu tiplerde sap hücresi kısadır. *Acinos*L *Ajuga* L. *Ballota* L. *Cyclotrichium*(Boiss.)Manden.Et Scheng. *Elsholtzia* Willd. *Glechoma* L. *Hymenocrater* Fisch. & C.A.Mey. *Lallemantia* L. *Lavandula* L. *Leonitis*L. *Lycopus* L. *Mellitis* L. *Mentha* L. *Micromeria* Benth. *Nepeta* L. *Ocimum* L. *Origanum* L. *Orthosiphon* Benth. *Otostegia* Benth. *Plectranthus* L. *Prunella* L. *Rosmarinus* L. *Salvia* L. *Sideritis* L.	 *Thymus vulgaris* [29]  *Cyclotrichium sp.* [14]

Stachys L. *Tetradenia* Benth. *Teucrium* L. *Thymus* L. *Zataria* Boiss. *Zizphora* L.	
3. Başı bir hücreli, sapı bir hücreli olan uzun salgı tüyleri Başı ve sapı bir hücreli uzun olan bu salgı tüylerinde baş hücresi yuvarlak, oval veya armut şeklinde olabilirler. *Chelenopsis* Miq. *Lavandula* L. *Mellitis* L. *Nepeta* L. *Salvia* L.	 *Chelenopsis odontochila* var. *odontochila* [15]
4. Başı bir hücreli, sapı iki hücreli olan kısa salgı tüyleri Başı bir ve sapı iki hücreli kısa olan bu salgı tüylerinde baş hücresi yuvarlak, oval veya armut şeklindedir. *Acinos* L. *Ballota* L. *Chelenopsis* Miq. *Coleus* Benth. *Cyclotrichium*(Boiss.)Manden.Et Scheng. *Hyptis* Jacq. *Hymenocrater* Fisch. & C.A.Mey. *Lallemantia* L. *Lamium* L. *Leonitis* L. *Mentha* L. *Nepeta* L. *Ocimum* L. *Origanum* L. *Plectranthus* L. *Phlomis* L. *Rosmarinus* L. *Salvia* L. *Satureja* Tourn. ex Mill. *Sideritis* L. *Tetradenia* Benth. *Teucrium* L. *Ziziphora* L.	 a. *Acinos* [9] b. *Nepeta sibthorpii* [30] c. *Phlomis fruticosa* [24] d. *Rosmarinus officinalis* [31] e. *Teucrium polium* [32]
5. Başı bir, sapı iki hücreli olan uzun salgı tüyleri Salgı oluşturan bir baş hücresi ve iki sap hücresine sahip uzun salgı tüyleri yuvarlak, oval veya armut şeklinde bir baş hücresine sahip olabilirler. Bazen bir boyun hücresi ile baş ve sap hücresi bağlı olabilir. *Acinos* L. *Dracocephalum* L. *Mellitis* L. *Nepeta* L. *Plectranthus* L. *Salvia* L.	  *Acinos* [7] *Plectranthus ornatus* [33]

6. Başı bir, sapı üç hücreli olan kısa salgı tüyleri Başı bir ve sapı kısa üç hücreden oluşan salgı tüyleri yuvarlak, oval veya armut şeklinde bir baş hücresine sahip olabililer. *Plectranthus* L. *Rosmarinus* L. *Salvia* L.	 a. *Salvia bracteata* [34] b. *Plectranthus ornatus* [33]
7. Başı bir hücreli, sapı üç hücreli olan uzun salgı tüyleri Başı bir sapı üç hücreden oluşan uzun salgı tüyleri yuvarlak, oval veya armut şeklinde bir baş hücresine sahip olabilirler. *Acinos* L. *Ballota* L. *Dracocephalum* L. *Mellitis* L. *Origanum* L. *Otostegia* Benth. *Plectranthus* L. *Salvia* L. *Stachys* L. *Tetradenia* Benth. *Teucrium* L.	 a. *Acinos* [9] b. *Dracocephalum moldavicum* [10] c. *Plectranthus barbatus* [33]
8. Başı bir, sapı dört hücreli olan uzun salgı tüyleri Başı bir sapı dört hücreden oluşan uzun salgı tüylerinin baş kısmı yuvarlak, oval veya armut şeklinde olabilirler. *Salvia* L. *Tetradenia* Benth. *Teucrium* L.	 a. *Salvia glutinosa* [35] b. *Teucrium salviastrum* [37] c. *Salvia smyrnea* [37]
9. Başı bir hücreli, sapı beş, altı ve yedi hücreli olan salgı tüyleri Salgı oluşturan bir baş hücresi ve beş, altı, yedi hücreden olusan uzun sap taşıyan salgı tüylerinin baş kısmı yuvarlak, oval, armut şeklinde olabilirler. *Salvia* L. *Stachys* L.	 *Salvia glutinosa* [35]

10. Başı iki hücreli, sapı bir hücreli olan kısa salgı tüyleri Başı iki, sapı bir hücreli salgı tüylerinin baş kısmı bazen yuvarlak bazen daha elips şekilde olabilirler. *Ajuga* L. *Ballota* L. *Dracocephalum* L. *Elsholtzia* *Hymenocrater* Fisch. & C.A.Mey. *Lallemantia* L. *Lamium* L. *Lavandula* L. *Leonitis* L. *Nepeta* L. *Ocimum* L. *Origanum* L. *Otostegia* Benth. *Phlomis* L. *Perovskia* Kar. *Plectranthus* L. *Prassium* L. *Prunella* L. *Rosmarinus* L. *Salvia* L. *Sideritis* L. *Stachys* L. *Tetradenia* Benth. *Teucrium* L. *Thymus* L.	 *Dracocephalum moldavicum* [10]  *Leonitis leonurus* [27]
11. Başı iki hücreli, sapı iki hücreli olan kısa salgı tüyleri Başı iki, sapı iki hücreden oluşan kısa salgı tüylerinin baş kısmı bazen yuvarlak bazen daha elips şekilde olabilirler. *Ballota* L. *Cyclotrichium*(Boiss.)Manden.Et Scheng. *Dracocephalum* L. *Leonitis* L. *Origanum* L. *Otostegia* Benth. *Phlomis* L. *Salvia* L. *Sideritis* L. *Stachys* L.	 a. *Otostegia fruticosa* var. *fruticosa* [12] b. *Dracocephalum moldavicum* [10] c. *Leonitis leonurus* [27]
12. Başı iki hücreli, sapı iki hücreli olan uzun salgı tüyleri Başı iki, sapı bir hücreden oluşan salgı tüylerinin baş kısmı bazen yuvarlak bazen daha elips şekilde olabilirler. *Salvia* L. *Sideritis* L.	 *Salvia halophila* [38]

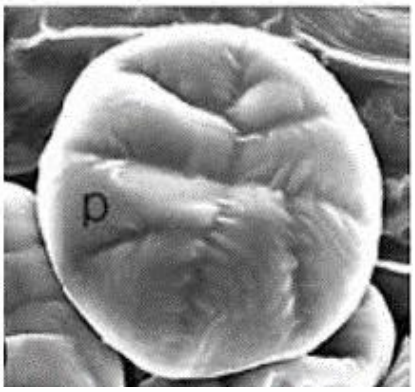
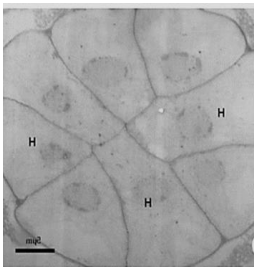
13. Başı iki hücreli, sapı üç-dört hücreli olan kısa salgı tüyleri Başı iki, sapı üç-dört hücreden oluşan kısa salgı tüylerinin baş hücreleri genellikle yuvarlak, ovalimsi şekillidir. Bu tip tüylere rastlanan cinsler: *Otostegia* Benth. *Salvia* L.	 *Otostegia fruticosa* var. *fruticosa* [12]
14. Başı iki hücreli, sapı üç-dört hücreli olan uzun salgı tüyleri Başı iki, sapı üç-dört hücreden oluşan uzun salgı tüylerinin baş hücreleri genellikle yuvarlak, ovalimsi şekillidir. *Ballota* L. *Salvia* L. *Sideritis* L. *Stachys* L.	 a. *Ballota nigra* subsp. *nigra* [8] b. *Stachys neurocalycina* [13]
15. Başı dört hücreli, sapı kısa olan salgı tüyleri Sapı çok kısa olan başı dört hücreli salgı tüyleri genelde epidermaya gömülü halde bulunurlar. Baş hücre genellikle yanlara doğru hafifçe genişlemiş ovaldir. *Ballota* L. *Chelenopsis* Miq. *Eltsholtzia* Willd. *Glechoma* L. *Lallemantia* L. *Lamium* L. *Lavandula* L. *Leonitis* L. *Mentha* L. *Nepeta* L. *Orthosiphon* Benth. *Otostegia* Benth. *Perovskia* Kar. *Prasium* L. *Rosmarinus* L. *Salvia* L. *Satureja* Tourn. ex Mill. *Sideritis* L. *Stachys* L. *Teucrium* L. *Zataria* Boiss.	 *Ballota nigra* subsp. *nigra* [8]   *Leonotis leonurus* [27]

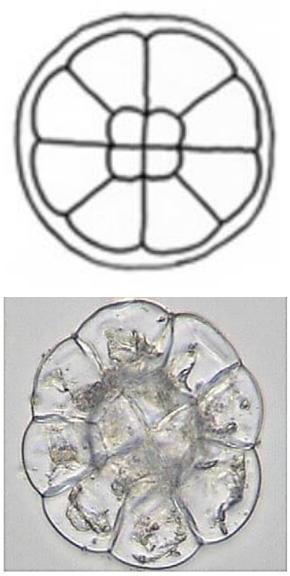
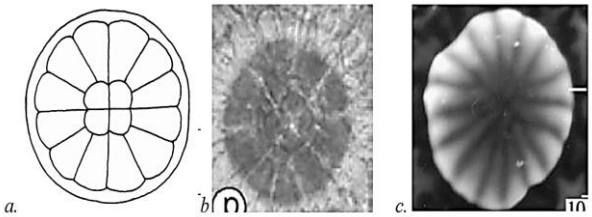
16. Başı dört, sapı iki hücreli uzun salgı tüyleri Başı dört, sapı iki hücreli ve uzun salgı tüylerinde baş hücre şekli genellikle yanlara doğru hafifçe genişlemiş ovaldir. *Leonitis* L. *Salvia* L. *Sideritis* L. *Stachys* L.	 *Leonotis leonurus* [27]
17. Başı sekiz, sapı iki hücreli uzun salgı tüyleri Başı sekiz, sapı bir hücreli ve uzun olan salgı tüyleri familyanın nadir görülen tüy tiplerinden birisi olup, bu şekilde bulunan cinsler: *Salvia* L. *Stachys* L.	 *Stachys* sp. [13]
18. Çatallı salgı tüyleri (Bifurkat) Bazen salgı tüyleri ortak bir sap hücresinin üzerinde çatal yaparak iki baş hücresi geliştirebilirler. Bu tip salgı tüyleri familya için son derece karakteristik olup araştırmamızda sadece *Lavandula*'da görülmüştür. *Lavandula* L.	  *Lavandula pedunculata* [39]
19. Yıldız salgı tüyleri Kapitat salgı tüyleri bazen tabandan dallanarak birçok tüyün çıkmasıyla yıldız şeklinde bulunabilirler. Bu tip salgı tüyleri familya için son derece karakteristik olup araştırmamızda sadece *Hyptis*'de görülmüştür. *Hyptis* Jacq.	 *Hyptis suaveolens* [26]

Peltat Salgı Tüyleri

Peltat salgı tüyleri bir taban hücresi, sapsız ya da sap geniş ve kısa hücre ile büyük ve yuvarlak bir baş hücresinden oluşur. Baş hücreleri ortak merkezli iki daire üzerinde dizilebildiği gibi tek bir daire üzerinde de dizilim gösterebilirler. Bu tip tüylerde baş hücreleri 4, 8, 12, 16 veya daha fazla hücreden meydana gelmiştir [6,7]. Peltat salgı tüyleri aşağıdaki Tablo 3'de listelenmiştir.

Tablo 3. Lamiaceae familyasındaki peltat salgı tüyleri

1. Başı dört hücreli, sapsız salgı tüyleri Sapı olmayan veya çok kısa olan başı dört hücreli salgı tüyleri genelde epidermaya gömülü halde bulunurlar. Baş hücre genellikle yanlara doğru hafifçe genişlemiş ovaldir. *Ballota* L. *Chelenopsis* Miq. *Eltsholtzia* Willd. *Glechoma* L. *Lallemantia* L. *Lamium* L. *Lavandula* L. *Leonitis* L. *Mentha* L. *Nepeta* L. *Ocimum* L. *Orthosiphon* Benth. *Perovskia* Kar. *Prasium* L. *Rosmarinus* L. *Salvia* L. *Satureja* Tourn. ex Mill. *Sideritis* L. *Stachys* L. *Teucrium* L. *Thymus* L. *Zataria* Boiss.	 *Salvia blepharophylla* [11]
2. Başı sekiz hücreli, sapsız salgı tüyleri Başı sekiz hücreli, sapı olmayan veya çok kısa salgı tüyleri 2 farklı tipten oluşur. Birinci tipte tek halka üzerinde sekiz hücre bulunurken, ikinci tipte merkezde iki, çevrede altı hücreden meydana gelmiştir. *Acinos* L. *Ajuga* L. *Ballota* L. *Chelenopsis* Miq. *Dracocephalum* L. *Lamium* L. *Lavandula* L. *Leonitis* L. *Lycopus* L. *Mentha* L. *Micromeria* Benth. *Nepeta* L. *Ocimum* L. *Origanum* L. *Otostegia* Benth. *Perovskia* Kar. *Rosmarinus* L. *Salvia* L. *Sideritis* L. *Stachys* L. *Teucrium* L. *Thymus* L.	 *Lycopus rubellus* [40]   *Salvia viridis* [5] *Rosmarinus officinalis* [31]

3. Başı oniki hücreli, sapsız salgı tüyleri Başı oniki hücreli salgı tüyleri 2 farklı tipten oluşur. Birinci tipte tek halkada oniki hücre bulunurken (bu tip çok nadirdir) ikinci tipte merkezde dört, çevrede sekiz hücre şeklinde bulunurlar. *Cyclotrichium* (Boiss.) Manden. Et Scheng. *Dracocephalum* L. *Leonitis* L. *Mentha* L. *Origanum* L. *Salvia* L. *Satureja* Tourn. ex Mill. *Stachys* L. *Thymus* L.	 *Salvia chrysophylla* [31]
4. Başı onaltı hücreli veya daha fazla olan, sapsız salgı tüyleri Başı onaltı hücreli salgı tüyleri 2 farklı tipten oluşur. Birinci tipte tek halkada onaltı hücre bulunurken, ikinci tipte merkezde dört, çevrede oniki hücre şeklinde bulunurlar. Bazense baş hücresi onaltı hücreden daha fazla da olabilir. *Leonitis* L. *Prostanthera* Labill. *Salvia* L. *Satureja* Tourn. ex Mill. *Stachys* L. *Thymus* L. *Ziziphora* L.	 a. *Salvia argentea* [7] b. *Satureja thymbra* [41] c. *Prostanthera ovalifolia* [42]

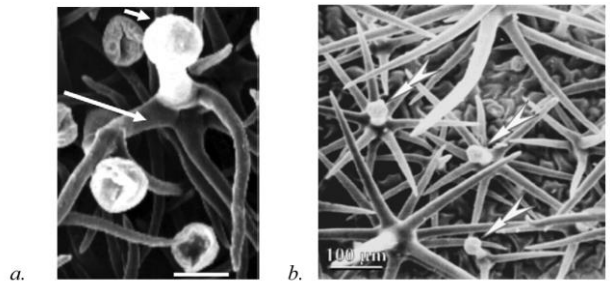
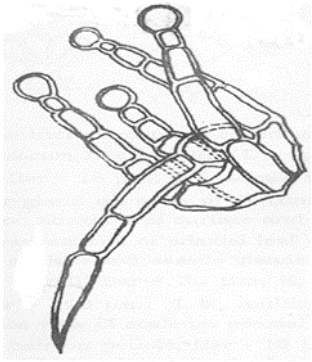
Karışık (örtü ve salgı tüyü birleşmiş-mixed) Tüyler

Karışık tüyler; örtü ve salgı tüylerini bir arada bulundururlar. Oluşum şekline göre dallanmış karışık tüyler ve yıldızsı karışık tüyler olmak üzere iki alt gruba ayrılırlar [7,24,26,39]. Karışık tüyler Tablo 4'de listelenmiştir.

Lamiaceae familyasında tüylerinin morfolojisi (özellikle salgı tüyleri), dağılımı ve yoğunluğu altfamilya ve bazı cins düzeyinde ayırt edici karakterler olarak kullanılmaktadır. Bu derleme makalede Lamiaceae familyasının tüy morfolojik özellikleri tanımlanmış ve tüylere ait şekiller ilgili literatürden sağlanmıştır. Öncelikli olarak Türkiye'de yayılışı olan 48 cinse ait tüylerin morfolojileri taranmış ve bu 48 cinsten *Coridothymus, Dorystoechas, Eremostachys, Galeobdolon, Hyssopus, Marrubium, Molucella, Pentapleura, Wiedemannia* cinsleri hariç diğer cinslerin tüyleri ile ülkemizde yayılışı olmayan *Chelonopsis, Coleus, Hyptis, Orthosiphon, Otostegia, Perovskia, Plectranthus, Pogostemon, Prostanthera, Tetradenia,* olmak üzere 10 cinse ait tüylerin morfolojileri araştırmamızda yer almıştır. Derlemenin sonuçlarına göre Şekil 1'de görüldüğü gibi toplamda 36 farklı tüy tipi belirlenmiştir. Temelde familya tüy tipleri; örtü tüyleri, salgı tüyleri ve karışık tüyler olarak 3 ana gruba ayrılırlar. Örtü tüyleri; kendi içinde basit (tek hücreli, çok hücreli) ve dallanmış (üstte dallanmış, tabanda dallanmış), salgı tüyleri; kapitat ve peltat ve karışık tüyler de; dallanmış ve yıldız tip olmak üzere alt gruplarda

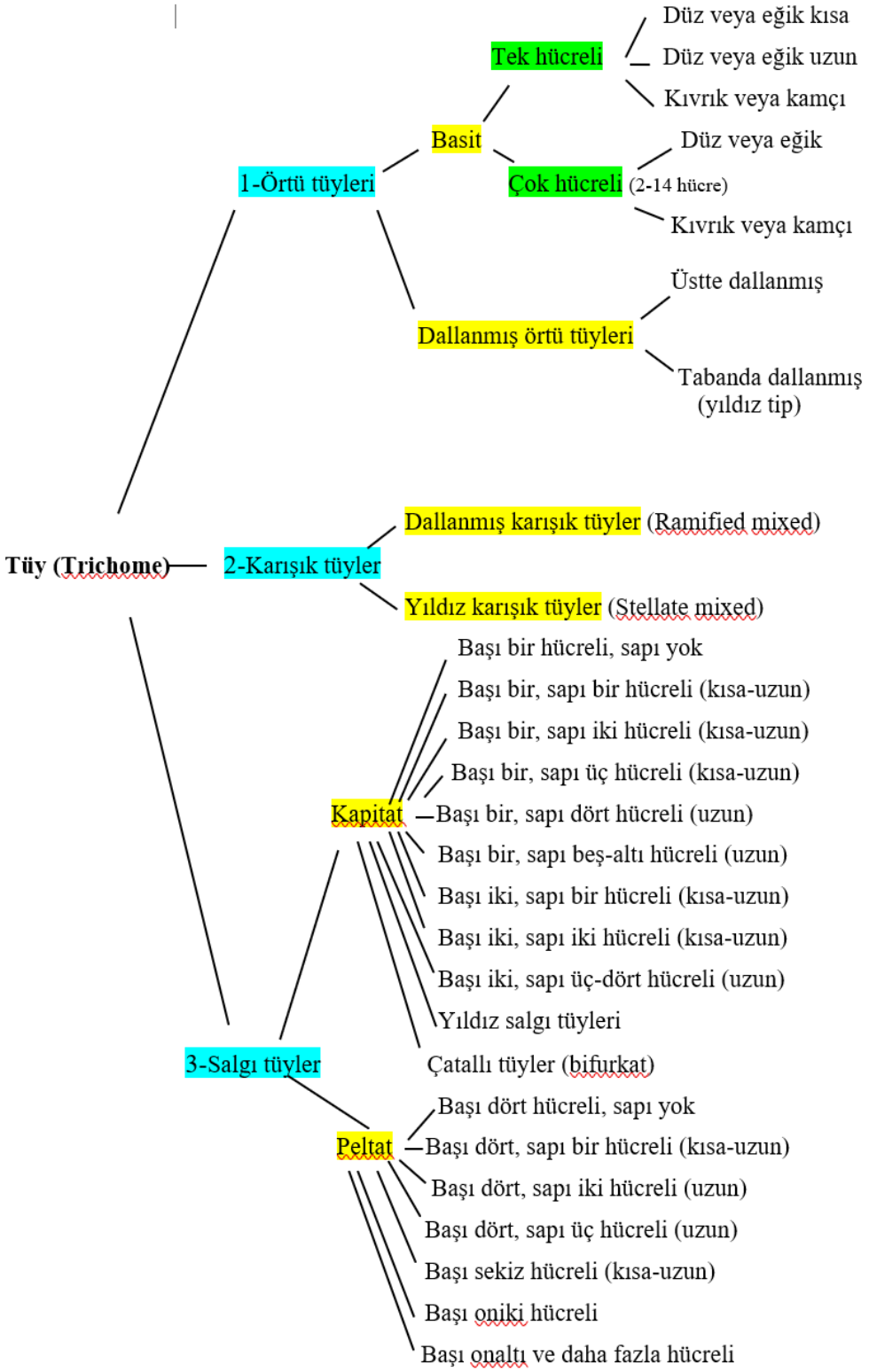
incelenebilirler. Örtü ve salgı tüylerinin alt gruplarıda Şekil 1'de görüldüğü gibi kendi içlerinde 3. seviyede alt gruplara ayrılabilirler.

Tablo 4. Lamiaceae familyasındaki karışık (mixed) tüyler

1. Dallanmış karışık tüyler (Ramified mixed) Dallanmış karışık tüyler; tabandan çıkan örtü tüyü üzerinde bir salgı tüyü taşır. *Lavandula* L. *Phlomis* L.	 a. *Lavandula pedunculata* [39] b. *Phlomis fruticosa* [24]
2. Yıldız karışık tüyler (Stellate mixed) Yıldız karışık tüylerde; tabandan çıkan çok sayıda örtü tüyü ve salgı tüyü bir aradadır. Bu tip karışık tüye sadece *Hyptis*'te rastlanmıştır. *Hyptis* Jacq.	 *Hyptis suaveolens* [26]

Çalışmamızın sonuçlarına göre tek ve iki hücreli düz veya eğik kısa örtü tüyleri, çok hücreli düz veya eğik örtü tüyleri, başı bir, sapı bir-iki hücreli kısa salgı tüyleri ile sapı olmayan veya çok kısa olan ve başı dört-sekiz hücreli salgı tüyleri son derece yaygın olup hemen hemen tüm cinslerde gözlenmiştir. Buna karşılık bir-on hücreli kamçı örtü tüyü, tabanda dallanmış örtü tüyü (yıldız tip), başı bir, sapı beş-yedi hücreli salgı tüyleri, başı iki, sapı bir-dört hücreli uzun salgı tüyleri, başı sekiz sapı bir uzun salgı tüyleri, çatallı salgı tüyleri (bifurkat), kapitat tipte yıldız salgı tüyleri, dallanmış karışık tüyler (Ramified mixed) ve yıldız karışık tüyler (Stellate mixed) ise familyada nadir olarak gözlenmiştir.

Derleme sonuçlarına göre; *Salvia, Teucrium, Stachys* cinslerinde tüy tiplerinin hemen hemen çoğu bulunurken, *Hyptis, Coleus, Elsholtzia* cinslerinde de daha az sayıda tüy tiplerine rastlanılmıştır. Familyanın taksonomisinde önemli bir karakter olan tüy morfolojisi bu çalışmayla ayrıntılı olarak ortaya konmuştur. Eczacılık açısından da son derece önemli bir familya olan Lamiaceae familyasının tüy morfolojileri elde ettiğimiz verilere göre bazı cinslerin ayrımında önemli bir karakter olarak kullanılabilirler.



Şekil 1. Lamiaceae familyasında tüylerin sınıflandırılması

TEŞEKKÜR

Eczacı Merve Konya'ya verdiği destek için teşekkür ederim.

YAZAR KATKILARI

Kavram: A.K.; Tasarım: A.K.; Denetim A.K.; Kaynaklar: A.K.; Malzemeler A.K.; Veri Toplama ve/veya İşleme A.K.; Analiz ve/veya Yorumlama: A.K.; Literatür Taraması: A.K.; Makalenin Yazılması: A.K.; Kritik İnceleme: A.K.; Diğer: A.K

ÇIKAR ÇATIŞMASI BEYANI

Yazar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan eder.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1236-1247, 2024
Doi: 10.33483/jfpau.1481724

DERLEME MAKALE / REVIEW ARTICLE

GIDA AZO BOYALARININ ERKEK REPRODÜKTİF TOKSİSİTESİ YÖNÜNDEN DEĞERLENDİRİLMESİ

EVALUATION OF FOOD AZO DYES IN TERMS OF MALE REPRODUCTIVE TOXICITY

Büşra KORKUT ÇELİKATEŞ[1]*, Merve BAYSAL[1]

[1]Anadolu Üniversitesi, Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, 26470, Eskişehir, Türkiye

ÖZ

Amaç: *Sentetik gıda boyaları, endüstriyel ve ticari ürünlerin görünümünü iyileştiren ve lezzetini artıran önemli bileşenlerden biridir. Ancak, çoğunlukla azo fonksiyonel gruplar ve aromatik halkalar içeren kimyasal yapılarından dolayı, bu maddelerin insan sağlığına potansiyel olarak zararlı olabileceği konusunda endişeler bulunmaktadır. Birçok çalışma, azo grubuna dahil olan boyaların aşırı duyarlılık reaksiyonlarına neden olabilen nitro türevleri olduğunu, bağırsak mikroflorası tarafından oluşan metabolitlerin mutajenez ve karsinojenez çalışmalarının odak noktasında yer aldığını belirtmiştir. Çeşitli çalışmalar, azo boyaların erkek reprodüktif sistemi üzerinde, özellikle de sperm parametrelerinde olumsuz etkileri olduğunu göstermiştir. Bu etkilere serum testosteron konsantrasyonundaki azalma da eşlik etmiştir.*
Sonuç ve Tartışma: *Gıda azo boyalarıyla ilgili çalışmalara olan ilginin artarak devam etmesi olası toksisite mekanizmalarını aydınlatma ihtiyacını beraberinde getirmiştir. Bu derlemede yaygın kullanılan gıda azo boyalarıyla ilgili genel bilgiler sunulmuş ve erkek reprodüktif sistem üzerindeki olumsuz etkilerine dair yapılan in vivo çalışmalar sonuçlarıyla değerlendirilmiştir.*
Anahtar Kelimeler: *Erkek reprodüktif sistem, gıda azo boyaları, toksisite*

ABSTRACT

Objective: *Synthetic food dyes are one of the important ingredients that enhance the appearance and taste of industrial and commercial products. However, concerns exist about their potential harm to human health due to their chemical structures, which often contain azo functional groups and aromatic rings. Many studies have stated that dyes belonging to the azo group are nitro derivatives that can cause hypersensitivity reactions, and that the metabolites produced by intestinal microflora are the focus of mutagenesis and carcinogenesis studies. Various studies have shown that azo dyes have negative effects on the male reproductive system, especially on sperm parameters. These effects were accompanied by a decrease in serum testosterone concentration.*
Result and Discussion: *The increasing interest in studies on food azo dyes has brought about the need to elucidate possible toxicity mechanisms. In this review, general information about commonly used food azo dyes is presented and the results of in vivo studies on their negative effects on the male reproductive system are evaluated.*
Keywords: *Food azo dyes, male reproductive system, toxicity*

* **Sorumlu Yazar / Corresponding Author:** Büşra Korkut Çelikateş
e-posta / e-mail: busrakorkutcelikates@anadolu.edu.tr, **Tel. / Phone:** +902223350580/3753

Gönderilme / Submitted : 10.05.2024
Kabul / Accepted : 03.08.2024
Yayınlanma / Published : 10.09.2024

GİRİŞ

Renk, gıdaların lezzet algısını oluşturan ve kabul edilebilirliğini etkileyen önemli bir faktördür [1,2]. Bu nedenle gıda endüstrisi, işlenmiş gıdaların görsel çekiciliği artırmak ve teknolojik farklılıkları maskelemek için doğal veya sentetik boyalar kullanmaktadır [3,4]. 19.yy ortalarından önce renklendirici özelliklere sahip maddeler doğal kaynaklardan elde edilmiştir. Ancak doğal kaynaklardan elde edilen boyaların çoğunun stabil olmaması ve gıdanın işlenmesi sırasında kolaylıkla bozulması sebebiyle 20. yy başlarında doğal kaynakların yerini neredeyse tamamen sentetik boyalar almıştır [5]. Sentetik boyalar, stabiliteleri, düşük maliyetleri, geniş renk yelpazeleri, kolay sentez edilebilirlikleri gibi avantajları nedeniyle de yaygın olarak kullanılmaktadır [6,7]. Gıda endüstrisinde kullanılan sentetik boyalar arasında pazarın %60'tan fazlasını oluşturan azo boyaların önemlerinin gelecekte daha da artacağı düşünülmektedir [8,9]. Azo boya endüstrisinin yıllık küresel üretimi 1 milyon tondan, değeri ise 15 milyar dolardan fazladır. 100.000'den fazla sentezlenen farklı azo boyanın 1000'den fazlası kullanımdadır [10].

Gıda Azo Boyalarının Kimyasal Yapıları

Azo boyalar düşük molekül ağırlıklı ve suda çözünürlüğü yüksek sentetik organik bileşiklerdir [11]. Azo grubu, aromatik halkaları birbirine bağlayan, genellikle amino ($-NH_2$) veya sülfonat ($-SO_3-$) grupları gibi diğer fonksiyonel gruplarla birlikte N atomları (N=N) arasında bir çift bağ ile karakterize edilir [12]. Mene, 1861 yılında birincil aromatik aminin diazotizasyonunu ve birleştirmeyi kullanarak ilk azo boyasını sentezlemiştir [13]. Sentezlenen azo bileşikleri güçlü canlı renkler sunar ve gıdaların yanı sıra tekstil boyaları, dövme pigmentleri ve ilaçlar gibi çok çeşitli sentetik kimyasallarda da bulunur [14]. En yaygın kullanılan gıda azo boyaları Tablo 1'de gösterilmiştir [15].

Tablo 1. Gıda endüstrisinde yaygın kullanılan azo boyaları

Azo Boyası	ADI (mg/kg/gün)	Renk	Azo Boyası	ADI (mg/kg/gün)	Renk
TRZ (E102)[1] (C.I. 19140)[2] (FD&C Yellow 5)[3] $C_{16}H_9N_4Na_3O_9S_2$	7.5		Ponzo 4R (E124)[1] (C.I. 16255)[2] $C_{22}H_{16}N_4O_{13}S_4$	0.7	
GS (E110)[1] (C.I. 15985)[2] (FD&C Yellow 6)[3] $C_{16}H_{10}N_2Na_2O_7S_2$	1		AK (E129)[1] (C.I. 16035)[2] (FD&C Red 40)[3] $C_{18}H_{14}N_2Na_2O_8S_2$	7	
KAR (E122)[1] (C.I.14720)[2] (FD&C Red 10)[3] $C_{20}H_{12}N_2Na_2O_7S_2$	4		PM (E133)[1] (C.I. 42090)[2], (FD&C Blue 1)[3] $C_{37}H_{34}N_2Na_2O_9S_3$	6	
AMA (E123)[1] (C.I.16185)[2] (FD&C Red 2)[3] $C_{20}H_{11}N_2Na_3O_{10}S_3$	0.15		ÇK (E155)[1] (C.I. 20285)[2] $C_{27}H_{18}N_4Na_2O_9S_2$	1.5	

* TRZ: Tartrazin, GS: Günbatımı sarısı; KAR: Karmosin; AMA: Amarant; AK: Allura kırmızısı; PM: Parlak mavi; ÇK: Çikolata kahverengi; ADI: Günlük kabul edilebilir alım miktarı; [1] Avrupa numaralandırma sistemi; [2] Uluslararası renk indeksi; [3]Amerika Birleşik Devletleri Gıda ve İlaç İdaresi onaylı terminoloji

Gıda Azo Boyaları ile İlgili Yasal Düzenlemeler

Sentetik gıda boyalarının insan sağlığı üzerindeki olumsuz etkileri konusunda giderek artan bir endişe vardır. Modern yaşamdaki büyük önemlerine rağmen, azo bileşiklerinin genel olarak karsinojenitesi, genotoksisitesi ve metabolizmasına ilişkin bilgi eksikliği devam etmektedir [16]. Çoğu durumda azo bileşiklerinin metabolitleri, boyaların kendisinden daha fazla toksikolojik öneme sahiptir;

ancak bu metabolitlerin toksisitesi düzenleyici kurumlar tarafından sistematik olarak ele alınmamıştır [17].

FAO (Food and Agriculture Organization/Gıda Tarım Örgütü) ve WHO (World Health Organisation/Dünya Sağlık Örgütü) bünyesinde 1956 yılında gıda katkı maddeleri konusunda ortak bir otorite olarak JECFA (Joint FAO/WHO Expert Committee on Food Additives/ FAO/WHO Gıda Katkı Maddeleri Ortak Uzman Komitesi) kurulmuştur. Bu komite gıda boyalarının küresel düzeyde güvenlik değerlendirmesi standartlarını belirlemiştir. Katkı maddeleri üzerinde yürütülen çalışmaların ele alındığı periyodik toplantılarda ADI (Acceptable Daily Intakes/Günlük Kabul Edilebilir Alım Miktarı) değerleri belirlenmektedir. ADI, bir kişinin tüm yaşamı boyunca kayda değer bir sağlık riski olmaksızın günlük olarak tüketebileceği miktarı ifade etmektedir. Birimi günde miligram/kilogram (mg/kg) vücut ağırlığıdır [18,19]. Tüketicilere güvenli gıda ürünleri sunmak amacıyla da FDA (Food and Drug Administration/Amerika Birleşik Devletleri Gıda ve İlaç İdaresi), EFSA (The European Food Safety Authority/Avrupa Gıda Güvenliği Otoritesi) ve JECFA kullanım izinlerini düzenlemektedir [19]. Riskin tanımlanmasını, kontrolünü ve azaltılmasını kolaylaştırmak amacıyla "E numaraları" adı verilen bir numaralandırma sistemi kurulmuştur. Örneğin gıda azo boyalarından tartrazin (TRZ) E102 koduyla, günbatımı sarısı (GS) E110 koduyla bilinmektedir. E kodu altında sınıflandırılan gıda renklendiricileri izin verilen miktarlarına göre kullanılmaktadır [18].

Gıda Azo Boyalarının Toksik Etkileri

Sentetik renklendiricilerin ADI değerlerinde alımı güvenli bulunsa da yüksek konsantrasyonlarda tüketilmesi, özellikle çocuklarda düşük vücut ağırlıklarından dolayı birçok rahatsızlığa neden olabilmektedir [20,21]. Gıda üreticilerinin ortalama %48'i izin verilen maksimum sınır olan 100 mg/kg'a uyarken, geri kalan %52'si belirlenen sınırın fazlasını kullanmaktadır. Bireysel yanıt yalnızca doz, yaş, cinsiyet, beslenme durumu ve genetik faktörlerle değil aynı zamanda düşük dozlara uzun süreli maruz kalmayla da ilişkilendirilmektedir [22].

Memelilerde, azo boyaların redüksiyonu başlıca alt gastrointestinal sistemin anaerobik ortamında bakteriyel azoredüktaz enzimleri tarafından kataliz edilen metabolizmaya bağlıdır [23,24]. Bu redüksiyonla, azo boyasına göre daha kolay absorbe edilen ve dışkıyla atılabilen sülfanilik asit ve aminopirazolon metabolitleri oluşur [25,26]. Değişmeden atılan azo boyalarının idrarla atılımı çok düşüktür veya ihmal edilebilir düzeydedir [15]. Azo boyaların metabolitleri, olumsuz hematolojik/biyokimyasal etkilerin yanı sıra reaktif oksijen türlerinin (ROT) neden olduğu oksidatif stres aracılığıyla karaciğer hasarına yol açabilir [24]. Sülfanilik asit, hücre bölünmesini etkileyebilir ve rejeneratif hiperplaziye neden olabilir, bu da potansiyel olarak karsinojeneze katkıda bulunabilir [27]. Ancak toksisite doza bağlıdır ve genel olarak "toksik olmayan" olarak kabul edilen bazı boyalar yüksek dozlarda toksik hale gelebilir [28]. ABD ve bazı Avrupa ülkelerinde bazı azo boyaları toksik, mutajenik ve karsinojenik etkileri nedeniyle gıda katkı maddesi olarak yasaklanmıştır [29]. Gıda azo boyaları burun akıntısından anafilaktik şoka kadar değişen alerjik reaksiyonlardan sorumlu tutulmuştur [30]. Ayrıca nörotoksisiteyi [31,32], hepatotoksisiteyi [22,33], nefrotoksisiteyi [34], genotoksisiteyi [35,36] ve reprotoksisiteyi indüklediğine dair çalışmalar mevcuttur [30,37].

Özellikle çocuklarda kontakt dermatit, ürtiker, gastrointestinal intolerans, bronkospazm, eozinofili, anjiyoödem ve hiperaktivite gibi etkilere sebep olabileceği ifade edilmiştir [38,39]. Yapılan *in vivo* çalışmalarda azo gıda boyalarının nefrotoksisiteye, hepatotoksisiteye, anemiye, lökopeniye neden olduğu rapor edilmiştir. Ayrıca bu boyaların proteinlerle etkileşerek protein konfigürasyonunun bozulmasına yol açtığı belirtilmiştir. Literatür incelendiğinde sentetik gıda boyalarının neredeyse tamamının toksik ve karsinojen olan kömür katranından elde edildiği görülmüştür [35,40,41].

Erkek infertilitesi tüm dünyada devam eden ciddi sağlık sorunlarından biridir. Erkek reprodüktif bozukluklarının görülme sıklığı gün geçtikçe artmaktadır. Genetik faktörler, yaşam tarzı, mesleki etkenler, ilaçlar gibi birçok faktör infertiliteyi etkilemektedir. Özellikle hazır gıda, abur cubur ve meşrubat tüketimi modern yaşam tarzının bir parçası haline gelmiştir [42]. Ayrıca çevresel faktörlerin de önemli bir rol oynadığı düşünülmektedir [43-45].

Gıda Azo Boyalarının Erkek Reprodüktif Sistem Üzerine Toksisitelerinin Değerlendirilmesi

Hayvanların vücut ağırlığının takibiyle elde edilen genel sağlık durumu bilgileri reprodüktif

sağlığın yorumlanması için de önemli olabilir [37]. Farklı dozlarda TRZ uygulanan hayvanlarda görülen anlamlı vücut ağırlığı artışları, boyanın diyabeti indükleyebileceği ile ilişkilendirilmiştir [37,46,47]. Ancak TRZ uygulamasının vücut ağırlığındaki anlamlı azalışlara neden olduğu bildirilen çalışmalar da mevcuttur. Bu durum TRZ toksisitenin bir göstergesi olarak kabul edilmiştir [30,42,48]. Benzer şekilde çikolata kahverengi (ÇK) uygulanan sıçanlarda vücut ağırlığında doza bağlı (200 mg/kg ve 400 mg/kg) bir azalış gözlenmiştir [49].

Erkek reprodüktif sistemde testis ve epididimis ağırlık kaybı, sperm sayısında ve motilitesinde azalma ksenobiyotiklerin spermatogenez üzerindeki olumsuz etkilerini tespit etmek için önemli kabul edilen göstergelerdir [37]. Testis ve epididimis ağırlığı spermatojenik hücrelerin ağırlığıyla ilişkili olduğundan spermatogenezdeki hasar testis ağırlığında da azalmaya sebep olabilir [46]. Daha önceki çalışmalarda testis ağırlığı ile sperm üretimi arasındaki pozitif korelasyondan bahsedilmiştir [50,51]. TRZ ve GS ile yürütülen çalışmalarda deney gruplarının testis ve epididimis ağırlıklarında kontrol grubuna göre anlamlı azalmalar gözlenmiştir [37,42,46,48,50]. Bununla birlikte, TRZ'nin testis ve epididimis ağırlıklarında anlamlı bir değişikliğe neden olmadığı da bildirilmiştir [30].

Sperm analizi erkeklerde reprodüktif sağlığın değerlendirilmesinde birincil kriterdir [42]. Sperm farklılaşması sırasında maruz kalınan toksik etkinin neden olduğu morfolojik anomaliler, üreme hücreleri üzerindeki kimyasal toksisitenin bir başka göstergesidir [46]. Sperm sayısının azalması ve anormal sperm yüzdesinin artması, spermatogenezdeki aksama ve spermatozoa apoptozu ile ilişkilendirilmiştir [48,51,52]. Ayrıca spermdeki morfolojik anomalilerin sperme şeklini veren yapısal proteinlerdeki hasarla bağlantılı olabileceği ifade edilmiştir [46]. Boussada ve arkadaşları sperm morfolojik değerlendirmesinde hasarı daha çok sperm kuyruğunda gözlemlemelerini TRZ'nin teorikte olgunlaşma veya spermatogenez süreci sırasında flagellum gelişiminde bozulmaya neden olabilme ihtimaline dayandırmıştır [30].

Testosteron (T) testis gelişimini, sperm olgunlaşmasını ve sperm apoptozunu kontrol ederek sağlıklı üreme aşamalarını sürdüren önemli bir faktördür; bu androjenin düşük konsantrasyonları genellikle Leydig hücrelerinin T üretimini düzenleyen hipotalamus-hipofiz-gonad ekseninin bozulmasıyla ilişkilidir [53]. Hipoandrojenik ortamın sebep olduğu epididimal fonksiyon bozuklukları spermatogenez ve steroidogenez başarısızlığıyla bağlantılıdır [42]. Testiste düşük T düzeyi, esas olarak aşırı sperm apoptozuna neden olur ve spermin hayatta kalmasını azaltır. Hipofiz tarafından salgılanan gonadotropin salgılatıcı hormon (GnRH), folikül uyarıcı hormon (FSH) ve luteinleştirici hormon (LH) ise sırasıyla spermatogenez ve steroidogenezin düzenlenmesinden sorumludur [54]. Hem androjenler hem de FSH, destekleyici somatik hücreler olan Sertoli hücrelerindeki reseptörler üzerinde etki göstererek optimal sperm üretimi için gereken fonksiyonları uyarır [53]. Deney hayvanlarına TRZ [30,42,48,55,56], GS [50,57], karmosin (KAR) [58], ÇK [49,59] ve parlak mavi (PM) [60] uygulamasıyla deney gruplarında T seviyelerinde anlamlı düşüşler görülmüştür. Bu düşüşler azo boya maruziyetinden etkilenen adenohipofizde GnRH seviyelerinde ve buna bağlı LH seviyelerindeki azalmanın, anormal sperm yüzdesindeki artışın ve yüksek ROT üretiminin neden olduğu oksidatif hasara bağlı Leydig hücre dejenerasyonunun bir sonucu olarak yorumlanmıştır [48,56].

Androjenlerin ana öncüsü olarak bilinen kolesterol seviyesindeki değişiklikler, T üretimini önemli ölçüde etkileyebilir. Yapılan bir çalışmada testiküler kolesterolde görülen azalma testisteki düşük T üretimiyle ilişkilendirilmiştir [30].

Herhangi bir antropojenik ajanın toksisitesi belirlenirken dokulardaki histolojik değişikliklerin de incelenmesi oldukça önemlidir [48,61]. Sıçanlara TRZ uygulamasıyla testis dokularında görülen histolojik değişikliklerin, Leydig ve Sertoli hücre fonksiyonlarındaki değişikliği ve spermatogenez sürecindeki aksamaları düşündürdüğü ifade edilmiştir [56].

Gıda azo boyalarının bağırsaktaki metabolizmasının ROT üretimini indüklediği gösterilmiştir [24,62,63]. ROT oluşumunun bir sonucu olarak oksidatif stres meydana gelmektedir [64]. Oksidatif stres, ROT üretimi ile antioksidan enzimlerin (süperoksit dismutaz (SOD), katalaz (KAT) ve glutatyon peroksidaz (GPX), glutatyon redüktaz (GR)) aktivitesi arasındaki dengesizlikten kaynaklanır. ROT miktarları aşırı olduğunda hücre hasarı meydana gelir. Bu artışın, sperm membran hasarı ile doğrudan ilişkili olabileceği öne sürülmüştür [65,66]. Testis hücrelerindeki lipid makromoleküllerin hasarı, dokuda lipid peroksidasyonun son ürünü olan malondialdehit (MDA) düzeyinin artmasıyla da ortaya konmaktadır [49]. Literatürdeki çalışmalarda sıçanların reprodüktif sisteminde antioksidan enzimlerin

varlığı rapor edilmiştir. Bu enzimlerin aynı zamanda spermatogeneze de katılarak spermatozoayı koruduklarını öne sürülmektedir [67,68]. Farklı gıda azo boyaları ile yapılan çalışmalarda testis dokusunda antioksidan enzimlerin azaldığı ve lipid peroksidasyon sürecinin arttığı raporlar sunulmuştur [30,42,48,49,58,64]. Bu çalışmaların bazılarında görülen yüksek MDA konsantrasyonlarının nedeni olarak da gıda azo boyalarının metabolizma ürünleri olan aromatik aminlerin hücre zarlarındaki çoklu doymamış yağ asitleri ile etkileşime giren *N*-hidroksi türevlerine oksitlenebilmesi gösterilmiştir [30,42,58]. Fare ve sıçanlarda gıda azo boyalarının erkek reprodüktif sistem üzerindeki olası etkilerini değerlendiren araştırmaların sonuçları Tablo 2'de özetlenmiştir.

Tablo 2. Deney hayvanlarında gıda azo boyalarının erkek reprodüktif sistem üzerindeki etkilerini araştıran çalışmalar

Azo Boyası	Tür	Uygulanan Doz (Oral)	Maruziyet Süresi	Sonuçlar	Referans
TRZ	4 haftalık Swiss albino fare	0,2 g/kg/gün 0,4 g/kg/gün	30 gün	Vücut ağırlığı ↑ Testis ve epididimis ağırlığı ↓ Sperm sayısı ve motilitesi ↓ Sperm anomalileri ↑	[46]
TRZ	90 günlük Wistar albino sıçan	72 mg/kg/gün	60 gün	SOD ve KAT aktivitesi ↓ GR ve GPX aktivitesi ↓ Testiste Cu ve Zn içeriği ↓ Testiste Fe ve Mn içeriği ↑ Seminifer tübüllerde histolojik hasar	[64]
TRZ	4 haftalık Swiss albino fare	İçme suyunda %0,1; 1 ve 2,5 oranlarında	13 hafta	Sperm sayısı ↓ (%2,5) Sperm motilitesi ↓ (%1 ve 2,5)	[37]
TRZ	Seksüel açıdan uygun Wistar albino sıçan	300 mg/kg/gün	30 gün	Vücut ağırlığı ↓ Testis ve epididimis ağırlığı ↔ Sperm anomalileri ↑ T düzeyi ↓ Total ve testiküler kolesterol düzeyi ↓ LDH ve ASP düzeyi ↑ Total protein düzeyi ↓ MDA düzeyi ↑ GR ve KAT aktivitesi ↓	[30]
TRZ	Sıçan[1]	100 mg/kg/gün 300 mg/kg/gün 500 mg/kg/gün	60 gün	Testis ve epididimis ağırlığı ↓ Sperm sayısı ve motilitesi ↓ T düzeyi ↓ FSH ve LH düzeyi ↑ MDA düzeyi ↑ SOD, GR, GPX, KAT aktivitesi ↓	[42]
TRZ	5 haftalık Swiss albino fare	10 µg/g/gün	30 gün	Vücut ağırlığı ↓ Testis ağırlığı ↓ Sperm sayısı ↓ Sperm anomalileri ↑ T düzeyi ↓ Total antioksidan kapasite ↓ Seminifer tübüllerde histolojik hasar	[48]
TRZ	Sıçan[1]	2,5-20 g/kg/gün	30, 60, 90 gün	T düzeyi ↓[2] Seminifer tübüllerde histolojik hasar[2]	[55]
TRZ	Wistar albino sıçan[1]	500 mg/kg/gün	60 gün	Sperm sayısı ve motilitesi ↓ Sperm anomalileri ↑ T ve LH düzeyi ↓ Seminifer tübüllerde histolojik hasar	[56]
TRZ	4 haftalık Swiss albino fare	100 mg/kg/gün	72 gün	Vücut ağırlığı ↑ Sperm sayısı ↓ Seminifer tübüllerde histolojik hasar	[47]
TRZ+ERT (1:1)	8 haftalık Wistar albino sıçan	2,5 mg/kg/gün 5 mg/kg/gün 10 mg/kg/gün 20 mg/kg/gün	23 gün	T, LH düzeyi ↑ FSH düzeyi ↑ (5 mg/kg) FSH düzeyi ↔ (10 mg/kg) FSH düzeyi ↓ (20 mg/kg) Seminifer tübüllerde histolojik hasar	[61]

Tablo 2 (*devamı*). Deney hayvanlarında gıda azo boyalarının erkek reprodüktif sistem üzerindeki etkilerini araştıran çalışmalar

Azo Boyası	Tür	Uygulanan Doz (Oral)	Maruziyet Süresi	Sonuçlar	Referans
GS	Sıçan[1]	250 mg/kg/gün 1500 mg/kg/gün	90 gün	Sperm sayısı ↓ Seminifer tübüllerde histolojik hasar	[51]
GS	Sprague-Dawley sıçan[1]	157,5 mg/kg/gün 315 mg/kg/gün	6 hafta	Bcl-2 gen mRNA ekspresyon düzeyi ↓ Kaspaz-3 gen mRNA ekspresyon düzeyi ↑ GPX gen mRNA ekspresyon düzeyi ↓ TST1 gen mRNA ekspresyon düzeyi ↑	[72]
GS	Swiss albino fare[1]	30 mg/kg/gün	60 gün	Testis ağırlığı ↓ Sperm motilitesi ↓ Sperm anomalileri ↑ T düzeyi ↓ Seminifer tübüllerde histolojik hasar	[50]
GS	Wistar albino sıçan[1]	5xADI (5 mg/kg/gün)	60 gün	T düzeyi ↓	[57]
KAR	Sprague-Dawley sıçan[1]	ADI (4 mg/kg/gün) 5xADI (20 mg/kg/gün) 10xADI (40 mg/kg/gün)	15, 30, 45 gün	Sperm sayısı ↓[2] Seminifer tübüllerde histolojik hasar[2] Testin gen mRNA ekspresyon düzeyi ↑ (15 gün) Testin gen mRNA ekspresyon düzeyi ↓ (30 gün, 45 gün) GDNF gen mRNA ekspresyon düzeyi ↑ (15 gün) GDNF gen mRNA ekspresyon düzeyi ↓ (45 gün) FSHR gen mRNA ekspresyon düzeyi ↓[2] PRKA3 gen mRNA ekspresyon düzeyi ↓ (30 gün, 45 gün) SPATA7 gen mRNA ekspresyon düzeyi ↓ (30, 45 gün) SSEA1 gen mRNA ekspresyon düzeyi ↑ (15 gün) SSEA1 gen mRNA ekspresyon düzeyi ↓ (30 gün, 45 gün) C-KIT gen mRNA ekspresyon düzeyi ↑ (15 gün) C-KIT gen ekspresyon düzeyi ↓ (30 gün, 45 gün)	[73]
KAR	12-14 haftalık sıçan[1]	250 mg/kg/gün	60 gün	T, FSH, LH, GnRH düzeyi ↓ MDA düzeyi ↑ SOD, GR, KAT aktivitesi↓	[58]
ÇK	Sıçan[1]	200 mg/kg/gün 400 mg/ kg/gün	8 hafta	T, FSH, LH, GnRH düzeyi ↓	[59]
ÇK	12-14 haftalık sıçan[1]	100 mg/kg/gün 200 mg/ kg/gün 400 mg/kg/gün	30 gün	Vücut ağırlığı ↓ Gonadosomatik indeks ↓ (200 mg/kg, 400 mg/kg) Sperm sayısı ve motilitesi ↓ (200 mg/kg, 400 mg/kg) T, FSH, LH düzeyi ↓ MDA düzeyi ↑ SOD, GR, GPX, KAT aktivitesi ↓ Seminifer tübüllerde histolojik hasar	[49]
PM	Sıçan[1]	0,08 g/kg/gün 0,4 g/kg/gün	15, 30, 45 gün	T düzeyi ↓[2] ASP düzeyi ↓[2] Seminifer tübüllerde histolojik hasar[2]	[60]

* TRZ: Tartrazin, ERT: Eritrosin; GS: Günbatımı sarısı; KAR: Karmosin; ÇK: Çikolata kahverengi; PM: Parlak mavi; ADI: Günlük kabul edilebilir alım miktarı; SOD: Süperoksit dismutaz; KAT: Katalaz; GR: Glutatyon redüktaz; GPX: Glutatyon peroksidaz; MDA: Malondialdehit; LDH: Laktat dehidrogenaz; ASP: Asit fosfataz; T: Testosteron; FSH: Folikül uyarıcı hormon; LH: Luteinleştirici hormon; GnRH: Gonadotropin salgılatıcı hormon; Cu: Bakır; Zn: Çinko; Fe: Demir; Mn: Mangan; GDNF: Glial hücre dizisi kökenli nörotrofik faktör; FSHR: Folikül uyarıcı hormon reseptörü; PRKA3: Protein kinaz A-sabitleyici protein 3; SPATA7: Spermatogenez ile ilişkili protein 7; SSEA1: Seviye spesifik embriyonik antijen-1; C-KIT: Tirozin kinaz reseptörü; ↑: Artış; ↓: Azalış; ↔: Değişiklik gözlenmemiştir; [1] Türe ait soy ve/veya yaş bilgisi verilmemiştir; [2] Tüm maruziyet sürelerinde değişiklik gözlenmiştir

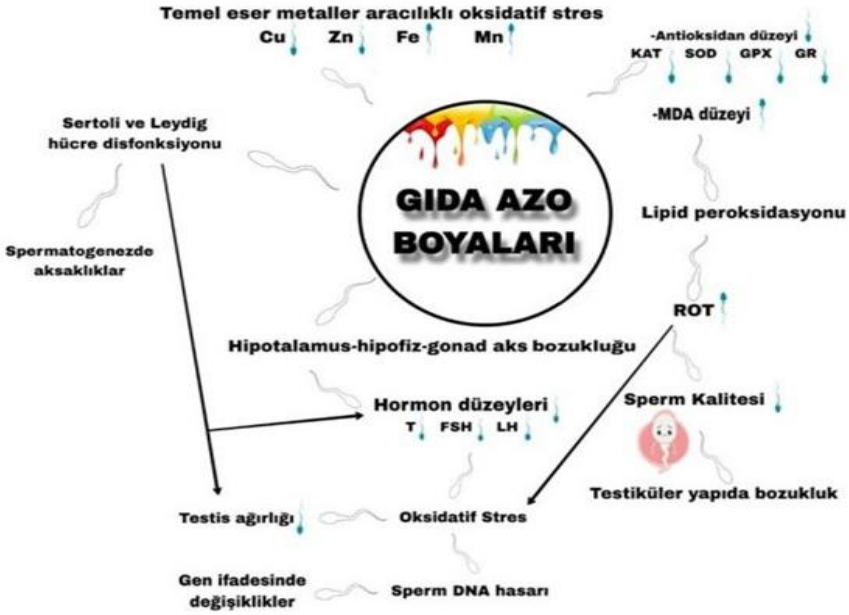
Enzimlerin katalitik ve antioksidan aktivitelerinde önemli rol oynayan bakır (Cu), demir (Fe), mangan (Mn) ve çinko (Zn) gibi eser metallerin konsantrasyonları infertilite ile ilişkilendirilmiştir. Hücresel düzeyde Zn'nin varlığı, gonadlarda hücre büyümesi ve bölünmesi için gereklidir [69]. TRZ maruziyetiyle sıçanlarda testiküler yapıdaki Cu ve Zn düzeylerinde görülen azalmalar seminifer tübüllerdeki hasar ve bozulmuş spermatogenezle ilişkilendirilmiştir. Fe düzeylerindeki artışın sebebi olarak da Cu ile arasındaki karşılıklı antagonizma gösterilmiştir. TRZ'nin testislerden başlattığı Zn mobilizasyonunun antioksidan enzim sisteminin depresyonuyla ve testiküler yapının bozulmasıyla sonuçlandığı ifade edilmiştir [64].

B-hücreli lenfoma-2 (Bcl-2), kaspaz aktivasyon blokajı ile hücre ölümünü önleyerek apoptoz ve nekrozu inhibe eden anahtar bir proteindir [70]. Testis spesifik taşıyıcı (TST1) ise gonad hücrelerinde seks steroidlerinin taşınması ve spermatogenezin düzenlenmesinde rol oynayan taşıyıcı bir sistemdir [71]. Sıçanlara GS'nin uygulanması testis dokusunda Bcl-2 ve GPX'in mRNA ekspresyonunda azalışa ve kaspaz-3 ve TST1 mRNA ekspresyonunda artışa sebep olmuştur. Gen ifadelerindeki bu değişikliklerin apoptozu tetiklediği ve hücresel detoksifikasyon sistemini aksattığı ifade edilmiştir [72].

Spermatogenezi kontrol eden genlerin ekspresyonuyla ilgili detaylı bulguların sunulduğu başka bir çalışmada KAR uygulanan sıçanlarda testin, glial hücre dizisi kökenli nörotrofik faktör (GDNF), folikül uyarıcı hormon reseptörü (FSHR), protein kinaz A-sabitleyici protein 3 (PRKA3), spermatogenez ile ilişkili protein 7 (SPATA7), tirozin kinaz reseptörü (C-KIT) ve seviye spesifik embriyonik antijen-1 (SSEA1) gen ifadelerinde doza (ADI, 5 x ADI, 10 x ADI) ve maruziyet zamanına (15, 30, 45 gün) göre değişkenlik gösteren, kontrol grubuna göre anlamlı farklılıkların bulunduğu ifade edilmiştir [73].

Sertoli hücrelerinin lizozomlarında mevcut olduğu bilinen asit fosfataz (ASP) enzimi spermatogenez sürecinin normal işleyişini teşvik eder [74]. PM'nin farklı dozlarda ve farklı maruziyet zamanlarında sıçanlara verilmesiyle tüm deney gruplarında görülen anlamlı testiküler ASP ve T düzeyleri düşüşünü birbiriyle ilişkilendirmiştir [60]. Bu çalışmadaki azo boyanın ASP üzerindeki etkisinin aksine başka bir çalışmada ise 300 mg/kg TRZ uygulanan sıçanlarda 30 günlük maruziyet sonucu ASP düzeylerinde artış gözlenmiştir. Doku ve hücrelerdeki toksisite düzeyini değerlendirmek için yaygın olarak kullanılan laktat dehidrogenaz (LDH) artışının da eşlik ettiği bu durum prostat ve veziküler bezlerdeki TRZ toksisitesi ile bağdaştırılmıştır [30].

Bahsi geçen *in vivo* çalışmaların ışığında gıda azo boyalarının erkek reprodüktif sistemi hangi mekanizmalarla etkileyebileceği Şekil 1'de görselleştirilmiştir.



Şekil 1. Gıda azo boyaları ile indüklenen erkek reprodüktif sistem toksisitesi olası mekanizmaları

SONUÇ VE TARTIŞMA

Gıda ve ilaç endüstrisinde kullanımı onaylanmış olmasına rağmen sentetik gıda azo boyaları sağlık açısından risk oluşturabilir. Son zamanlarda gıda azo boyalarının erkeklerde reprodüktif fonksiyonlar açısından etkilerini inceleyen deneysel çalışmaların sayısında artış gözlenmiştir.

Yapılan çalışmalar, gıda azo boyaları uygulanan hayvanların sperm konsantrasyonunda ve motilitesinde belirgin bir azalmanın yanı sıra anormal sperm yüzdesinde bir artışın olduğunu ortaya koymuştur. Bu boyaların hayvanlarda erkek reprodüktif fonksiyonları, oksidatif stres yoluyla testiküler dokuya zarar vererek, ön hipofizden salınan GnRH, FSH ve LH'ın plazma seviyesini azaltarak, Leydig hücrelerinden T salgılanmasını inhibe ederek baskıladığı görülmüştür. T seviyelerindeki azalmalar, gıda azo boyalarının erkek reprodüktif sistemde bozulan işlevler için anahtar bir mekanizmayı temsil edebilir. Mevcut çalışmalardan, gıda azo boyalarının doza ve zamana bağlı olarak erkek reprodüktif sistem üzerinde farklı düzeylerde toksik etkileri olduğu sonucuna varılabilir.

Sağlıklı hayvanlarda az sayıda çalışma yapılması ve klinikteki çalışmaların uygulanabilirliğinin tartışmalı olması reprodüktif patolojilerde boyaların rolünün net olarak anlaşılmasını mümkün kılmamaktadır. Bu nedenle azo boyaların neden olabileceği erkek reprodüktif sistem toksisitesinde rol oynayan mekanizmaları belirlemek için daha fazla çalışmaya ve araştırmaya ihtiyaç vardır. Ayrıca reprodüktif çalışmaların daha çok TRZ üzerine odaklanması, literatürde diğer boyaların birkaçına dair kısıtlı çalışmanın yer alması dikkat çekicidir.

Üretim ve kullanım kolaylığı açısından doğal gıda boyalarına göre avantajlarının bulunması nedeniyle gıda azo boyaları yaygın olarak kullanılmaktadır. Bu nedenle reprodüktif çağdaki erkekler üzerindeki olumsuz etkilerini araştırmanın, zararlı etkileri konusunda üretici/tüketici farkındalığını oluşturmanın, hükümet politikalarında yapılan düzenlemelerle gıdalara eklenen konsantrasyonlarını belirterek uygun şekilde etiket hazırlamanın konuya dikkat çekmek için yardımcı olacağı düşünülebilir.

YAZAR KATKILARI

Kavram: B.K.Ç.; Tasarım: B.K.Ç., M.B.; Denetim: M.B.; Kaynaklar: - ; Malzemeler: - ; Veri Toplama ve/veya İşleme: B.K.Ç.; Analiz ve/veya Yorumlama: B.K.Ç., M.B.; Literatür Taraması: B.K.Ç.; Makalenin Yazılması: B.K.Ç., M.B.; Kritik İnceleme: M.B.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1248-1263, 2024
Doi: 10.33483/jfpau.1488042

DERLEME MAKALE / REVIEW ARTICLE

BİTKİ SEKONDER METABOLİTLERİNİN BİYOSENTEZİNİ VE AKÜMÜLASYONUNU ETKİLEYEN FAKTÖRLER

FACTORS AFFECTING THE BIOSYNTHESIS AND ACCUMULATION OF PLANT SECONDARY METABOLITES

Cennet ÖZAY[1]*, Ecenur PEHLİVAN[2]

[1]İzmir Kâtip Çelebi Üniversitesi, Eczacılık Fakültesi, Eczacılık Temel Bilimleri Anabilim Dalı, 35620, İzmir, Türkiye
[2]İzmir Kâtip Çelebi Üniversitesi, Eczacılık Fakültesi, 35620, İzmir, Türkiye

ÖZ

Amaç: *Primer metabolitler, bitkinin temel yaşamsal işlevlerinde doğrudan etkiliyken, sekonder metabololitler ise doğrudan etkili olmayan, bitkinin hayatta kalması ve bulunduğu ortama uyum sağlaması ile ilgili görevleri olan biyoaktif bileşiklerdir. Fonksiyonel gıdalarda, kozmetik ürünlerde, tarım ve zirai uygulamalarda, aromaterapide, boyalar ve diğer endüstriyel ürünlerde kullanım alanlarının olmasının yanında birçok ilaç ve sağlık ürünü de bitkisel kaynaklı sekonder metabolitlerden elde edilir. Bitkilerin çevresel etkileşimlerine yanıt olarak oluşturdukları bileşikler olan sekonder metabolitler, bitkilerin savunma mekanizmalarında, iletişimde ve çeşitli biyolojik süreçlerde rol oynarlar. Hem bitkilerin kendileri, hem de insanlar açısından faydalı olan bu bileşiklerin biyosentezlerini ve akümülasyonlarını etkileyen faktörlerin araştırılması önemlidir.*
Sonuç ve Tartışma: *Bitki sekonder metabolitlerinin biyosentezi ve akümülasyonu üzerinde etkili olan çeşitli genetik, ontogenik, morfogenetik ve çevresel faktörler incelenmiştir. Diğer tüm faktörler sabit kalsa da sadece bir faktördeki değişiklik bitkilerde sekonder metabolitlerin içeriğini ve miktarını seçici olarak değiştirebilmektedir. Ayrıca, bitki büyümesini ve verimliliğini, bunun sonucunda da sekonder metabolit üretimini artırmak için, metabolomik, proteomik ve transkriptomik gibi yeni yöntemler kullanılarak çeşitli çevresel etkenlerin sinerjistik etkilerini moleküler seviyede anlamak amacıyla daha fazla araştırmaya ihtiyaç vardır.*
Anahtar Kelimeler: *Bitki sekonder metabolitleri, biyosentez, biyotik ve abiyotik faktörler*

ABSTRACT

Objective: *While primary metabolites are directly effective in the basic vital functions of the plant, secondary metabolites are bioactive compounds that are not directly effective and have functions related to the survival and adaptation of the plant to its environment. In addition to their use in functional foods, cosmetic products, agricultural applications, aromatherapy, dyes, and other industrial products, many drugs and health products are also obtained from plant-derived secondary metabolites. Secondary metabolites, which are compounds formed by plants in response to environmental interactions, play a role in plant defense mechanisms, communication, and various biological processes. It is important to investigate the factors affecting the biosynthesis and accumulation of these compounds, which are benefiial for both plants themselves and humans.*
Result and Discussion: *Various genetic, ontogenic, morphogenetic and environmental factors affecting the biosynthesis and accumulation of plant secondary metabolites have been examined.*

* **Sorumlu Yazar / Corresponding Author:** Cennet Özay
e-posta / e-mail: cennet.ozay@ikcu.edu.tr, **Tel. / Phone:** +902323293535/6163

Gönderilme / Submitted : 22.05.2024
Kabul / Accepted : 08.08.2024
Yayınlanma / Published : 10.09.2024

Although all other factors remain constant, changes in only one factor can selectively alter the content and amount of secondary metabolites in plants. Additionally, further research is needed to understand the synergistic effects of various environmental factors at the molecular level using new methods such as metabolomics, proteomics and transcriptomics to increase plant growth and productivity, and subsequently secondary metabolite production.
Keywords: *Biosynthesis, biotic and abiotic factors, plant secondary metabolites*

GİRİŞ

Bitki metabolitlerinin kullanımı M.Ö. 2600 yıllarına kadar dayanmakta olup, takip eden 4000 yılda bitkilerden kaynaklanan sekonder metabolitler (SM) gıdanın yanı sıra ilaç ve zehir amaçlı olarak da kullanılmaya başlanmıştır. Morfin, 1806 yılında afyon haşhaşından (*Papaver somniferum*) izole edilen ilk doğal ürün olmuş ve ikincil metabolit araştırmalarında yeni bir dönem açmıştır. Daha sonra bitki ekstraktı aktivitesinin, kendi kimliğine sahip olan ve aynı zamanda saflaştırılabilen tek bir organik bileşiğe atfedildiği tespit edildi. Böylece, bu araştırma sonuçları doğal ürün araştırmalarını başlattı ve bugüne kadar bitki SM'leri, tıbbi ürünlerin %30'undan fazlasının doğrudan veya dolaylı olarak bu ürünlerden türetildiği gerçeğiyle ortaya konan önemli bir rol oynadı [1].

Bitkisel sekonder metabolit (BSM)'ler, çeşitli fizyolojik aktivitelere sahip primer metabolitlerden türetilir. Bitkilerin hayatta kalmaları ve çevreye adaptasyonları için hayati bir role sahiptirler. BSM'lerin bu özelliği onları birincil metabolitler kadar önemli kılar. SM'ler bitkilerin hayatta kalmasında doğrudan bir etkiye sahip olmamakla birlikte uzun vadeli bir etkiye sahiptirler [2]. Bitkiler alemi, belirli taksonomik gruplarla sınırlı olan 100.000'den fazla SM üretmektedir. Yapılan çalışmalarda BSM'lerin organdan organa, bazen de bitki ve türleri arasında farklılık gösterdiği ve bunların bitki sınıflandırmasında taksonomik karakter olarak kullanılabileceği bildirilmiştir. Karbon iskeletlerindeki farklılıklar ve fonksiyonel grupların çeşitliliği onların çok farklı sınıflara ayrılmasını gerekli kılar. BSM'ler terpenler, fenolik bileşikler, azot içeren bileşikler ve kükürt içeren bileşikler olmak üzere dört ana gruba ayrılır [3].

SM'ler, bitkiler tarafından üretilen ve onları kendi ortamlarında rekabetçi kılan maddelerdir. Bu fitokimyasal bileşikler, bitkinin kendisi ve diğer canlı organizmalar üzerinde çok çeşitli etkiler gösterir. Bu derlemede, BSM'lerin verim ve içeriğinin genetik, ontogenik, morfogenetik ve çevresel faktörlerin varlığında nasıl değişebileceği tartışılmıştır.

Sekonder Metabolitler ve Önemi

Canlılık için (büyüme, gelişme, çoğalma, vb.) mutlak gerekli olan karbonhidratlar, yağlar, proteinler, vitaminler, enzimler ve nükleik asitler primer metabolitler olarak adlandırılırken, temel yaşamsal işlevlerle doğrudan ilişkisi olmayan maddeler ise sekonder (ikincil) metabolitler olarak adlandırılmaktadır [4].

BSM'ler, primer metabolitlerden türetilir ve çok sayıda biyolojik özelliğe sahiptir [5]. BSM'ler adaptasyon, hayatta kalma, büyüme ve diğer temel işlevlere katkıda bulunma gibi özelliklerinden dolayı bitkilerdeki hayati bileşenlerdir. Bitkileri, biyotik (örneğin bakteri, mantarlar, nematodlar, böcekler veya otlatma yoluyla hayvanlar) ve abiyotik (daha yüksek sıcaklık ve nem, yaralanma veya ağır metallerin varlığı gibi) streslere karşı korumada önemli rol oynarlar [6]. Bitkiler, tozlaşma ve tohum dağılımı için böcekleri veya diğer hayvanları çekmek amacıyla SM'leri kullanırlar. Bu metabolitler arasında uçucu yağlar, renkli flavonoidler ve tetraterpenler gibi bileşikler bulunur. Bu şekilde, SM'ler bitkiler için sinyal bileşikleri olarak işlev görerek çevrelerindeki canlıları çekmeye yardımcı olurlar. SM'ler, özellikle ilaç, tat, koku, böcek öldürücü ve boya gibi kimyasal ürünlerde yüksek ekonomik değere sahip oldukları için kullanılmaktadırlar. Biyoaktif özellikli kimyasal bileşikler olan SM'ler tıp ve eczacılık alanlarında sıklıkla kullanılsa da, fonksiyonel gıda bileşeni ve besin takviyesi olarak kullanılabilmeleri dolayısıyla da önemlidir [6].

Mevcut bulgular, doğal bileşiklerin insan sağlığını geliştirmek için potansiyel kullanımını kanıtlamaktadır ve geniş SM yelpazesi, onları yenilikçi farmasötikler için değerli bir rezervuar haline getirmektedir [7]. Bu bileşikler, ilgili bitkilerle ilişkili tıbbi özelliklerden sorumludur ve tüm bitki türlerindeki toplam SM sayısı yüz bini aşmaktadır [8]. Ağırlıklı olarak meyve ve sebzelerde bulunan,

fito-bileşikler olarak da isimlendirilen SM'ler; kanserler, kardiyovasküler hastalıklar, inflamatuvar ve bağışıklık bozuklukları ve nörodejeneratif bozukluklar gibi farklı durumlarda duyarlılığı azaltmada önemli bir rol oynamaktadır [9]. Bu bileşikler genellikle nöroprotektif, antioksidan, anti-enflamatuar, anti-diyabetik, anti-kanser ve ilaç geliştirme için başlangıç malzemesi olarak kullanılan diğer özellikler dahil olmak üzere oldukça spesifik biyolojik aktivitelere sahiptir [10].

Esasen bitkinin kimyasal savunma mekanizmalarından organik savunma alt başlığına dahil olan SM'ler, bitkiyi herbivor, bakteriyal ve fungal patojen saldırılarına karşı korumakla birlikte aynı ortamdaki diğer bitkilerle rekabet güçlerini de artırırlar [11]. SM'ler düşük molekül ağırlıklı bileşiklerdir ve genellikle belirli organ, doku ve hücrelere özgü olarak oluşurlar. Bu bileşikler, aynı bitki popülasyonu içinde bile miktar ve tür bakımından farklılık göstebilirler. Aynı türdeki spesifik bileşikler kemotaksonomide belirteç olarak kullanılmaktadırlar [6].

Sekonder Metabolit Çeşitleri ve Biyosentezleri

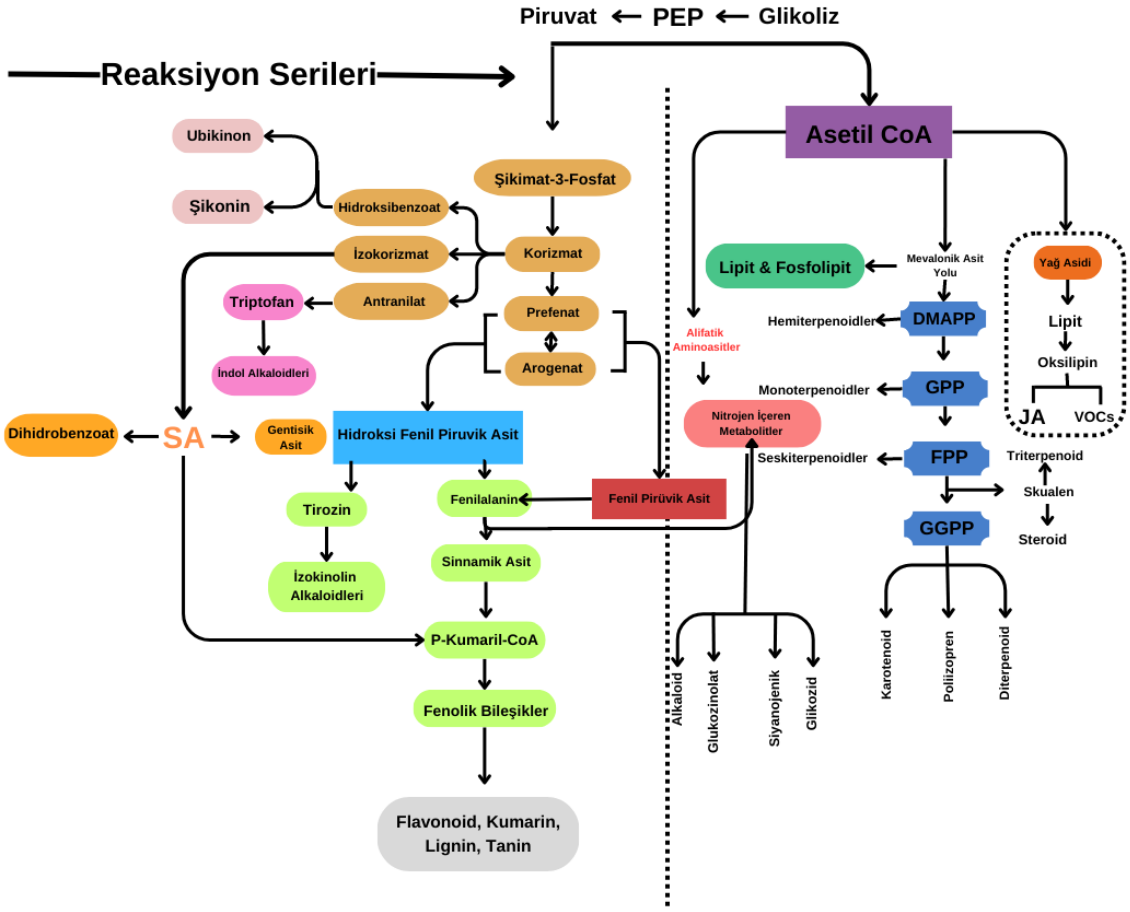
İskelet çeşitliliği ve ilaç adayı olma potansiyeli nedeniyle literatürde ilgi çekici bulunan BSM'ler, dört ana sınıfa (Şekil 1) ayrılmaktadır: i) Fenolik bileşikler, ii) Terpenler, iii) Azot içeren bileşikler, iv) Kükürt içeren bileşikler [2].

Şekil 1. Bitki sekonder metabolitlerinin genel sınıflandırılması

Bu metabolitlerin neredeyse tamamı bitkinin stresle karşılaşma durumunda kendini savunması bakımından önemli ölçüde katkıda bulunurlar. Bitkilerde SM'lerin biyosentezi, hem birincil hem de ikincil metabolizmayı birbirine bağlayan karmaşık yolları içermektedir (Şekil 2). Bu bileşikler strese adaptasyonda ve çeşitli fizyolojik işlevlerde önemli roller oynamakta, bu da onları bitkinin hayatta kalması ve savunması için gerekli kılmaktadır [12].

SM'lerin kritik öncülleri primer metabolitlerdir. SM'lerin sentezi türler arasında bile çeşitlilik gösterebilirken, primer metabolitlerin sentezi canlılar arasında hemen hemen aynıdır. Biyosentez yollarına dayanarak, SM'ler üç ana gruba ayrılabilir: şikimat yolunda sentezlenen fenolik bileşikler, mevalonik yolda sentezlenen terpenler ve trikarboksilik asit döngüsü yolunda sentezlenen azot içeren bileşikler [13]. Şikimat yolunun öncüsü olan şikimik asit, fosfoenolpiruvat (glikolitik yoldan) ve eritroz 4-fosfatın (pentoz fosfat yolundan) bir kombinasyonundan üretilir. Protein sentezinin yapı taşları olan fenilalanin, tirozin ve triptofan, fenolikler ve azot içeren bileşikler gibi bitki SM'leri için ortak öncüllerdir ve şikimat yolunda üretilirler [14]. Fenilalanin; flavonoidlerin, lignanların, ligninlerin,

kondanse tanenlerin ve fenilpropanoid/benzenoid tipindeki uçucu maddelerin ortak öncüsüdür. Tirozin; izokinolin alkaloitleri, pigment betalainleri ve kinonları (örn. plastokinon) üretirken triptofan ise alkaloitlerin, fitoaleksinlerin, indol glukozinolatların ve bitki hormonu oksinin öncüsüdür [15].



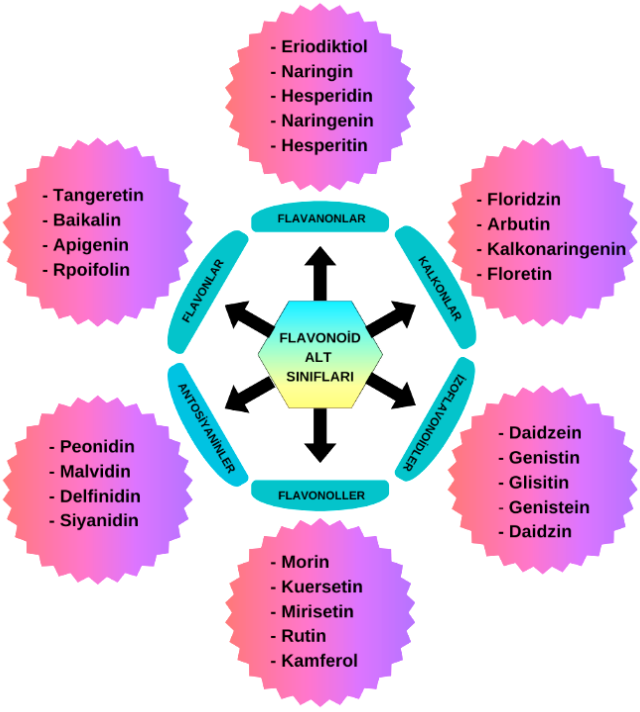
Şekil 2. Bitki sekonder metabolitlerinin biyosentez yolakları [12]

Fenolik Bileşikler

Sekonder metabolitlerin önemli bir grubu olan fenolik bileşikler, bitkilerde ve bitki kaynaklı gıdalarda bulunmakla birlikte parazit ve zararlılara karşı bitki savunmasında gereklidir [16]. En az bir fenol halkası içeren yapıları ile karakterize edilirler. Yapısal olarak oldukça çeşitli olan fenolik bileşikler, basit moleküller (örneğin, vanilin, gallik asit ve kafeik asit) ve polifenolleri (örneğin, stilbenler, flavonoidler ve polimerler) içerirler. Fenolik bileşikler bitkilerde çözünür veya bağlı formlarda bulunurlar. Çözünür fenolik bileşikler genellikle endoplazmik retikulumda sentezlenip vakuollerde depolanırken, bağlı fenolik bileşikler ise hücre duvarındaki selüloz, pektin, protein gibi yapılara glikosidik ve ester bağlarıyla kovalent olarak bağlanırlar [17].

Kimyasal yapılarına göre; fenolik asitler (hidroksibenzoik ve hidroksisinnamik asitler), kumarinler, flavonoidler, stilbenler, lignanlar ve tanenler olmak üzere alt gruplara ayrılmaktadırlar. Düşük moleküler ağırlıklı fenolik bileşiklerin çeşitlilik gösteren farklı gruplarından oluşan flavonoidler, insan beslenmesinde bitkisel besinler formunda bulunur ve bitkiler aleminde oldukça yaygındırlar (bugüne kadar ~ 8000 flavonoid molekülü rapor edilmiştir). Yüksek yapılı bitkilerdeki SM'lerin en karakteristik sınıflarından birini oluştururlar. Flavonoidlerin kimyasal yapısı genellikle fenil halkası ve heterosiklik halka içeren 15 karbonlu bir iskeletten oluşur. Bitki savunması ve pigmentasyon sistemlerinde hayati fonksiyonlara sahip olan flavonoidler, sağlığı geliştirici çeşitli özelliklere sahiptirler

ve farmasötik, tıbbi ve kozmetik ürünlerin de önemli bir bileşenidirler. Örneğin, antiinflamatuvar, antioksidatif, antikarsinojenik ve antimutajenik özelliklerinden dolayı önemli hücresel enzimatik fonksiyonları değiştirebilirler ve çok sayıda enzimin etkili inhibitörleridirler [18]. Bitki yaşamı için çok önemli olan flavonoidler Şekil 3'te sınıflandırılmıştır.



Şekil 3. Bitki flavonoidlerinin sınıflandırılması [12]

Klasik olarak diyet antioksidanları olarak tanımlanan büyük bir difenolik bileşik grubu olan izoflavonoidler; izoflavonları, izoflavanları ve pterokarpanları içerir. Bir heterosiklik C-halkası ile birleştirilmiş bir fenil halkasından ve C3 pozisyonunda kaynaşmış başka bir B-halkasından oluşurlar [19]. İzoflavonoidler sınırlı bir bitki grubunda dağılım gösterirler ve çoğunlukla baklagil türlerinde bulunurlar. Bunlar azot bağlayıcı nodül oluşumu ve dolayısıyla simbiyotik *Rhizobium* bakterilerinin teşviği için önemli olan bileşiklerdir [20].

Terpenler

Bitkilerdeki SM'lerin ana sınıfı olan terpenler veya terpenoidler, glikolitik veya asetil CoA ara ürünlerinin türevlerinden oluşurlar. Lipitte çözünürler ve geniş bir yapısal çeşitliliğe sahiptirler (50.000'den fazla farklı yapı). Terpenler, çeşitli kompleks fitohormonların, sterollerin ve pigmentlerin temel yapı taşlarıdır ve onların aroma özellikleri ile fizyolojik etkilerinden birincil olarak sorumludurlar. Bazı terpenler hoş bir aromaya sahip olmaları ve tozlaşmayı sağlayıcı cezbediciler olarak işlev görmelerine rağmen, çoğu savunma toksinleri ve herbivorları caydırıcı olarak işlev görürler [21].

Terpenler, içerdikleri izopren birimlerinin sayısına göre; monoterpenler, seskiterpenler, diterpenler, triterpenler, tetraterpenler ve politerpenler olarak altı gruba ayrılmaktadır. Uçucu yağların ana bileşenleri olmalarının ve bitkilerde temel roller oynamalarının yanı sıra, birçok terpen aromalardan kokulara ve ilaçlara kadar farmasötik ve endüstriyel uygulamalarda yaygın olarak kullanılmaktadır. Birçok çalışma, kanser kemopreventif etkileri, antimikrobiyal, antiviral, analjezik, anti-inflamatuar, antifungal, antiparazitik ve diğer aktiviteler de dahil olmak üzere terpenlerin biyolojik özelliklerinin çeşitliliğini göstermiştir [22].

Bitkilerin antagonistik ve mutualistik etkileşimler için kullandıkları bilgilendirici ve savunma araçları olarak işlev gören terpenler, biyotik (patojenik mikroplar, herbivor zararlılar ve yabani otlar) ve

abiyotik (su, sıcaklık, ışık ve tuz) streslere karşı koymak için bitkiler tarafından üretilirler. Fitoantisipinler, patojen efektörlerin yokluğunda yapısal olarak salgılanırken, fitoaleksinler ise indüklenebilir patojenik mikroplara veya bitkiyle beslenen otçullara karşı yanıt olarak üretilirler. Sentetik pestisitlerin insanlar ve çevre üzerindeki olumsuz etkilerinden dolayı, doğal pestisit geliştirmede aktif bir bileşen olarak terpen araştırmaları, düşük riskleri nedeniyle ilgi duyulan araştırmalar arasındadır [23].

Azot İçeren Bileşikler

Azot, canlı hücrelerin büyüme ve gelişiminde yer alan metabolik süreçler için önemli bileşenlerden biridir. Bitkiler, azot bağlayıcı bakterilerin yardımıyla köklerinden azot elde ederler. Bitki büyümesi için ikincil azot kullanımı ve talebi konakçı bitkilerde çok yaygındır. Azotun hidrojen ve oksijen gibi diğer gazlarla kimyasal olarak bağlanmasıyla çeşitli azotlu bileşikler oluşur. İlaç olarak kullanılan hemen hemen her ilacın ana bileşenlerinden biri azottur [24].

Büyük miktarlarda azot içeren bitkiler, azotlu ikincil metabolitler de üretirler. Azot içeren SM'ler, başlıca üç ana kategoriye ayrılır: alkaloitler, siyanojenik glikozitler ve protein olmayan amino asitler. Yapısal olarak çok çeşitli formlara sahip olabilen alkaloitlerin kesin bir tanımı yoktur ve alkaloitleri temel bileşen olarak azot içeren diğer doğal metabolitlerden ayırmak zordur [24]. Kimyasal yapılarına ve doğal kaynaklarına göre sınıflandırılan alkaloitler genellikle C-N iskeletlerinin dağılımına göre diterpenoid, steroidal, kinazolin, indol, izokinolin, kinolin, piridin, pirolidin ve diğer alkaloitler gibi büyük gruplara ayrılırlar. Alkaloitler, azot içeren heterosiklik halkalara sahip organik bazlar olup non-nükleozid ve non-peptidik bileşikler olarak da tanımlanırlar. Düşük molekül ağırlıklı bileşikler olup alkaloit içeren familyalarda 12.000'den fazla azot içeren içeren bileşik tanımlanmıştır [25]. Alkaloitlerin çoğu toksiktir ve tadları acıdır, bu da onları bitkilerin omurgasız haşere saldırılarına, mikrobiyal patojenlere ve otçullara karşı savunma ajanları olarak kullanılmalarını mümkün kılar. Sitotoksik, karsinojenik veya mutajenik aktivite, antifungal, antiviral ve antibakteriyel aktivitelere sahip olduğu bilinen alkaloitler, terapötik potansiyele sahiptirler [26].

Siyanojenik glikozitler, alfa-hidroksinitril (syanohidrin) tipi bir aglikon ve bir şeker molekülünden (çoğunlukla D-glukoz) oluşur. Bitkiler aleminde siyanojenik glikozitler içeren taksonların sayısı en az 2500'dür ve bu taksonların çoğu Fabaceae, Rosaceae, Linaceae, Compositae ve diğer familyalara aittir. Siyanojenik glikozitler bitki savunma mekanizmalarında önemli bir rol oynamaktadır [27]. Bitkilerde, temel 20 amino asit dışında, proteinlere asimile edilmeyen 200'den fazla serbest bitki hücresi amino asidi vardır. Bu serbest amino asitlere protein olmayan amino asitler denir. Bitkilerdeki başlıca işlevleri çeşitli çevresel streslere yanıt vermektir [28].

Kükürt İçeren Bileşikler

Kükürt içeren ikincil metabolitler, nispeten küçük bir bitki SM grubudur. Bu grup; glukozinolatlar, fitoaleksinler, allininler, tiyoninler ve defensinleri içerir [29]. Glukozinolatlar lahana, brokoli, turp gibi sebzelerde koku ve tattan sorumludur ve esas olarak Brassicaceae familyasına ve diğer ilgili familyalarda bulunurlar. Keskin kokulu uçucu bileşenlerin (tiyosiyanatlar, izotiyosiyanatlar, nitriller gibi) bitkilerden salınması, glukozinolatların mirosinaz adı verilen hidrolitik enzimler ile parçalanıp, glukozun yapıdan uzaklaştırılmasıyla sağlanır. Parçalanma ürünleri, herbivorlara karşı toksin ya da onları uzaklaştırıcı olarak savunmada görev alırlar. Hardal yağı glikozitleri olarak bilinen glukozinolatlar ve parçalanma ürünlerinin bazı kanser türleri (akciğer, mide, kolon, prostat vb.) ve kalp-damar hastalıklarının önlenmesinde önemli rollere sahip olduğu bilinmektedir [30].

Kükürt içeren SM'ler bitki sağlığı üzerinde önemli bir etkiye sahiptir, çünkü bitkiler yeterli kükürde sahip olmadan azotu düzgün bir şekilde kullanamazlar. Kükürt ve azotun ideal miktarları bitkinin çeşitli çevresel streslerle başa çıkma yeteneğini arttırmaktadır. Fitoaleksinler, enfeksiyon bölgesi çevresinde birikerek istilacı patojenlerin yayılmasını sınırlamaya yardımcı olan bakteriyel veya fungal enfeksiyona ve diğer stres formlarına yanıt olarak sentezlenmektedir. Defensinler, tiyoninler ve lektinler ise mikrobiyal saldırı ve benzeri durumlardan sonra sentezlenen ve kükürt metabolitleri bakımından zengin, depolanmayan bitki proteinleridir [31].

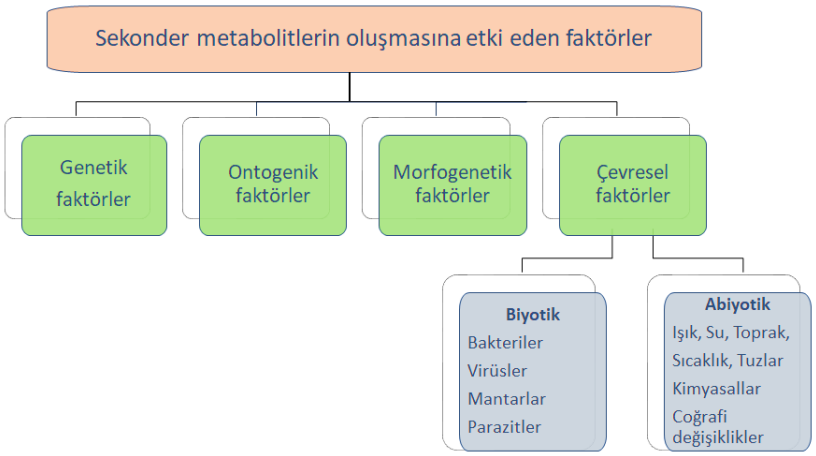
Sekonder Metabolitlerin Rolleri

BSM'ler, bitkilere koku, tat ve renk katan, onları olumsuz koşullarda patojenlerden, herbivorlardan ve çevresel streslerden bir savunma mekanizması olarak koruyan anahtar bileşiklerdir. BSM'lerin ilaç, beslenme, kozmetik endüstrisi gibi pek çok sektöre önemli katkıları vardır [32]. Bitkiler tarafından üretilen SM'ler, birçok hastalığın tedavisinde ilaç etken maddesi olarak kullanıldığı için oldukça değerlidir. Bununla birlikte, bitkiler ve insanlar için bu kadar önemli bir yere sahip olan BSM'lerin üretimi, iklimsel ve diğer faktörler nedeniyle sonraki nesillerde dalgalanmalar göstermektedir. Çeşitli BSM'lerin içeriği türden türe ve hatta aynı türe ait bitkilerde bile değişiklik gösterir [33].

Biyosentetik bir yolla üretilen BSM'lerin nihai bölgeye taşınması ve depolanması, biyokimyasal ve hücresel faktörlerden etkilenir. BSM'lerin sentezi ve birikimi için spesifik hücresel yapıların oluşması ve farklılaşması da gelişimsel faktörlerden etkilenir. BSM'lerin konsantrasyonları sıcaklık, kuraklık, tuzluluk, mevsimsellik, sirkadiyen ritim, rakım, ışık, UV radyasyonu, metal iyonları, yaralanma ve besin eksiklikleri gibi diğer birçok abiyotik faktörden etkilenir [34]. Bunların yanı sıra, yetiştirme koşulları ve ilgili BSM'lerin metabolik yolakları da konsantrasyonlarını etkilemektedir [32]. BSM'lerin biyosentezinin ve yüksek konsantrasyonlarının indüklenmesi, konukçu bitkilerin savunmaya karşı koymasını sağlamak için patojen saldırısına (biyotik faktörler) verilen yanıta da bağlıdır. Farklı faktörlerden etkilenen BSM'lerin biyosentezi ve konsantrasyonu, çeşitli çalışmalara dayanarak birçok araştırmacı tarafından tanımlanmıştır. Fenolik bileşikler gibi SM'ler, besin mevcudiyeti ve ışık yoğunluğu gibi çevresel faktörlere yanıt olarak farklı genetik, fizyolojik ve gelişimsel seviyelerde değişiklik gösterirler [35]. BSM'lerin konsantrasyonu ve birikimi genetik düzeyde de etkilenmektedir. Biyosentetik yolda yer alan genler genellikle çeşitli transkripsiyon faktörleri tarafından transkripsiyon düzeyinde kontrol edilir ve bu faktörler farklı BSM'lerin konsantrasyonu, birikimi ve biyosentezinin düzenlenmesinde önemli bir rol oynar [36]. Farklı BSM'lerin üretimindeki artış, konsantrasyonlarının değiştirilebileceğini açıkça gösteren elisitörler ve bitki büyüme düzenleyicileri kullanılarak *in vitro* doku kültürü yoluyla elde edilebilir.

Sekonder Metabolitlerdeki Değişiklikleri Etkileyen Faktörler

Yapılan çalışmalarda genel olarak, SM'lerin diğer kalitatif ve kantitatif özellikler gibi stabil kalmadığı gözlenmiştir. Dolayısıyla, BSM'lerdeki dalgalanmadan sorumlu olan faktörler dört ana gruba ayrılabilir: (i) genetik, (ii) ontogenik (iii) morfogenetik ve (iv) çevresel faktörler (Şekil 4). Bu dört temel faktörün tümü BSM'lerin üretiminde veya akümülasyonunda önemlidir [8].



Şekil 4. Bitki sekonder metabolitlerinin oluşmasına etki eden faktörler

Genetik Faktörler

BSM'lerin biyosentetik yolları hakkında çok az bilgi mevcut olduğu için bu konu hala araştırma konusudur; ancak bazı genetik çalışmalar bitkilerde SM üretiminin genetik kontrol altında

gerçekleştiğini göstermiştir. Bitki genomlarında bulunan birkaç bin genin sadece %15-25'inin BSM'lerin sentezine yol açan ikincil metabolizmaya katkıda bulunduğu varsayılmaktadır. Bu genler, yolak genlerinin gen ifadesini etkileyerek metabolik akışı etkileyen farklı transkripsiyon faktörleri tarafından düzenlenir [36]. Belirli bir bitki türünün, bitkiler aleminin tüm ikincil metabolitlerini sentezlemek için gerekli olan yalnızca birkaç enzimi kodladığı anlaşılmıştır [37].

Sekonder metabolizmada, sınırlı sayıda enzim tarafından çeşitli substratlardan birçok ürün oluşturulur; bu da tek bir enzimin farklı substratlardan birden fazla ürünün sentezini katalize ettiği anlamına gelir. Bununla birlikte, evrim yoluyla enzimatik reaksiyonlar mevcut substrata göre değişmiş, bu da değişmiş enzimlerin ortaya çıkmasına neden olmuştur. Değişmiş enzimler, belirli bir bitki türünün ilgili genlerinin ifadesini değiştiren birkaç amino asitte değişiklik yapmaktadır. Bununla birlikte, bitkilerin ikincil metabolizmasında enzimlerin substrata özgü olduğu ve bir substrattan tek bir ürün ürettiği araştırılmıştır. Bir enzim, eski substrata benzeyen yeni bir substratın ortaya çıkmasıyla birkaç amino asidin yer değiştirmesiyle kendini değiştirebilir. Eğer değiştirilmiş enzim bitki için faydalı bir ürün üretirse, bu genetik değişiklikler benimsenir ve sentezini artırarak sorumlu genin ifadesinde değişikliklere yol açar [37]. Çeşitli çalışmalar, bazı spesifik BSM'lerin yalnızca bir bitki türüyle sınırlı olduğunu göstermiştir. Bu durum, sadece belirli türlerin bu BSM'leri sentezleme yeteneğine sahip olduğunu göstermektedir. Bu durum, bir türe özgü yeni ikincil metabolizma enzimlerinin ortaya çıkmasına neden olan değişim sürecinde meydana gelmiştir. Beş farklı genin ifadesi, abiyotik stres ve savunma sinyalleri altında *Cistus creticus*'ta terpenoid biyosentetik yolağında yer almaktadır. Bu genler arasında *CcHMGR* (mevalonat yolu ile ilgili), *CcDXS* ve *CcDXR* (metilentritol 4-fosfat yolu ile ilişkili) ve her iki biyosentetik yola katılan enzimleri kodlayan ancak bitkinin farklı dokularında ifade edilen iki geranilgeranil difosfat sentaz enzimi (*CcGGDPS1* ve *CcGGDPS2*) bulunmaktadır. Tüm genler farklı dokularda farklı ifade profilleri izler. Bu genlerin artan seviyesi, *C. creticus*'ta izoprenoid birikiminin artmasına katkıda bulunur [38]. Çalışma, genlerin ve enzimlerin, BSM'lerin biyosentetik yolaklarına aktif olarak katıldığını açıkça göstermektedir. Bununla birlikte, çeşitli transkripsiyon faktörleri de bitkilerde savunma mekanizmasının düzenlenmesinde rol oynar ve farklı ailelerde (*ERF, bZIP, MYB, bHLH* ve *WRKY*) gruplanırlar. Bunların arasında *MYC2* transkripsiyon faktörü *bHLH* (temel Helix-Loop-Helix) ailesine aittir. *MYC2*, savunma mekanizmasının jasmonik asit-bağımlı yolunun düzenlenmesinde rol oynar. *MYC2* ayrıca bitkilerde kuraklık toleransı, ışık sinyali, kök büyümesi ve sirkadiyen saat gibi diğer süreçlerde de yer alır. Transkripsiyon faktörü *NaMYC2, Nicotiana attenuata*'nın savunma yanıtındaki biyosentetik yolların düzenlenmesinde önemli bir rol oynamaktadır [39].

Herbivor saldırısına yanıt olarak bitkiler, bitki savunmasında yer alan çeşitli transkripsiyon faktörlerinin indüklendiği bir düzenleyici mekanizma geliştirir, aynı şekilde *MYC2* transkripsiyon faktörü de indüklenir. Bu çalışmalar, BSM'lerin biyosentezinin şüphesiz farklı düzenleyici genler, enzimler, transkripsiyon faktörleri tarafından etkilendiğini ve patojenlerin neden olduğu streslerin farklı BSM'lerin birikiminin veya üretiminin yetersizliğine yol açtığını kanıtlamıştır. Bu BSM'lerin düzeyi, bitkilerin olumsuz koşullarda hayatta kalabilmesi için savunma molekülü olarak ihtiyaçları doğrultusunda değişir [8].

Ontogenik Faktörler

Ontogeni, bir organizmanın gelişiminde yer alan tüm olaylar dizisidir. Tohumdan başlar ve fide aşaması, vejetatif aşama, olgunlaşma aşaması gibi farklı gelişim evrelerinden geçerek yaşlanma aşamasıyla son bulur. Her bir aşama ile ilişkili bazı spesifik karakterler vardır; örneğin fide aşaması depolanmış tohum rezervlerine bağlıdır, tohum rezervleri gençlik aşamasında tükenirken olgunlaşma aşaması (çiçeklenme ve meyve verme) üreme altında gerçekleşir. Farklı ontogenik aşamalar, savunmaya yanıt olarak alkaloitler, fenolikler, terpenoidler, siyanojenik glikozitler, savunma proteinleri ve enzimler gibi çeşitli birincil ve ikincil bitki ürünlerinin konsantrasyonunu etkileyebilir [40]. *Papaver somniferum*'da farklı gelişim aşamalarında farklı konsantrasyonlarda morfin (alkaloit) içeriği tespit edilmiştir [41]. Benzer şekilde, *Astragalus compactus*'un farklı ontogenik aşamalarda içerdiği BSM'lerin (uçucu yağ, fenoller ve saponinler) konsantrasyonu ve antioksidan aktivite değişkenlik göstermektedir. Çiçeklenme aşamasında bitkinin içerdiği SM'lerin değiştiği rapor edilmiştir. Bu aşamada fitol ana bileşen iken, dokosanol, hidrokarbonlar ve steroller ise düşük miktarlarda bulunmuştur. Farklı

hidrokarbonlar ve sterollerin yüksek konsantrasyonu fruktifikasyon aşamasında mevcut olmaya devam ederken, fitol ve alkollerin bu aşamada görülmediği, ancak toplam fenolik madde (TFM) miktarının fruktifikasyon aşamasında en yüksek bulunduğu belirtilmiştir. Antioksidan aktivite için IC_{50} değeri, çiçeklenme ve vejetatif aşamalarda düşük kalırken, fruktifikasyon aşamasında ise en yüksek olarak gözlenmiştir. TFM ve antioksidan aktivite arasında fruktifikasyon aşamasında pozitif korelasyon rapor edilmiştir [42]. BSM'lerin üretimi veya birikimine yönelik farklı ontogenik çalışmalara dayanarak, bitki yaşam döngüsü sırasındaki farklı gelişim aşamalarının ikincil bitki metabolitlerinin içeriğini etkilediği sonucuna varılabilir. İkincil bitki metabolitleri tüm bitki yaşamı boyunca sabit bir konsantrasyona sahip değildir, bunun yerine ihtiyaca göre farklı çevresel koşullarda değişirler [8].

Morfogenetik Faktörler

Bitkiler, salgılama, depolama, mekanik destek vb. gibi özel işlevleri yerine getiren bazı dokulara sahiptir. Doğal kimyasalların çoğu damarlı bitkilerin salgı dokuları tarafından üretilir. Salgı dokuları yapı, konum ve salgılanan materyal bakımından çeşitlilik göstermektedir ve bu dokular, ürettikleri materyale göre şu şekilde sınıflandırılırlar: latisiferler, nektarlar, kanallar (sakız ve reçineler), tuz bezleri, hidatodlar, müsilaj salgılayan hücreler, enzim salgılayan hücreler, yağ hücreleri, yağ salgılayan trikomlar, flavonoid salgılayan dokular. Bu farklılaşmış dokular aynı zamanda metabolik yollarında da farklılıklar içerir [43]. Bir grup gen, hücre bölünmesi, farklılaşması vb. gibi farklı işlevleri yerine getirmek için birbirleriyle koordine olur ve bu genlerin ifadesi hücre tipine, bölgeye ve organa özgüdür. Bazı bitki türleri, alkaloitler, terpenler ve glikozitler gibi ikincil ürünlerin sentezi için gerekli biyosentetik gen kümelerine sahiptir [44]. BSM'lerin biyosentezi genellikle belirli bir doku ile sınırlıdır [37]. Latisiferlerde afyon haşhaş lateksinin sentezi ve birikimi bu varsayıma iyi bir örnektir. Morfin, kodein, tebain ve narkotin alkaloitleri büyük ölçüde tam gelişmiş kapsüllerde birikir ve kapsüllerin tüm boyutlarında maksimum düzeyde bulunur. Başlangıçta köklerde bu alkaloitler çok az miktarda bulunur, bu da kapsüllerin çeperinde maksimum latisiferöz hücre ağının bulunması nedeniyle kapsüllerin diğer bitki kısımlarına göre maksimum miktarda alkaloit biriktirdiğini gösterir. Üreme organları, bitkinin vejetatif kısımlarına göre alkaloitlerin çoğunu biriktirir. Afyon haşhaşında tüm bitki hücreleri morfin sentezi genlerini barındırmasına rağmen, bu enzimler sadece birkaç hücre tarafından ifade edilir [45].

Çevresel Faktörler

Bitkiler hayatta kalmak için çevreyle etkileşime girer ve bu nedenle BSM'lerin biyosentezini düzenleyen hem biyotik hem de abiyotik uyarıcılar dahil olmak üzere çeşitli çevresel faktörlerden etkilenir. Farklı ortamlarda yetişen aynı türe ait bitkiler, belirli bir ikincil metabolitin konsantrasyonunda farklılıklara sahip olabilir. [46]. Abiyotik ve biyotik faktörler, bu faktörlerden kaynaklanan olumsuz koşulların bir sonucu olarak bitkilerde abiyotik ve biyotik streslere neden olur. Bitkiler bu streslere karşı koymak için spesifik ikincil metabolitler üretirler. Dolayısıyla, çevresel faktörler, BSM'lerin biyosentezinde belirleyici faktörlerdir. Bitkilerdeki biyotik stres; bakteri, virüs, mantar, parazit gibi diğer canlı organizmaların bitkilere verdiği zararın bir sonucu olarak ortaya çıkarken, abiyotik stresler ise; bitkilerin kalitesini ve verimliliğini etkileyen ışık yoğunluğu, su mevcudiyeti, sıcaklık (sıcak ve soğuk), toprak türü ve bileşimi vb. nedenlerden kaynaklanır [46]. Radyasyon (ışık, UV) ve pestisitler, metaller (Ni, Cd, Co, Cr, Fe, Zn, Mn, vb.), mineraller, oksijen ve ozon gibi gaz halindeki toksinler, kirleticiler, bitki büyüme düzenleyicileri (2,4-D, IAA ve NAA) ve tuzlar gibi kimyasalların neden olduğu stresler de bitkilerde abiyotik strese yol açar [32].

Biyotik Faktörler

Bitkiler, mantarlar, virüsler, bakteriler ve nematodlar gibi çeşitli biyolojik ajanların fiziksel saldırılarına maruz kalır ve bu da biyotik stres olarak bilinen strese neden olur. Bitkiler sesil doğaları nedeniyle kendilerini korumak ve savunmak için hareket edemezler. Ayrıca hayvanlarda olduğu gibi bir bağışıklık sistemine de sahip olmadıkları için, ancak ikincil metabolitler aracılığıyla patojenlere karşı direnç gösterirler. Bazı BSM'ler (fitoaleksinler), bitkilerin patojenlere karşı savunma sistemi olarak işlev gören antimikrobiyal aktivitelere sahiptir [47]. Patojenlere karşı savunma sırasında, bitkilerin yüksek konsantrasyonlarda BSM'lere ihtiyaç duyması, bunların biyosentezini hızlı bir biçimde uyarır. Acı baklada (*Lupinus angustifolius*) *Colletotrichum lupine* tarafından oluşturulan fungal enfeksiyona karşı

fenolikler gibi sekonder metabolitlerin miktarında değişiklikler gözlenir [48]. Bitkiler patojen saldırısına yanıt olarak doğuştan gelen bir bağışıklık sistemi geliştirir ve bu sistem bazal bağışıklık ve efektör tetiklemeli bağışıklık olmak üzere iki mekanizma ile tetiklenir. Bazal bağışıklık sisteminde, mikropla ilişkili moleküler kalıplar, hücre yüzeyindeki bu kalıpları tanıma reseptörleri tarafından algılanırken, efektör tetiklemeli bağışıklıkta ise, mikrobiyal protein veya peptitler, fitotoksinler ve düşük moleküler ağırlıklı doğal ürünlerde olduğu gibi etkilere neden olur. Bu efektörler bitkiler tarafından bir enfeksiyon sinyali olarak ayırt edilir ve bitkinin bu efektörlere karşı tepkisi çeşitli metabolik yolları aktive ederek farklı ikincil metabolitlerin sentezlenmesine ve bitkiyi korumak için patojen saldırısına yanıt olarak yükselen ve daha sonra azalan BSM konsantrasyonuna neden olur [49].

Abiyotik Faktörler

Ontogenez sırasında bitkiler çevreleriyle etkileşime girer ve su, ışık, sıcaklık, toprak ve kimyasallar (mineraller, gübreler) gibi farklı abiyotik bileşenlerle temas ederler. Bitkiler, gelişimsel büyümeleri ve hayatta kalmaları için bu bileşenlere uygun miktarlarda ihtiyaç duyarlar. Bununla birlikte, bu abiyotik bileşenlerin az ya da çok olması bitkilerde strese neden olur ve sonuçta BSM'lerin üretiminde ya da akümülasyonunda değişikliklere yol açar [8].

Kuraklık Stresi

Su, metabolitlerin ve besin maddelerinin bitkinin tüm kısımlarına taşınması için bir ortam olarak işlev gördüğünden, bitkinin fizyolojik faaliyetlerinde anahtar bir moleküldür. Su kaynağı sınırlayıcı hale geldiğinde veya bitkilerde terleme oranı arttığında kuraklık stresine ve tuzluluk stresine neden olur. Kuraklık; bitkilerin su potansiyelini ve turgorunu, normal fizyolojik faaliyetleri etkileyecek derecede düşüren, bitkilere yeterli miktarda su sağlanamamasından kaynaklanır [50]. Kuraklık bitki büyümesini ve fotosentezi etkileyerek bitkinin biyokimyasal özelliklerini değiştirir [51]. Stoma kapanması, membran hasarı ve diğer bozulan enzim aktiviteleri (ATP sentezi), fotosentez oranında düşüşe neden olur. Kuraklıktan kaynaklanan ozmotik stres aynı zamanda tahıl ürünlerinin verimliliğini de etkiler [52]. Çeşitli BSM'ler, bitkilerin bu koşullar altında hayatta kalmalarına yardımcı olur. Kuraklık stresi nedeniyle *Artemisia annua, Hypericum perforatum* ve *Catharanthus roseus* gibi birçok tıbbi bitkide stress nedeniyle BSM üretiminde artış tespit edilmiştir [53].

İki *Adonis* türünün kuraklık stresine tepki mekanizması incelendiğinde hem *A. amurensis* hem de *A. pseudoamurensis*'in kuraklık stresine karşı adaptif değişiklikler gösterdiği bulunmuştur. Fizyolojik düzeyde, su stresi bitki yapraklarının su içeriğini azaltmış ve *Adonis* türleri kuraklık stresine toplam biyokütleyi azaltarak, ozmotik düzenleyicilerin seviyelerini ve antioksidan enzim aktivitelerini artırarak yanıt vermiştir. İkincil metabolitlerden flavonoidler ve toplam fenolik madde miktarı da kuraklığa tepki olarak önemli bir artış göstermiştir [54]. Papatya bitkisinde (*Matricaria chamomilla*) tuzluluk ve kuraklık streslerinin her ikisinin de bitkide uçucu yağ içeriğini, bitki başına düşen dal ve çiçek sayısını azalttığı tespit edilmiştir [55]. Su kıtlığı *Artemisia*'da artemisinin, *Hypericum brasiliense*'de ise betulinik asit, kuersetin ve rutin gibi ikincil metabolitlerin miktarını artırmaktadır. Su stresi, yaprakların fotosentetik oranını ve hiperisin ve psödohiperisin gibi sekonder metabolitlerin konsantrasyonunu düşürürken, ana sekonder metabolit hiperforinin konsantrasyonunu ise arttırmıştır [56]. Su stresi ayrıca tatlı fesleğen (*Ocimum basilicum*) ve Amerikan fesleğeni (*Ocimum americanum*) gibi bitkilerde vejetatif büyümeyi, toplam karbonhidratları, uçucu yağı, prolin, azot (N), fosfor (P), potasyum (K) ve protein içeriğini de etkilemektedir. Su stresi uçucu yağ yüzdesini artırırken N, P, K ve protein içeriğini azaltmaktadır. Her iki tür için de maksimum bitki verimi ve uçucu yağ verimi için tarla kapasitesinin %75'inin suyla muamelesi gereklidir [57]. Bu çalışmalar, suyun bitkilerin fizyolojik faaliyetlerinde önemli bir role sahip olduğunu ve BSM'lerin konsantrasyonunu ve biyosentezini değiştirebileceğini doğrulamıştır.

Tuzluluk Stresi

Toprakta bulunan Na^+ gibi tuz iyonlarının yüksek konsantrasyonu, tuzluluk stresine neden olur ve bu durum bitki dokularında SM birikimini önemli ölçüde etkiler. Topraktaki artan tuz seviyeleri beslenme dengesizliklerine ve hiper-ozmotik strese neden olurken bunun sonucunda bitkilerde fotosentez, büyüme ve besin alımı azalır. Tuz stresinin oluşturduğu hem iyonik hem de ozmotik stres,

bitki SM içeriğini artırabilir veya azaltabilir [58]. *Rauvolfia tetraphylla* ve *C. roseus'ta* sırasıyla reserpin ve vinkristin alkaloitlerinin konsantrasyonu tuz stresi altında artmaktadır. *Ricinus communis* bitkisinde risin alkaloit seviyesinin sürgünlerde arttığı, köklerde ise azaldığı gözlemlenmiştir [59]. *M. chamomilla*'da tuzluluk artışıyla birlikte farklı fenolik asitlerin (protokateşuik, klorojenik ve kafeik asitler) biriktiği görülmüştür. *Nigella sativa ve Mentha pulegium'*da fenollerin artışı tuz stresi koşullarında fark edilmiştir. Sonuç olarak bitkiler tarafından farklı iyonların biriktirilmesi, SM'lerin konsantrasyonunu değiştirebilmektedir. Bu çalışmalar, tuzluluğun BSM'lerin birikimini desteklediğini de açıkça göstermektedir.

Sıcak ve Soğuk Stresi

Bitkilerin büyümesi uygun sıcaklığa bağlıdır. Yüksek ve düşük sıcaklıklar, bitkiler üzerinde sıcak ve soğuk stresi oluşturan olumsuz bir etkiye sahiptir [60]. Yüksek sıcaklık bitkilerde stoma iletkenliğini azaltır ve sonuç olarak fotosentez ve büyümenin azalmasına neden olur. BSM'lerin üretimi de yüksek sıcaklıktan etkilenir. Yüksek sıcaklıkta fotosistem II, fotokimyasal etkinlikte azalma göstererek bitkilerde stresin artmasına neden olur. Bazı çalışmalar, yüksek sıcaklığa yanıt olarak BSM'lerin biyosentezinde artış gösterirken, bazı çalışmalar ikincil metabolitlerde azalma olduğunu göstermiştir [61].

Soğuk stresi, bitkilerin büyümesi ve gelişmesini olumsuz yönde etkileyen ve verimliliği önemli ölçüde kısıtlayan düşük sıcaklığı (< 20^{o}C) ifade eder. Doğrudan metabolik reaksiyonları ve dolaylı olarak da su alımını ve hücresel dehidrasyonu engelleyen, bitkilerin tam genetik potansiyelinin ortaya çıkmasını önler. Düşük sıcaklık aynı zamanda bitkilerde farklı fizyolojik, biyokimyasal ve moleküler değişikliklere neden olur ve bu da onları soğuğa alışma (düşük sıcaklık stresinde hayatta kalma ve yaralanmaya direnme yeteneği) olarak bilinen soğuk stresinde hayatta kalma konusunda daha dirençli hale getirir [62].

Ağır Metal Stresi

Ağır metaller, agroteknoloji ve endüstrinin gelişmesiyle birlikte yüksek biyoakümülasyon ve toksisitelerinin de etkisiyle bitkiler için ana abiyotik stres ajanlarından biri haline gelmiştir [63]. Ağır metallerin bitki büyümesi ve fizyolojisi üzerindeki etkileri hakkında önemli veriler mevcut iken SM üretimi üzerindeki etkileri hakkında daha az veri mevcuttur. Bitkilerin metabolik aktivitesindeki ağır metal kaynaklı değişiklikler şeker, fotosentetik pigment ve protein üretimini etkileyebilir. Bu etkiler, bu doğal ürünlerin üretiminde yer alan enzimlerin inhibisyonu nedeniyle ortaya çıkar [64]. Ağır metaller, ikincil metabolizmanın belirli yönlerini değiştirerek biyoaktif bileşiklerin sentezini de değiştirebilir. Örneğin, Fe, Ag, Ni ve Co gibi metallerin çeşitli bitkilerde SM sentezini tetiklediği bildirilmiştir. Benzer şekilde, *Brassica juncea* üzerinde yapılan bir çalışmada, metallerin (örneğin Fe, Cr, Zn ve Mn) etkili bir şekilde biriktirilmesi, bitkinin yağ içeriğini %35'e kadar önemli ölçüde artırmıştır [65].

Radyasyon Stresi

Işık, bitkiler tarafından fotosentez ve büyüme için gerekli olan ve BSM'lerin birikimini ve kalitesini etkileyen temel bir abiyotik bileşendir. Güneş ışığı *Mikania glomerata*'da kumarin birikimini kolaylaştırmaktadır. Işık periyodunun kısa olması bitkilerin yaprak ve gövdelerindeki kumarin seviyesini azaltırken, uzun ışık periyodu ise kumarin seviyesini önemli ölçüde artırmıştır. Kumarin içeriği yaprak ve gövdelerde fotoperiyottan önemli ölçüde etkilenmiştir [66]. Dolayısıyla, ışık yoğunluğu ve fotoperiyodun BSM'lerin birikimi üzerinde önemli etkileri vardır.

Kimyasal Stres

Bitkiler, BSM'lerin biyosentezi de dahil olmak üzere düzgün büyümeleri için besin maddeleri, gübreler, elisitörler, büyüme düzenleyicileri gibi farklı kimyasallara ihtiyaç duyarlar. Ancak bu kimyasallar uygun konsantrasyonda bulunmadığında kimyasal stres olarak bilinen strese yol açarlar. Kimyasal strese mineraller, ağır metaller, gübreler, kirleticiler, pestisitler, büyüme düzenleyicileri ve elisitörler gibi farklı kimyasallar neden olabilir. Mikrobesinlerin uygulanması, *Cassia angustifolia*'da ikincil metabolitleri artırabilecek birincil metabolitlerde artışa neden olmuş, klorofil, protein ve fenol içerikleri $FeSO_4$, $ZnSO_4$ ve $CuSO_4$ ilavesinden etkilenmiştir [67].

Azot ve fosfor bitki büyümesi ve gelişiminde önemli rol oynayan temel besin faktörleri olmalarının yanı sıra, b+unların miktarları bazı BSM'leri de etkilemektedir. Ayrıca gübre formundaki azot da *Arabidopsis* ve diğer bitki türlerinde olduğu gibi bazı genlerin gen ekspresyonlarının kontrol edilmesinde bitkiler için sinyal görevi görmektedir [68]. Bitkiler, çevredeki farklı konsantrasyonlarda azotun varlığına tepki vermek için çeşitli mekanizmalar geliştirmiştir. Genomik ve biyoinformatik gibi farklı yaklaşımlar, farklı azot konsantrasyonlarına karşı bitki tepkisini içeren düzenleyici yolu ortaya çıkarmak için kullanılır. Azotun mevcudiyetine yanıt olarak bitki adaptasyonları, gen ifadesinde değişikliklere neden olur ve metabolik, fizyolojik ve gelişimsel adaptasyonlar olarak kendini gösterir [68]. Bitkilerde büyümeyi ve SM'leri etkileyen fosfor, *Salvia officinalis*'te yaprak biyokütlesini, toplam fenolik ve rosmarinik asit konsantrasyonlarını arttırırken, uçucu yağ kalitesi ve miktarı üzerinde herhangi bir etkisi bulunmamıştır. Bu nedenle fosfor, bitkilerde büyüme ve SM biyosentezinde önemli bir role sahiptir [69]. SM'ler üzerindeki çeşitli kimyasal etkilere ilişkin bu tür çalışmalar, bitkilerin büyümesi ve gelişmesi için ihtiyaç duyduğu kimyasalların aynı zamanda BSM'lerin biyosentezini de etkilediğini, dolayısıyla BSM'lerin konsantrasyonunun bu kimyasallara tepki olarak değişebileceğini açıkça göstermektedir.

Mevsimsel Varyasyonlar

Bitkilerdeki sekonder metabolitlerin içeriği, genetik kontrolün, gen ifadesinin, genotiplerin [70], biyolojik ve çevresel faktörlerin yanı sıra biyokimyasal, fizyolojik, ekolojik ve evrimsel süreçlerin varlığına rağmen mevsimsel ve günlük varyasyonların yanı sıra tür içi ve türler arası özelliklere bağlı olarak değişebilmektedir. Afyon haşhaşı (*P. somniferum*), morfin, kodein, tebain, narkotin ve papaverin başta olmak üzere 80'den fazla alkaloit açısından zengin bir kaynaktır ve ilaç endüstrisinde yaygın olarak kullanılmaktadır. Bu alkaloitler, farklı gelişim aşamalarında içeriklerinde mevsimsel farklılıklar göstermektedir. Alkaloitin optimum hasat zamanı ve günlük değişim eş zamanlı olarak dikkate alınmalıdır, aksi takdirde ana bileşik dönüşür veya bozulur, geri dönüşü olmayan bir yol izleyen alkaloit biyosentetik yolundaki enzim aktivitesindeki artış veya azalmaya bağlı olabilir, bu nedenle bir kez başka bir bileşiğe dönüştürüldüğünde, önceki bileşik lateksten yok olur [34].

Bölge/Lokasyon Etkileri

Bitkilerdeki SM'lerin biyosentezi çeşitli biyotik ve abiyotik faktörlerden etkilenirken, bunların konsantrasyonları da diğer faktörlerden etkilenir. Konum/bölge, bitki türlerindeki ikincil metabolitlerin içeriğini değiştirebilir ve böylece bitkilerin varsayılan faaliyetlerini kesintiye uğratabilir [71]. Konum ve iklim koşulları *Nothapodytes nimmoniana*'daki kamptotesin (bir monoterpen indol alkaloit) içeriğini etkiler [72]. Farklı bölgelerden elde edilen *Mentha spicata*'da lokasyonların antibakteriyel aktivite üzerindeki etkisi verim farklılıklarına yol açmaktadır. Yüksek rakımlı bölgelerden toplanan bitkilerin antibakteriyel aktiviteleri, antibakteriyel aktivitelerden sorumlu ikincil metabolitlerin daha fazla üretimi nedeniyle daha çok etkilenmektedir. Farklı enlem, boylam, ortalama sıcaklık ve iklim koşullarına sahip farklı ekolojik bölgeler, *C. roseus*'taki antioksidan aktivite ve toplam fenolik bileşikler üzerinde etkilidir [73]. Bu çalışmalar, konumların ve çevresel koşulların BSM'lerin biyosentezi üzerinde kesinlikle etkisi olduğunu açıkça kanıtlamaktadır. Bazı yer/bölge veya iklim koşullarında, bitkiler kendilerini diğer koşullara göre daha iyi adapte etmektedir. Verimli iklim koşulları bitkilerin büyümesini ve gelişmesini desteklemekte, bu da SM'lerin konsantrasyonunun belirlenmesinde önemli bir rol oynamaktadır. Bu nedenle, aynı bitkinin farklı bölgelerdeki SM konsantrasyonları, bitkiler için elverişli veya elverişsiz iklim koşullarının mevcudiyetine bağlı olarak değişebilir. BSM'lerin birikimi veya üretimi, uygun veya uygun olmayan iklim koşullarına göre bitkilerin fizyolojik ihtiyaçlarına da bağlıdır.

SONUÇ VE TARTIŞMA

Bazı bitkiler, nutrasötik, koku, boya, kozmetik ve farmasötik alanlarda hammadde olarak kullanılan kimyasal olarak aktif bileşenlerin zengin kaynaklarıdır. Yaygın olarak SM'ler olarak bilinen bileşenler, çeşitli stres koşullarında bitki tarafından adaptasyon için kullanılır. Biyotik ve abiyotik stresler bitkiyi sadece yapısal ve anatomik olarak değiştirmekle kalmaz, aynı zamanda kimyasal bileşenlerin miktarlarında da dalgalanmalara neden olur. Farklı sekonder metabolitlerin içeriği nesilden

nesile sabit kalmayıp değişiklik gösterir. Bunun birinci sebebi; çeşitli biyotik ve abiyotik faktörlerin sekonder metabolit sentezinde ve düzenlenmesinde önemli bir rol oynadığıdır, ikinci olarak ise sekonder metabolitlerin, birincil metabolitler gibi bitkinin hayati fonksiyonları için mutlak gerekli olmadığıdır.

Bu derlemede, çeşitli genetik, ontogenik, morfogenetik ve çevresel faktörlerin bitki sekonder metabolitlerinin biyosentezi üzerindeki etkileri ve bunların içeriğindeki dalgalanmalar tartışılmıştır. BSM'lerin biyosentezi çeşitli biyotik ve abiyotik faktörlere bağlıdır ve diğer faktörler sabit kalsa da sadece bir faktördeki değişiklik BSM'lerin içeriğini değiştirebilir. Bununla birlikte, farklı stres durumları bitkilerdeki çeşitli SM'lerin içeriğini de seçici olarak değiştirebilir. Birçok farmakolojik özellikle (antioksidan, antiinflamatuvar, antikanser, antimikrobiyal, antidiyabetik, nöroprotektif, antihipertansif vs.) ilişkili olan bitki SM'leri, ilaç keşfi için zengin bir kaynak durumundadır. Doğada yetişen bitkilerin çok fazla sayıda değişkenle baş ederek hayatta kalma yarışında, ürettikleri SM'leri elde etmek masraflı ve zaman alıcı bir işlem olmasının yanında bitki kaynaklarının geleceğini de tehlikeye sokmaktadır. Stres koşulları altında yetişen bitkilerde SM üretiminin artması ve bunların terapötik ilaçların biyolojik keşfindeki potansiyelleri göz önüne alındığında, bitki doku kültürü yöntemleriyle kontrollü şartlarda istenilen SM'lerin biyotik ve abiyotik elisitörler yardımıyla doğaya zarar vermeden büyük miktarlarda üretilmesi önem kazanmaktadır. Sonuç olarak bitkilerde SM sentezinin farklı stresler tarafından değiştirilebileceği örneklerle gösterilmiştir. Bununla birlikte, bitkilerin büyüme ve verimliliğinin iyileştirilmesi için metabolomik, proteomik ve transkriptomik gibi yeni teknikler kullanılarak çoklu çevresel faktörlerin sinerjistik etkisini anlamak için moleküler düzeyde daha fazla araştırmaya ihtiyaç vardır.

YAZAR KATKILARI

Kavram: C.Ö.; Tasarım: C.Ö.; Denetim: C.Ö.; Toplama ve/veya İşleme: C.Ö., E.P.; Analiz ve/veya Yorumlama: C.Ö., E.P.; Literatür Taraması: C.Ö., E.P.; Makalenin Yazılması: C.Ö., E.P.; Kritik İnceleme: C.Ö., E.P.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 48(3): 1264-1280, 2024
Doi: 10.33483/jfpau.1527648

DERLEME MAKALE / REVIEW ARTICLE

DNA METİLASYONUNA NEDEN OLAN MİKOTOKSİNLERİN ELEKTROANALİTİK YÖNTEMLERLE ANALİZİ

ANALYSIS OF MYCOTOXINS CAUSING DNA METHYLATION BY ELECTROANALYTICAL METHODS

Manolya Müjgan GÜRBÜZ[1,2], Tülay ÇOBAN[3]*, Burcu DOĞAN TOPAL[4]

[1]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, 06110, Ankara, Türkiye
[2]Lokman Hekim Üniversitesi, Eczacılık Fakültesi, Analitik Kimya Anabilim Dalı, 06510, Ankara, Türkiye
[3]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, 06560, Ankara, Türkiye
[4]Ankara Üniversitesi, Eczacılık Fakültesi, Analitik Kimya Anabilim Dalı, 06560, Ankara, Türkiye

ÖZ

Amaç: *DNA metilasyonunu etkileyen mikotoksinlerin analizi toksikolojide gıda güvenliği açısından oldukça önemlidir. Mikotoksin maruziyetinin kontrolü ile anlaşılması, gelişmiş gıda işleme teknikleri ve uygun depolama uygulamalarıyla birleştirildiğinde, gıda güvenliğinin artırılmasına olanak verir.*
Sonuç ve Tartışma: *Bu derleme, çeşitli yaygın mikotoksinlerin (aflatoksin B1, okratoksin A, vb.) neden olduğu DNA metilasyonundaki değişiklikleri ve bunların tespiti için kullanılan elektroanalitik yöntemleri özetlemektedir.*
Anahtar Kelimeler: *DNA metilasyonu, elektroanalitik yöntemler, mikotoksin*

ABSTRACT

Objective: *The analysis of mycotoxins affecting DNA methylation is of great importance in toxicology for food safety. Control and understanding of mycotoxin exposure, combined with improved food processing techniques and appropriate storage practices, allows for increased food safety.*
Result and Discussion: *This review summarizes the changes in DNA methylation caused by several common mycotoxins (aflatoxin B1, ochratoxin A, etc.) and the electroanalytical methods used for their detection.*
Keywords: *DNA methylation, electroanalytical method, mycotoxin*

GİRİŞ

Mikotoksinler mantarlar tarafından üretilen toksik kimyasal bileşiklerdir ve çesitli türleri bulunmaktadır. Mikotoksinlerin kanserojen, mutajenik, teratojenik ve nörotoksik etkileri bilinmektedir. Özellikle aflatoksin, okratoksin ve fumonisin gibi spesifik mikotoksinler patojenik rolleri ve karaciğer kanseri gibi hastalıklarla bağlantıları nedeniyle dikkat çekmektedir. Toksikolojik olarak, bu bileşikler ciddi sağlık sorunlarına yol açabilir. Aflatoksinler karaciğer kanseriyle ilişkilendirilirken, okratoksinler böbrek toksisitesine neden olabilir. Fumonisinler DNA hasarına ve kansere yol açabilirken, trikotesenler bağışıklık sistemini baskılar. Bu toksinler düşük seviyelerde bile birikici ve uzun vadeli etkiler gösterir,

* **Sorumlu Yazar / Corresponding Author:** Tülay Çoban
e-posta / e-mail: coban@pharmacy.ankara.edu.tr, **Tel. / Phone:** +903122033120

Gönderilme / Submitted : 03.08.2024
Kabul / Accepted : 12.08.2024
Yayınlanma / Published : 10.09.2024

insan sağlığı üzerinde önemli riskler oluşturur. Maruz kalma süresi ve miktarı gibi faktörler, mikotoksinlerin etkilerini belirler [1-3]

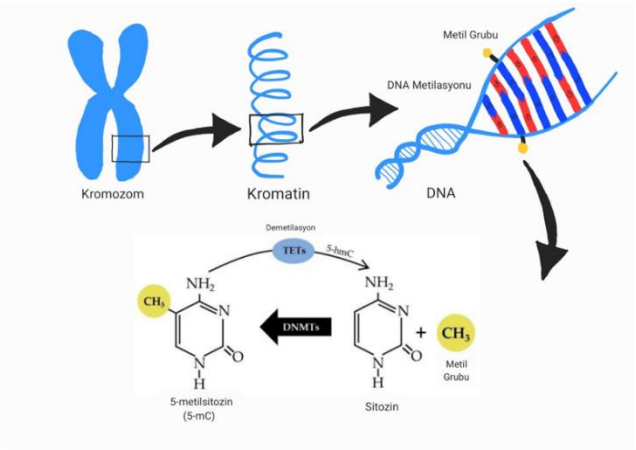
Mikotoksinler, MAPK, JAK2/STAT3 ve Bcl-w/kaspaz-3 ile ilişkili yollar yoluyla hücre döngüsü bozukluklarını, hücre proliferasyonunu, oksidatif stresi ve apoptozu indükleyebilir ve üreme toksisitesi, immünotoksisiteye ve genotoksisiteye neden olabilir. Mikotoksinlerin toksisite mekanizması DNA, RNA ve protein düzeylerinde epigenetik toksisiteye sahiplerdir. Bu mikotoksinlerin çeşitli gıdalardaki varlığı ve tespiti için kullanılan metodolojiler, bunların insan ve hayvan sağlığı üzerindeki potansiyel etkilerinin anlaşılması açısından kritik öneme sahiptir [4].

DNA metilasyonu, sitozin bazlarının metillenmesi yoluyla gerçekleşir ve gen ifadesinin baskılanmasında kritik bir rol oynar (Şekil 1). Bu süreç özellikle, gen promotor bölgelerinde yoğunlaşan CpG adalarında meydana gelir ve bu bölgelerin metilasyon durumu hücre tipine göre değişiklik göstermektedir [5].

DNA'daki sitozin bazlarına metil gruplarının eklenmesi, DNA metiltransferaz enzimleri tarafından gerçekleştirilir ve bu süreç, genlerin ifadesini düzenleyerek hücresel işlevleri doğrudan etkiler. Metilasyon, hücrelerin çevresel etkilere ve iç sinyallere nasıl tepki verdiğini belirler ve hücresel hafızanın bir parçası olarak işlev görür. Bu dinamik süreç, gelişim boyunca de novo DNA metilasyonunu ve demetilasyonu içerir. Sonuç olarak farklılaşmış hücreler dokuya özgü gen transkripsiyonunu düzenleyen stabil ve benzersiz bir DNA metilasyon modeli geliştirir [6].

Memeli genomlarında DNA metilasyon deseni bimodaldır; çoğu genom metillenmişken, CpG adaları olarak adlandırılan kısa DNA dizileri genellikle metilasyondan korunur. Metilasyon, genellikle baskılanmış bir kromatin durumu ve promotör aktivitesinin inhibisyonu ile ilişkilendirilir. Bu süreç, transkripsiyon faktörlerinin bağlanmasını engelleyerek veya kromatin yapısını etkileyerek gerçekleşir. DNA metilasyonu, memeli gelişimi için hayati öneme sahiptir ve çeşitli metiltransferaz eksikliği olan farelerde embriyonik ölümle sonuçlanır. Ayrıca, genomik imprinting ve X kromozomu inaktivasyonunda da doğrudan etkili olduğu görülmüştür [7].

Epigenetik, DNA dizisindeki değişikliklerle açıklanamayan gen fonksiyonundaki kalıtılabilir değişiklikleri inceleyen bir bilim dalıdır. DNA metilasyonu gibi epigenetik sistemler, hayvan gelişimini etkileyen ve kalıtılabilir özellik gösteren mekanizmalar arasında yer alır. Özellikle, DNA metilasyonunun embriyoda zaten baskılanmış olan genleri etkileyebileceği ve embriyonik transkripsiyonun DNA metilasyon makinesinin dışlanmasına yol açabileceği tartışılmaktadır. Bu süreçlerin anlaşılması, hastalıkların tedavisinde ve sağlık bilimlerinde yeni yollar açabilir [8].



Şekil 1. 5-mC için metilasyon ve demetilasyon şeması

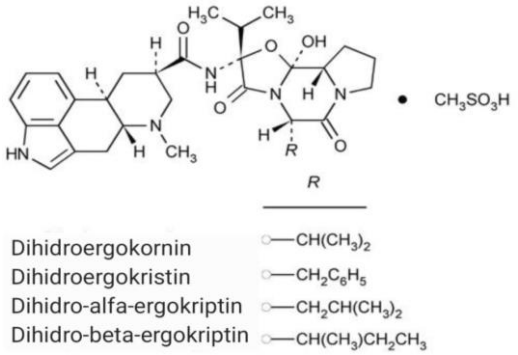
Mikotoksinler, mantarlar tarafından üretilen toksik kimyasal bileşikler olup, yüzlerce çeşidi bulunmaktadır. Bunlar, mantarın vücut yapısına doğrudan katkıda bulunmazlar; ancak, ekolojik nişlerinde rekabet avantajı sağlamak, bitki savunmalarına karşı koymak veya böcek öldürücü olarak işlev görmek gibi çeşitli amaçlarla üretilirler. Mikotoksinlerin bazıları, penisilin gibi antibiyotik olarak kullanılırken, aflotoksinler gibi bazıları ise güçlü kanserojenlerdir. Tarım ürünlerinde yıllardır var olan

bu toksinler, yıl ve bölgeye göre kontaminasyon ve problem seviyesi açısından farklılık gösterir [9].

Mikotoksinlerin yol açtığı sağlık sorunlarının şiddeti, maruz kalınan mikotoksin türüne, miktarına ve süresine, bireyin yaşı, sağlığı ve cinsiyetine, ayrıca genetik, diyet durumu ve diğer toksik etmenlerle olan karmaşık etkileşimlere bağlıdır. Mikotoksin zehirlenmesi, vitamin eksikliği, kalori yoksunluğu, alkol kötüye kullanımı ve enfeksiyon hastalığı gibi faktörlerle kötüleşebilir. Ayrıca, mikrobiyal hastalıklara karşı hassasiyeti artırabilir ve malnütrisyon etkilerini şiddetlendirebilir [1].

Mikotoksinlerin kanserojen, mutajenik, teratojenik ve nörotoksik etkileri bilinmektedir. Özellikle aflotoksinler, okratoksinler ve fumonisinler gibi spesifik mikotoksinler, hastalık yapıcı rolleri ve karaciğer kanseri gibi hastalıklarla olan bağlantılarıyla dikkat çeker. Bu mikotoksinlerin çeşitli yiyeceklerdeki varlığı ve tespiti için kullanılan metodolojiler, insan ve hayvan sağlığı üzerindeki potansiyel etkilerinin anlaşılmasında kritik öneme sahiptir [10].

Ergot, Claviceps purpurea tarafından üretilen iki genel alkaloid biyomolekül sınıfı içerir: amin alkaloidler ve amino asit alkaloidler. Ergot alkaloidleri, tahıllarda büyüyen zehirli bir mantar olan C. purpurea'dan elde edilir. Amin alkaloidleri serotonin reseptörlerinin antagonistidir, amino asit alkaloidleri ise serotonin reseptörleri için daha az seçicidir ve diğer monoamin reseptörlerinde etki gösterir. Amin alkaloidlerinin bilişsel güçlendirici özellikleri vardır. Ergot'taki biyomoleküller arasında, dört alkaloid içeren ergotoksin türevleri (ergoloid mesilatlar): ergokristin, ergokornin, x-ergokriptin ve B-ergokriptin bulunur (Şekil 2). Hiderjin, ergotoksinin dört dihidro türevini içerir. Bu biyomoleküllerin her biri çeşitli farmakolojik aktivitelere sahiptir ve birlikte hydergine etkisini üretmek için sinerjik olarak hareket ederler [11].



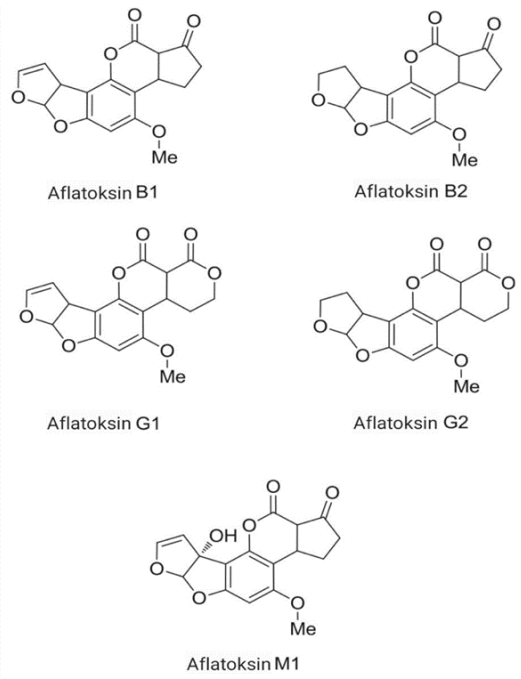
Şekil 2. Ergoloid mesilat yapılarının moleküler gösterimi [12]

Aflatoksinler, Aspergillus ailesinin çeşitli türleri tarafından üretilen mikotoksinlerdir. İnsan ve hayvan gıdalarında, büyüme sırasında ve genellikle hasat sonrası depolama sürecinde mantar kontaminasyonu sonucu bulunurlar. Aflatoksinlerin insanlarda etkileri artık şüphe götürmez ve gıdalardaki aflatoksinler için yasal sınırlar oldukça düşüktür. Aflatoksinler en yaygın olarak yer fıstığı, kuru meyve, ağaç yemişleri (badem, ceviz, Antep fıstığı ve Brezilya fıstığı gibi), baharatlar, incir, ham bitkisel yağlar (yer fıstığı yağı, hindistancevizi yağı), kakao çekirdekleri ve en önemlisi mısır, pirinç, pamuk tohumu ve kopra gibi çeşitli tarımsal ürünlerle ilişkilidir. Yağlı tohumlarda ve bitkisel yağlarda bulunabilecek aflatoksinler B1, G1, B2 ve G2 olup, B1 ve G1 en yaygın olanlarıdır (Şekil 3). Genel olarak, tohumlar, yer fıstığı ve kopradaki aflatoksinlerin %10'undan fazlası, presleme ve ekstraksiyon sonrasında çıkarılan yağa geçmez, çünkü aflatoksinler ağırlıklı olarak proteine bağlıdır.

Okratoksinler, Aspergillus ve Penicillium cinslerine ait mantarlar tarafından üretilen mikotoksinlerdir. En yaygın olanı Okratoksin A (OTA) (Şekil 4) olup, diğer formları Okratoksin B (OTB) ve Okratoksin C (OTC) bulunur. OTA, hem insan hem de hayvan sağlığı için ciddi riskler taşır ve bu nedenle kapsamlı bir şekilde araştırılmıştır. OTA, yiyeceklerde ve özellikle tahıllarda bulunabilir ve insan sağlığı üzerinde çeşitli zararlı etkilere sahiptir [14].

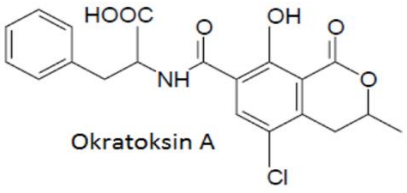
OTA'nın sağlık üzerindeki zararları arasında nefrotoksisite ön plana çıkar. Bu toksin, böbrek hücrelerine zarar vererek, kronik böbrek hastalıklarının riskini artırabilir. Bu etkisi, özellikle Balkan endemik nefropatisi (BEN) ile bağlantılıdır, bu hastalık böbrek yetmezliğine kadar ilerleyebilen kronik bir durumdur. Bunun yanı sıra, OTA'nın kanserojen etkileri de bilinmektedir. DNA hasarına yol açarak

ve oksidatif stresi artırarak çeşitli kanser türlerinin gelişimine katkıda bulunabilir. Bu toksin, aynı zamanda immün baskılayıcı özelliklere sahip olup, vücudun enfeksiyonlara karşı savunma mekanizmalarını zayıflatabilir, bu da maruz kalınan bireylerde enfeksiyon riskini artırabilir [15].



Şekil 3. Aflatoksin B1, B2, G1, G2 ve M1 yapılarının moleküler gösterimi [13]

OTA, genotoksik etkilere de sahiptir; hücre döngüsünü durdurabilir ve apoptoza (programlanmış hücre ölümü) yol açabilir. Bu etkileriyle genetik stabiliteyi bozarak hastalıkların temelinde yatan mekanizmalara müdahale edebilir. OTA'nın bu zararlı etkileri, protein sentezinin inhibisyonu, enerji üretiminin baskılanması, oksidatif stresin indüklenmesi ve DNA aduktlarının oluşumu gibi çeşitli biyokimyasal mekanizmalarla gerçekleşir. Ayrıca, OTA, insan ve hayvan albüminine güçlü bir şekilde bağlanarak bu etkilerini artırır [16].



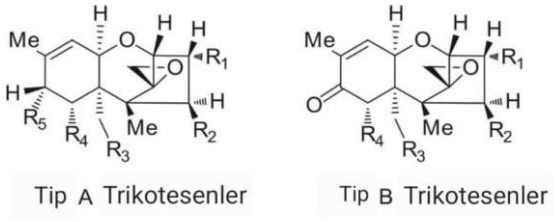
Şekil 4. Okratoksin A (OTA) için moleküler gösterim [15]

Böyle geniş çaplı ve ciddi sağlık sorunlarına yol açabilen OTA, gıdaların uygun olmayan depolama koşullarında oluşabilir ve uzun süreli maruziyetler son derece tehlikeli sonuçlar doğurabilir. Bu nedenle, OTA kontaminasyonunu önlemek ve gıdaların güvenliğini artırmak için çeşitli yöntemler araştırılmaktadır. Özellikle, uygun depolama pratikleri ve gelişmiş gıda işleme teknikleri, OTA'nın gıdalarda oluşumunu ve birikimini azaltmada kritik rol oynar [16].

Trikotesen mikotoksinler, özellikle Fusarium, Stachybotrys, Myrothecium, Trichothecium ve diğer bazı mantar cinsleri tarafından belirli iklim koşullarında üretilen seskiterpenoid bileşiklerdir. Bu mikotoksinler, genellikle tahıl tanelerini enfekte eden Fusarium mantarı tarafından üretilir ve deoksinivalenol (veya vomitoksin), nivalenol, T-2 toksin, HT-2 toksin ve diacetoksiskirpenol gibi çeşitli formlar içerir. Monogastrik hayvanlar bu toksinlere özellikle hassas olup, özellikle genç domuzlar yüksek duyarlılık gösterirken, kümes hayvanları ve ruminantlar, gastrointestinal sistemdeki mikrobiyal

metabolizma yoluyla bazı trikotesenlere karşı daha az duyarlıdır [17].

Trikotesenlerin kimyasal yapısı (Şekil 5), özellikle 12, 13-epoksi halka yapısına sahiptir ve bu yapı, biyolojik aktivite ve toksisite için kritik öneme sahiptir. Yan zincirlerin yapısı ve pozisyonu gibi özellikler de biyolojik etkinlikleri üzerinde etkilidir. Kimyasal sınıflandırmaya göre, trikotesenler Tip A, Tip B, Tip C ve Tip D olmak üzere dört gruba ayrılır. Tip A trikotesenleri, aralarında T-2 toksin ve onun metaboliti HT-2 toksinin de bulunduğu en toksik trikotesenler arasındadır. Bu toksinlerin üretimi, amino asit sentezi ve yağ asidi metabolizması gibi biyolojik yollarla benzerlik gösterir. Bazı trikotesenler, özellikle makrosiklik yapıdaki olanlar, havada taşınabilir ve bu durum "hastalıklı bina sendromları" ile ilişkilendirilebilir [17].



		R1	R2	R3	R4	R5
Tip A	T-2 toksin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
	HT-2 toksin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
	Diasetoksiskirpenol (DAS)	OH	OAc	OAc	H	H
	Neosolaniol (NEO)	OH	OAc	OAc	H	OH
Tip B	Nivalenol (NİV)	OH	OH	OH	OH	-
	Deoksinivalenol (DON)	OH	H	OH	OH	-
	Fusarenon X (FUSX)	OH	OAc	OH	OH	-
	15-Asetil-deoksinivalenol (15-as-DON)	OH	H	OAc	OH	-

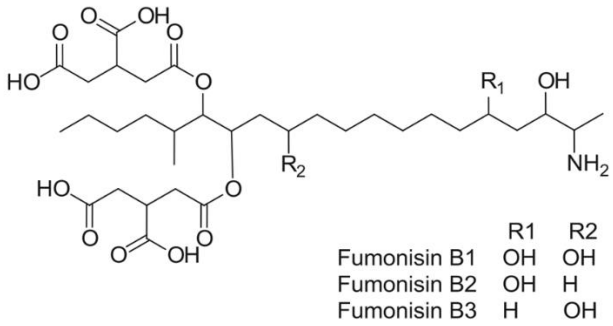
Şekil 5. Tip A ve Tip B Trikotesenler için moleküler gösterimleri

Fumonisinler (Şekil 6), ağırlıklı olarak *Fusarium verticillioides* ve *F. proliferatum* tarafından üretilen mikotoksinlerdir. Bu toksinler dünya genelinde mısır ve mısır bazlı gıdalarda bulunur. Fumonisin B1 en yaygın olanıdır ve laboratuvar ile çiftlik hayvanlarında türüne özgü toksisiteler gösterir, bunlar arasında kemirgenlerde karaciğer ve böbrek kanseri yer alır. Fumonisinlerin toksikolojik ve kanserojen etkilerinin altında yatan genotoksik olmayan mekanizma, seramid sentazın inhibisyonu ve sfingolipid metabolizmasının bozulmasıdır. Fumonisin B1 veya diğer fumonisinlerin insan sağlığı üzerindeki etkisi tam olarak anlaşılamamış olmasına rağmen, epidemiyolojik ve deneysel kanıtlar, bu toksinlerin büyük miktarlarda kontamine mısır bazlı gıdalar tüketen popülasyonlarda özofagus kanseri ve nöral tüp defektleri için bir risk faktörü olduğunu göstermektedir. Bu konuda yapılan seçilmiş toksikolojik araştırmalar, insan maruziyetini ve potansiyel riski daha iyi anlamak için temel oluşturan bulgular sunar. Bu araştırmalar, sıçanlarda böbrek toksisitesinin biyoyas olarak kullanılmasını ve alkali pişirmenin (masa ve tortilla yapımında kullanılan geleneksel yöntem olan nixtamalizasyon) ve ekstrüzyonun fumonisinle kontamine olmuş mısırın toksisitesini etkili bir şekilde azalttığını göstermektedir. Ayrıca, epidemiyolojik çalışmalarda kullanılmak üzere sağlam maruziyet biyobelirteçlerinin geliştirilmesi de bu kapsamda yer alır. Gelecekteki girişimler, fumonisinler ve insan sağlığı arasındaki ilişkiyi daha iyi anlamak için idrarda fumonisin B1 konsantrasyonu gibi biyobelirteçlerin doğrulanmasına ve hangi hayvan modellerinin insanlar için en uygun olduğunu belirlemek için karşılaştırmalı çalışmalara odaklanmalıdır [18].

Elektroanalitik yöntemler, analitik kimyada kullanılan güçlü tekniklerdir ve özellikle farmasötik, çevresel ve gıda güvenliği alanlarında etkili bir şekilde kullanılmaktadır. Bu yöntemler, yüksek hassasiyet, hız ve seçicilik sağlayarak çeşitli bileşiklerin tespiti ve analizinde önemli rol oynar. Elektrokimyasal hücre içindeki reaksiyonları izleyerek analitler hakkında değerli bilgiler sunan bu teknikler arasında voltametri, potansiyometri ve impedans spektroskopisi bulunur.

Voltametri, çözelti içindeki analitlerin redoks davranışlarını çalışmak için kullanılan en yaygın

elektroanalitik tekniklerden biridir ve dönüşümlü voltametri (DV), doğrusal taramalı voltametri (DTV), diferansiyel puls voltametri (DPV) ve kare dalga voltametri (KDV) gibi çeşitli formları içerir. Potansiyometri, bir elektrokimyasal hücre içindeki potansiyel farkı ölçerek analit konsantrasyonunu belirler ve özellikle iyon seçici elektrotlar (ISE) ile sıklıkla kullanılır. Elektrokimyasal impedans spektroskopisi (EİS), elektrot yüzeyindeki elektrokimyasal süreçlerin kinetik ve termodinamik özelliklerini incelemek için kullanılır [20].



Şekil 6. Fumonisin B1, B2 ve B3 için moleküler gösterimi [19]

Elektroanalitik yöntemlerin avantajları arasında, düşük konsantrasyonlardaki analitleri bile tespit edebilme kapasitesi, analiz için gereken zamanın minimize edilmesi ve düşük maliyetleri yer alır. Ayrıca, biyolojik sıvılar, çeşitli çevresel örnekler ve gıdalar gibi çeşitli matrislerde uygulanabilir olmaları, bu teknikleri oldukça esnek kılar. Gelişmiş puls yöntemlerinin geliştirilmesiyle, elektrokimyasal çalışmalar özellikle ilaç analizlerinde ve biyolojik örneklerde daha düzenli olarak kullanılmaya başlanmıştır. Bu teknikler, ilaçla ilgili birçok problemi yüksek doğruluk, hassasiyet ve seçicilikle çözebilir. Ayrıca, katı veya cıva bazlı elektrotlar gibi farklı çalışma elektrotları kullanılarak, bu yöntemlerin uygulama alanları genişletilebilir. Katı elektrotlar, mekanik stabilite ve daha geniş anot aralığı sağladığı için tercih edilir ve akış akımlarına kolayca uygulanabilirler [21].

Bu elektroanalitik yöntemlerin farmasötik, çevresel ve gıda güvenliği alanlarındaki uygulamaları, analitik kimya alanında devamlı bir ilerleme sağlamakta ve bu tekniklerin değerini artırmaktadır [22].

DNA metilasyonuna neden olan mikotoksinler arasında Aflatoksinler, Okratoksin A (OTA), fumonisinler ve trikotesenler bulunur. Aflatoksinler, özellikle Aflatoksin B1 (AFB1), Aspergillus flavus ve Aspergillus parasiticus tarafından üretilir ve genellikle yer fıstığı, mısır gibi tahıllarda bulunur. AFB1, güçlü kanserojen özellikleriyle bilinir ve DNA metilasyonundaki değişikliklerle ilişkilendirilir, böylece p53 tümör baskılayıcı geninde mutasyonlara neden olabilir. Araştırmalar, Aflatoksinlerin gen ekspresyonunu baskıladığını ve karaciğer kanseri riskini artırdığını göstermiştir [23-25].

Okratoksin A (OTA), Aspergillus ochraceus ve Penicillium verrucosum tarafından üretilir ve böbreklerde ciddi toksik etkilere neden olabilir. OTA'nın DNA metilasyonunu değiştirerek kanserojen etkiler gösterdiği ve hücre ölümüne yol açarak gen ekspresyonunu değiştirdiği bilinmektedir. OTA'nın böbrek ve karaciğer toksisitesi, DNA hasarına neden olarak ve oksidatif stresi artırarak gerçekleşir [1].

Fumonisinler, özellikle Fusarium verticillioides ve Fusarium proliferatum tarafından üretilen Fumonisin B1 (FB1), sfingolipid metabolizmasını bozarak DNA metilasyonunda değişikliklere yol açar. Fumonisinler, özellikle mısır ve mısır bazlı ürünlerde bulunur ve genotoksik olmamakla birlikte, epigenetik mekanizmalar üzerinden kanserojen etkiler gösterebilir. Araştırmalar, FB1'in DNA metilasyonunu değiştirerek gen ekspresyonunu bozduğunu ve böylece karaciğer kanseri gibi hastalıklara yol açabileceğini göstermektedir [1].

Trikotesenlerin DNA metilasyonu üzerindeki etkileri, gen ifadesinin düzenlenmesi ve çeşitli hastalıkların gelişimi ile ilgili olabilir. Özellikle T-2 toksin ve diğer Trikotesenler, hücre içi sinyal yollarını etkileyerek DNA metilasyon desenlerini değiştirebilir. Bu değişiklikler, özellikle uzun vadeli maruziyet durumlarında, genetik ifade düzeylerinde ve hücresel fonksiyonlarda kalıcı değişikliklere yol açabilir. Trikotesenler, ribozomları hedef alarak protein sentezini inhibe eder ve bu süreç hücresel stres

yanıtlarını tetikleyebilir. Bu stres yanıtları arasında reaktif oksijen türlerinin artışı ve DNA'ya zarar verme potansiyeli bulunur. DNA hasarı, hücrenin epigenetik düzenleme mekanizmalarını etkileyebilir ve bu da DNA metilasyon desenlerinde değişikliklere yol açabilir. Ayrıca, Trikotesenler doğrudan DNA metiltransferaz enzimlerini etkileyerek DNA metilasyon süreçlerini modüle edebilir [26].

DNA Metilasyonuna Neden Olan Mikotoksinler ile Yapılmış Elektrokimyasal Çalışmalar

Aflatoksinler

Kunene ve arkadaşları tarafından yürütülen bir çalışmada, anti-AFB1 antikoru-sığır serum albümini (SSA) konjügatının L-sistein (L-Sis) katmanı kullanılarak palladyum nanopartikül-bor nitrür (PdNP-BN) modifiye karbon keçe (KK) elektroda bağlanması suretiyle bir Aflatoksin B1 (AFB1) elektrokimyasal immünosensörünün üretimini tanımlamaktadır. İmmünosensörün her bir üretim adımı, DV ve elektrokimyasal impedans spektroskopisi (EİS) kullanılarak karakterize edilmiştir. Bu koşullarda, üretilen immünosensör için 1-10 ng ml^{-1} ($R^2 = 0.9987$) arası doğrusal aralık ve 0,834 ng ml^{-1} gibi düşük teşhis sınırı bulunmuştur. Üretilen elektrokimyasal immünosensör, şarap örneklerinde %93-106 arasında bir geri kazanım seviyesi ile AFB1 tespit edebilmiştir [27].

Bir diğer çalışmada, AFB1'e özgü aptamer ile işlevselleştirilmiş dikey hizalanmış gözenekli silika film (DGSF) nanokanallarından redoks probu ($Ru(NH_3)_6^{3+}$)'nun AFB1 kontrollü difüzyonuna dayalı olarak AFB1 tespiti için oldukça özgül bir elektrokimyasal aptasensör geliştirilmiştir. Önerilen bu elektrokimyasal aptasensör, 3 pg/ml ile 3 µg/ml aralığında doğrusal aralık göstermiştir ve AFB1 tespiti için düşük bir teşhis sınırı olan 2.3 pg/ml'e sahiptir. Yapılan elektrokimyasal aptasensör ile yer fıstığı ve mısır örneklerinde de tatmin edici sonuçlarla pratik analizler gerçekleştirilmiştir [28].

Baruah ve arkadaşları tarafından yürütülen bir çalışmada AFB1 tespiti için iletken polimer kompozit elektrot olan Poli (3,4-etilendioksitiyofen): polistiren sülfonik asit (PEDOT-PSS) tabanlı, sahada kullanılabilir, düşük maliyetli ve doğrudan elektrokimyasal bir sensör üzerinde çalışılmıştır ve en belirgin tekniği seçmek için, geçici kapasitans ve DPV olmak üzere iki farklı elektroanalitik teknik karşılaştırılmıştır. Geçici kapasitans tekniği kullanılarak 18.18–300.0 ng ml^{-1} aralığında doğrusallık ve 55.41 ng ml^{-1} (369 pM) teşhis sınırı elde edilmiştir. DPV tekniği ile de 18.18–342.85 ng ml^{-1} aralığında doğrusallık ve 435 pM teşhis sınırı göstermiştir. Bu sensörün en az örnek hazırlığı kullanılarak doğrudan uygulamasının gösterimi için, AFB1 tespiti beyaz düğme mantarları ve oda koşullarında saklanan bamya kullanılarak başarıyla onaylanmıştır. Gerçek örneklerle sensör yanıtı, sensörün depolanan çiftlik ürünlerini kolayca izlemek için kullanışlı olduğunu önermektedir [29].

Ong ve arkadaşları tarafından yürütülen bir çalışmada AFB1 tespiti amacıyla yüzey modifikasyonu yapılmış bir perde baskılı karbon elektrot (PBKE) üzerinden bir polianilin (PAni) destek matrisi bazlı impedimetrik aptasensör geliştirilmiştir. PAni bazlı aptasensörün üretim prosedürü, DV ve EİS yöntemleri ile karakterize edilmiştir. İmpedimetrik aptasensör, EİS tekniği kullanılarak optimize edilmiştir ve gerçek örnek matrislerinde AFB1 tespit etme yeteneği, iyi bir geri kazanım yüzdesi (87.9% ile 94.7% arası) ile ceviz, tarçın, karanfil, mısır ve soya fasulyesi gibi gıdalar ve yemlerde yapılan bir geri kazanım çalışması ile değerlendirilmiştir. Doğrusal aralık 3×10^{-2} ile 8×10^{-2} nM (R^2=0.9991) ve teşhis sınırı 0.01 nM olarak elde edilmiştir [30].

Shi ve arkadaşlarının 2020 yılında yürütmüş olduğu bir çalışmada, hibrit 4-aminobenzoik asit-indirgenmiş grafen oksit (PABA-iGO) nanokompozitleri ile Au^{3+} iyonları, aşırı PABA varlığında Au nanopartiküllere (AuNP'ler) indirgenerek Au-poli PABA (PPABA)-r-GO nanohibritlerini oluşturup bu yapı nanosensör olarak kullanılarak AFB1 antikorlarını kovalent olarak bağlayarak elektrokimyasal immünosensör hazırlanmıştır. $Fe(CN)_6^{3-/4-}$ kullanılarak impedans sinyali üzerinden sonuçlar elde edilmiştir. 0.01 ile 1 ng ml^{-1} ve 1 ile 25 ng ml^{-1} arasında iyi bir doğrusal aralık sergilemiştir ve teşhis sınırı 0.001 ng ml^{-1} (S/N = 3) olarak belirlenmiştir. Ayrıca bu immünosensör, bitkisel yağ örneklerinde AFB1'i başarılı bir şekilde tespit etmiştir [31].

Bir diğer çalışmada, çok kristalli altın nanopartiküllerle oluşturulmuş karboksilatlı grafen oksit (Au-COOH-GO) nanohibritler hazırlanmış ve AFB1 tespiti için elektrokimyasal immünosensör üretiminde kullanılmıştır. İmmün elektrot tasarımının her adımı, DV, EİS ve KDV teknikleri kullanılarak değerlendirilmiştir. Uygun koşullar altında, AFB1 için teşhis sınırı 0,05 ng ml^{-1} (S/N=3) olup, doğrusal AFB1 konsantrasyon aralığı 0,05 ile 25 ng ml^{-1} arasında değişmektedir. Gerçek pirinç

örneklerinde AFB1'i başarıyla tespit edebilmiştir [32].

Ben Abdallah ve arkadaşlarının 2019 yılında yürütmüş olduğu bir çalışmada, AFB1 tespiti için bir perde baskılı elektrot (PBE) kullanılarak elektrokimyasal immünosensör geliştirilip karakterize edilmiştir. Bu optimize edilmiş sensör, 50 fg/ml'den 5 ng/ml'ye kadar geniş bir doğrusal aralıkta AFB1 tespiti sağlamıştır ve yaklaşık 50 fg/ml teşhis sınırı elde edilmiştir [33].

Bir diğer çalışmada, AFB1 tespiti için manganez oksit nanopartiküller (Mn_2O_3np) bazlı bir elektrokimyasal immünosensör sunmaktadır. Bu immünoelektrotun AFB1'e karşı verdiği yanıt, 1 pg ml^{-1} ile 10 µg ml^{-1} arasında doğrusal aralığında ölçülmüştür ve sensör, 0.54 pg ml^{-1} ile en düşük teşhis sınırı göstermiştir. Mısır ekstresi içeren bir örneğin doğrusal aralıkta (1 pg ml^{-1} ile 10 µg ml^{-1}) yanıtı incelenmiş ve %98.6 geri kazanım oranı göstermiştir [34].

Süt ürünlerinde AFB1 tespiti için yürütülen bir çalışmada dispense baskılı elektrotlar kullanılarak esnek biyosensörler üretilmiş ve ardından tek duvarlı karbon nanotüpler (TDKNT'ler) ile fonksiyonelleştirilmiş ve duyarlılıklarını artırmak için spesifik antikorlarla kaplanmıştır. Daha sonra, immünosensör, bir tampon çözeltisinde ve doplanmış süt örneğinde AFM1'in tespiti için kronoamperometrik teknik kullanılarak test edilmiştir. Sonuçlar, sensörlerin çalışma aralığının tampon ve doplanmış süt örneğinde minimum 0.01 µg/l ve maksimum 1 µg/l olduğunu göstermiştir. TDKNT fonksiyonelleştirilmiş sensörün teşhis sınırı 0.02 µg/l bulunmuştur [35].

Zhang ve arkadaşlarının yürütmüş olduğu bir çalışmada tarım ürünlerinde AFB1 tespiti için ultra hassas bir elektrokimyasal immünosensör geliştirilmiştir. Ferrosen (Fs) ve çok duvarlı karbon nanotüpler (ÇDKNT'ler) iyi katalitik aktiviteye, kimyasal stabiliteye ve elektronik taşıma özelliklerine sahiptir. Fs, ÇDKNT'ler ve KS aracılığıyla PBKE yüzeyine sabitlenmiş ve ardından Fs@ÇDKNT nanoyapısı ile Fs/ÇDKNT/KS modifiye edilmiştir. Uygun koşullar altında, önerilen DV ve DPV kullanarak $1x10^{-3}$ ile 2×10^{4} ng/ml geniş bir doğrusal aralık ve 0.159 pg/ml düşük AFB1 teşhis sınırı elde etmiştir [36].

Bir diğer çalışmada, AFB1'in indirgenmesi yoluyla bizmut film elektrotlar kullanarak AFB1'in miktarını belirlemek için DPV tekniğinin geliştirilmesini ve optimize edilmesini önermektedir. Teşhis sınırı ve tayin alt sınırı sırasıyla 11.2 ng l^{-1} ve 37.3 ng l^{-1} olarak bulunmuştur. AFB1'in 38.46 ng l^{-1} çözeltisi kullanılarak yapılan ölçümlerde, %BSS değerleri gün içi ve günler arası sırasıyla %5.3 ve %4.6 olmuştur [37].

AFB1 tespiti için Wang ve arkadaşlarının 2019 yılında yürütmüş olduğu bir çalışmada, 3′-ucunda metilen mavisi (MM) redoks etiketi bulunan kısa bir anti- AFB1 aptameri, altın bir elektrotun yüzeyine immobilize edilmiştir ve AFB1'in yokluğunda, komplementer bir DNA (kDNA) zinciri, MM işaretli aptamerle hibritleşerek aptasensör oluşturulmuştur. Optimum koşullar altında, bu sinyal-açma elektrokimyasal aptasensörü kullanarak, 2 nM–4 µM doğrusal aralığında AFB1 tespiti gerçekleştirilmiştir. Bu sensör, 20 kat seyreltilmiş bira ve 50 kat seyreltilmiş beyaz şarapta AFB1 tespiti yapılmasını sağlamıştır [38].

Bir diğer çalışmada, grafen kuantum noktaları (GKN'lar) ve Au NP'ler ile hazırlanmış bir bileşik kullanan AFB1 için elektrokimyasal bir immünosensör tanımlanmıştır ve AFB1 karşı bir antikor ile modifiye edilmiştir. Elektrokimyasal prob olarak heksasiyanoferat kullanarak, sensör 0.1 ile 3.0 ng ml^{-1} AFB1 konsantrasyon aralığında ölçüm yapabilmiştir. Biyosensör, mısır örneklerinin (doplanmış) analizinde uygulanmıştır [39].

Kulikova ve arkadaşlarının yürütmüş olduğu bir çalışmada AFB1 tespiti için seçici DNA aptameri, CKE üzerine elektrokaplanmış iki ince PAni tabakası arasına yerleştirildi. DTV ve EİS yöntemleri kullanılmıştır. Elde edilen bulgulara göre DTV ve EİS yöntemlerinin ikisi için de doğrusal aralık 3 - 90 ng/l arasında bulunurken teşhis sınırı ise srasıyla 1 ng/l ve 5 ng/l olarak bulunmuştur. Süt örneklerinde 20 ng/l düzeyinde güvenilir bir şekilde uygulanabilirliği kanıtlanmıştır [40].

Bu çalışmada, AuNP ve GKN kullanımına dayalı bir nanokompozitin kolay bir sentezini PBE üzerine modifiye ederek AFB1 tayini için bir nanosensör geliştirilmiştir. Optimize edilmiş koşullar altında, 1.0–50.0 nmol l^{-1} doğrusal aralık ve sırasıyla 0,47 ve 1,5 nmol l^{-1} teşhis ve tayin alt sınırı değerlerine ulaşıldı. Maltlanmış arpa numunelerinde de gerekli analizler yapılmış ve geri kazanım değerleri %76-103 aralığında elde edilmiştir [41].

Wang ve arkadaşlarının 2014 yılında yürütmüş olduğu bir çalışmada AFB1 tayini için ÇDKNT'ler, Au/Pt bimetalik nanopartiküllerin (Au/PtNP'ler) ve ince bir baskılı filmin kademeli olarak

değiştirilmesiyle CKE üzerine modifiye edilerek elektrokimyasal sensör elde edildi. DV, DPV ve EİS yöntemleri kullanılarak yapılan bu çalışmada doğrusal aralık 1×10^{-10} ile 1×10^{-5} mol l^{-1} arasında bulunurken teşhis sınırı ise 0.03 nmol l^{-1} olarak bulunmuştur [42].

Okratoksinler

Gökçe ve arkadaşlarının yürütmüş olduğu bir çalışmada, OTA'nın analizi için kalem grafit elektrot (KGE) bazlı yeni bir impedimetrik aptasensör tanımlanmıştır. Uygun maliyeti ve yüksek aktif yüzey alanının avantajlarını kullanarak, KGE, OTA'nın hassas tespiti için spesifik bir aptamer ile modifiye edilmiştir. 0.1 ile 2.0 ng m l^{-1} arasında doğrusal aralık ve 0,1 ng m l^{-1} gibi düşük bir teşhis sınırı elde edilmiştir. Basit tasarımlı bu aptasensör, doplanmış bira örneklerinde başarıyla test edilmiş olup, OTA konsantrasyonları 0,4–1,6 ng m l^{-1} aralığında %93.4 ± 6.6 geri kazanım yüzdesi göstermiştir [43].

Bir diğer çalışmada, karbon siyahı-grafit pasta elektrot (KS-G-GPE) içeren bir elektrokimyasal sensör üretilmiştir. Bu sensör, DPV kullanılarak durum buğdayı matrislerinde OTA'yı tespit etmek için tamamen optimize edilmiştir. Geliştirilen elektrokimyasal sensör, OTA'yı başarıyla tespit etmiş ve teşhis ve tayin alt sınırı sırasıyla 57.2 nM (0.023 μg m l^{-1}) ve 190.6 nM (0.077 μg m l^{-1}) olarak belirlenmiştir [44].

OTA tespiti için yürütülen bir çalışmada, fonksiyonelleştirilmiş grafen (f-grafen) ile KS bazlı elektrokimyasal aptasensör geliştirilmiştir. Aptasensör, standart örnek için yaklaşık 1 fg/ml ve doplanmış örnek için 0.01 ng/ml OTA teşhis sınırı göstermiştir. Ayrıca, üretilen aptasensörün gerçek uygulaması üzüm suyu örneklerinde değerlendirilmiştir. OTA'nın geri kazanım oranları %90–101 aralığında elde edilmiştir [45].

Hou ve arkadaşlarının 2022 yılında yürütmüş olduğu çalışmada, OTA'nın spesifik aptameri elektrokimyasal bir aptasensör geliştirilmiştir Altın bir elektrot (AuE), OTA aptameri ve kDNA hibritizasyonu yoluyla elde edilen çift sarmallı DNA (çsDNA) kompakt bir katmanla modifiye edilmiştir ve bu katman, redoks probu $[Fe(CN)_6]^{3-/4-}$ sayesinde EİS tekniği ile ölçümler gerçekleştirilmiştir. Optimum koşullar, 0.05–10 ng/ml doğrusal aralık ve 0.05 ng/ml olarak teşhis sınırı belirlenmiştir. Doplanmış malt örneklerinde %74.8–105 geri kazanım oranları ve %8.86–13.09 arası bağıl standart sapmalar (BSS) elde edilmiştir [46].

OTA tespiti için yürütülen bir diğer çalışmada, yeşil bir elektrokimyasal immünosensör geliştirilmiştir. Çalışma Au elektrot yüzeyinde kompakt bir 2-merkaptoasetik asit (TGA) tek katmanı kendi kendine birleştirerek, Au/TGA/SSA-OTA/anti-OTA monoklonal antikor bileşik problarını oluşturmuş ve DPV analizine dayalı olarak çalışmıştır. Optimum koşullar altında, geliştirilen immünosensör OTA için 0.1–1.0 ng/ml aralığında doğrusal aralık ve 0.08 ng/ml teşhis sınırı sergilemiştir. Doplanmış malt örneklerinde gerçek uygulama, önerilen immünosensörün yüksek doğruluğunu ve matris girişimlerinin olmadığını doğrulamıştır [47].

Yürütülen bir diğer çalışmada, OTA'nın kantitatif tespiti için bir elektrokimyasal immünosensör önerilmiştir. Üretilen SSA/anti-OTA/PdNP'ler/KK immünosensörü, doplanmış kahve örneklerinde OTA'nın tespiti elektrokimyasal performans sergilemiştir. Uygun çalışma koşullarında, geliştirilen immünosensörün doğrusal aralığı 0.5−20 ng ml^{-1} (R^2 = 0.996) olup, teşhis sınırı 0.096 ng ml^{-1} olarak bulunmuştur [48].

Argoubi ve arkadaşlarının yürütmüş olduğu bir çalışmada, OTA tespiti için tasarlanmış tek kullanımlık bir aptasensör platformu geliştirilmiştir. Bu platform, ferrosen türevi ile işlevselleştirilmiş AuNP yapılı PBKE kullanmakta ve biyoreseptör bölgesi olarak bir anti-OTA aptameri içermektedir. Tespit, hedef molekül varlığında aptamerin katlanması ile indüklenen ferrosen elektrokimyasal sinyal değişimlerine dayanmaktadır. 0.5 ile 70 ng ml^{-1} aralığında doğrusal aralık ve 11 pg ml^{-1} teşhis sınırı bulunmuştur. Özellikle, sensör cihazı, kavrulmuş kahve çekirdekleri ve şarap gibi karmaşık ortamlarda numune ön işleme gerek kalmadan OTA'yı etkin bir şekilde tespit eder ve doğru geri kazanımlar sağlamıştır [49].

Yürütülen bir diğer çalışmada, iz seviyelerinde OTA'nın hassas belirlenmesi için bir elektrokimyasal aptasensör tasarlanmıştır. Sensörde OTA'yı yakalamak için bir DNA aptameri ve sinyal arttırıcı olarak gümüş metallizasyonu kullanılmıştır. Bağlanmamış aptamerleri sindirmek için ise Ekzonükleaz I kullanılmıştır. Sensör, 0.7 pg ml^{-1} teşhis sınırı, 2.48 pg ml^{-1} tayin alt sınırı ve 1 pg ml^{-1}

ile 0.1 μg ml^{-1} arasında doğrusal aralık (R^2 = 0.968) ile mükemmel analitik performans sergilemiştir [50].

Huang ve arkadaşlarının 2024 yılında yürütmüş olduğu bir çalışmada, OTA tespiti için çok duvarlı karbon nanotüp-modifiye niyobyum karbür (Nb_2C-ÇDKNT'ler) bazlı yeni bir moleküler baskılı polimer (MBP) elektrokimyasal sensör tasarladık. Bu tasarımda, bir camsı karbon elektrot (CKE) önce Nb2C-ÇDKNT'ler heteroyapısı ile modifiye edildi. Bu sensör 0.04 ile 10.0 μM arasında geniş bir doğrusal aralık ve 3.6 nM teşhis sınırı gösterdi. Gerçek numune analizinde, %89.77 ile %103.70 arasında değişen geri kazanım oranları bulunmuştur [51].

Yürütülen bu çalışmada, polioksometalat ($H_3PW_{12}O_{40}$, POM) fonksiyonelleştirilmiş iGO modifiye edilmiş CKE üzerinde yer alan gümüş nanopartiküllere (AgNP) dayalı yeni bir moleküler baskılı voltametrik sensör, OTA tayini için sunulmuştur. Yöntemin doğrusallık aralığı ve teşhis sınırı sırasıyla $5.0 \times 10^{-11} – 1.5 \times 10^{-9}$ M ve 1.6×10^{-11} M olarak hesaplanmıştır. Voltametrik sensör, iyi seçicilik ve geri kazanımla üzüm suyu ve şarap örneklerine uygulanmıştır [52].

Afzali ve arkadaşlarının 2016 yılında yürütmüş olduğu bir çalışmada, AuNP'ler kimyasal olarak modifiye edilmiş bir karbon pasta elektrot (KPE), OTA tayini için hassas bir elektrokimyasal sensör geliştirildi ve DPV yöntemi kullanıldı. Pik akımı, 0.5-100 nM aralığında OTA konsantrasyonuyla doğrusal olarak arttı. Teşhis sınırı 0,2 nM ve bağıl standart sapma %6.2 (n = 7) olarak bulundu. Yöntem, kahvaltılık gevrekler, gevrek bazlı bebek mamaları ve bira örnekleri gibi tahıl türevi ürünlerdeki okratoksin A'nın tayinine uygulandı [53].

OTA tespiti için yeni bir elektrokimyasal sensör, CKE üzerine ÇDKNT'ler ve MBP ile modifiye edilmesiyle üretilmiştir. Geliştirilen sensördeki OTA'nın elektrokimyasal oksidasyonu DV ve DPV yöntemleri ile araştırılmıştır. Geliştirilen MBP/ÇDKNT/CKE sensörü, DPV kullanıldığında, doğrusal aralık 0.050 ile 1.0 μM aralığında ve teşhis sınırları DV ve DPV yöntemleri ile sırasıyla 0.0041 μM (1.7 μg/l) ve 0.014 μM (5.7 μg/l) olarak bulunmuştur. Spike edilmiş bira ve şarap örneklerinde ise %84 ile %104 arasında geri kazanımlarla, OTA tayininde başarıyla uygulanmıştır [54].

Mishra ve arkadaşlarının yürütmüş olduğu bir çalışmada, DPV yöntemi aptasensör kullanarak kakao çekirdeklerinde OTA tespiti önerilmiştir. Geliştirilen aptasensör, 0.07 ng/ml teşhis sınırı ve %3.7 bağıl standart sapma ile 0.15–5 ng/ml aralığında iyi bir doğrusal aralık göstermiştir. Ayrıca bu aptasensör, %3.87 bağıl standart sapma ile %82.1–85 aralığında geri kazanım değerleri göstermiştir [55].

Fumonisinler

Munawar ve arkadaşlarının yürütmüş olduğu bir çalışmada, FB1 tanınması için nanoMBP'ler kullanarak yüksek hassasiyetli ve seçici bir elektrokimyasal sensör tasarlanmış ve üretilmiştir. Hem EİS hem de DPV teknikleri kullanılmıştır. 1 fM ile 10 pM arası (R^2 = 0.98) doğrusal aralık ve EİS ile DPV için sırasıyla teşhis sınırı 0.03 ve 0.7 fM'ye eşit olarak bulunmuştur. FB1 ile zenginleştirilmiş mısırdaki FB1 geri kazanımı yüksek (%96-102) olmuştur [56].

Yürütülen bu çalışmada, kDNA aynı anda OTA aptameri ve FB1 aptameri ile hibritleşecek şekilde tasarlanmış ve benzersiz Y-şekilli bir DNA yapısı oluşturarak eş zamanlı tespiti sağlamak için geliştirilmiştir. Altın nanorodlar (AuNRs), tiyolat Fs, tiyolat OTA aptameri (Apt1) ve tiyolat FB1 aptameri (Apt2) ile tiyolinden (Tiy) oluşan bir amplifiye sinyal elemanı ve tanıma elemanı oluşturmak için kullanılmıştır. Apt1-AuNR'lar-Tiy kompleksi ve Apt2-AuNR'lar-Fs kompleksi, kDNA ile hibritleşerek altın bir elektrot üzerinde benzersiz bir Y-DNA yapısı oluşturur. DPV tekniği ile yapılan ölçümler sonucunda 1,0 pg· ml^{-1} ile 100 ng· ml^{-1} doğrusal aralıklarında OTA ve FB1'in eş zamanlı olarak belirlenmesini sağlamıştır ve teşhis sınırları sırasıyla 0.47 ve 0.26 pg· ml^{-1}'dir. Doplanmış bira örneklerinde OTA ve FB1'in belirlenmesinde uygulanmış ve %89 ile %102 arasında geri kazanımlar elde edilmiştir [57].

Yürütülen bir diğer çalışmada, MBE fotoelektrokimyasal (MBE-FEK) sensör platformu, FB1 için ölçüm yapmak üzere GO-CdS heteroeklem ile modifiye edilmiş ITO elektrot bazında hazırlanmıştır. Bu çalışmada FB1 için 0.01 ile 1000 ng ml^{-1} aralığında doğrusal aralık ve 4.7 pg ml^{-1} teşhis sınırı göstermiştir [58].

FB1 tespiti için yürütülen bu çalışmada maskesiz litografi kullanılarak gümüş-CeO_2 formasyonu ve saf yapısal özelliklere sahip küresel yapılar (Ag) ile bir elektrokimyasal mikroakışkan biyosensör

platformu üretilmiştir. Doğrusal aralık, 10 pg ml^{-1} ile 100 ng ml^{-1} arasında elde edilmiştir. Teşhis ve tayin alt sınırları sırasıyla 1,5 pg ml^{-1} ve 3,9 pg ml^{-1} olarak elde edilmiştir. Gerçek gıda örneklerinde uygulanabilirlik, doplanmış mısır örnekleri kullanılarak değerlendirilmiş ve %85'e kadar doğrusal yanıt ile geri kazanım göstermiştir [59].

Wei ve arkadaşlarının yürütmüş olduğu bir çalışmada, DNA ve ekzonükleaz-I kullanarak bir elektrokimyasal aptasensör, FB1 tespiti için oluşturulmuştur. kDNA elektrot yüzeyine immobilize edilmiştir. Daha sonra, FB1 aptameri ile kDNA hibritleşerek çift sarmallı DNA oluşturulmuştur. Uygun koşullar altında, pik akımındaki değişim ile FB1'in logaritmik konsantrasyonu arasındaki doğrusal ilişki 1.0×10^{-3}–1000 ng ml^{-1} aralığında gözlenmiştir ve teşhis sınırı 0.15 pg ml^{-1} olarak bulunmuştur [60].

FB1 tespiti için yürütülen bir çalışmada, aptameri kontrollü bir şekilde sabitlemek ve hedefini tanıma verimliliğini artırmak için tetrahedral DNA nanoyapıları (TDN'ler) kullanan kağıt tabanlı bir elektrokimyasal aptasensör tasarlanmıştır. Ölçümler DPV yöntemi ile yapılmıştır. Doğrusal aralık 50 fg/ml ile 100 ng/ml arasında ve teşhis sınırı 21 fg/mL olarak bulunmuştur [61].

Sarpal ve arakadaşlarının 2023 yılında FB1 tespiti için yürütmüş olduğu bir çalışmada, elektrokimyasal biyosensörün üretiminde elektrokimyasal performansı artırmak amacıyla trimangan tetraoksit (Mn_3O_4) nanopartikülleri ve GO nanokompozit kombinasyonu kullanılmıştır. ITO kaplı cam substrat üzerinde elektrokimyasal biyosensör üretmek için, elektroforetik biriktirme tekniği kullanılarak GOMn3O4 nanokompozitinden ince bir film hazırlanmış ve seçici FB1 tespiti için antikorlar (ab-FB1) elektrota immobilize edilmiştir. Ölçümler için DPV tekniği kullanılmıştır. Biyosensörün duyarlılığı, 1 pg ml^{-1} ile 800 ng ml^{-1} doğrusal aralığında ve 0.195 pg ml^{-1} teşhis sınırı elde edilmiştir. Ayrıca, immünoelektrotların geri kazanımı tatlı mısır örneklerinde de gerçekleştirilmiş ve %98.91 olarak hesaplanmıştır [62].

Yürütülen bir çalışmada, FB1 tespiti için tek kullanımlık PBE kullanıldı ve anti-toksin antikor immobilizasyonu için, geliştirilmiş elektriksel iletkenlik ve biyouyumluluk için AuNP'ler ve PPy ile elektrokimyasal iGO nanokompozit film ile modifiye edildi. Optimize edilmiş test koşulları altında, FB1 için elde edilen teşhis sınırı ve doğrusal aralık sırasıyla 4.2 ppb ve 0.2 ile 4.5 ppm olarak bulunmuştur. Bağıl standart sapma değeri ise %4.9 olarak tespit edilmiştir. Geliştirilen bu sensör spike edilmiş mısır örneklerine de uygulanmıştır [63].

Naghshbandi ve arkdaşlarının 2023 yılında yürütmüş olduğu bir çalışmada, mısır örneklerinde FB1 tespit etmek için DNA-aptamer tanıma ve elektrokimyasal tekniğe dayalı yeni bir aptasensör geliştirildi. Tiyol modifiyeli tek zincirli bir DNA, AuNP'ler ile elektrokaplanmış bir PBKE üzerine immobilize edilmiştir. Aptamer-FB1 etkileşimi sonucunda FB1'in teşhis sınırı 0.14 ng/ml ve doğrusal aralığı 0,5–500 ng/ml arasında bulunmuştur [64].

Bu çalışmada, FB1 için impedimetrik bir immünosensör, CKE yüzey üzerine paladyum tellür kuantum noktalarıyla katkılanmış poli(2,5-dimetoksianilin)-ÇDKNT'lere dayalı olarak ve FB1 antikoru kullanılarak geliştirildi. Geliştirilen bu immünosensör ile EİS yöntemi kullanarak ölçümler gerçekleştirildi ve 7 ile 49 ng l^{-1}'lik doğrusal aralık ve 0.46 pg l^{-1} teşhis sınırı bulundu [65].

Trikotesenler

Sangu ve arkadaşlarının 2023 yılında yürütmüş olduğu bir çalışmada, MXene, DON için yüksek duyarlılık ve seçiciliğe sahip bir biyosensör sistemi oluşturmak için kullanılmıştır. MXene, hidroklorik asit-lityum florür (HCl-LiF) kullanılarak titanyum alüminyum karbür (Ti3AlC2-MAX) tozundan alüminyumun seçici olarak aşındırılmasıyla hazırlanmıştır. Hazırlanan MXene yüzeyi, özel olarak tasarlanmış DON aptameri ile immobilize edilmiştir ve DON'a olan DTV kullanılarak ölçülmüştür. Bu sensör ile DON için 1 fg ml^{-1} teşhis sınırı bulunmuştur [66].

Bir diğer çalışmada, gıda örneklerinde kalan DON mikotoksinini tespit etmek için elektrokimyasal bir immünosensör geliştirilmiştir. Cihaz, mikotoksini tespit etmek için elektrokimyasal kadmiyum sülfür nanopartikül – DON antikoru (CdSNP-AbDON) ve sığır serum albümini manyetik mikro-parçacıklar (DON-SSAMP) kullanır. CdSNP-AbDON, DON'a özgü antikorların CdS nanopartikülleri (CdSNP'ler) ile işaretlenmesiyle hazırlanmıştır. Ölçüm tekniği olarak anodik sıyırma voltametrisi kullanılmıştır. Teşhis sınırı 342.4 µg kg^{-1}, doğrusal aralık ise 610 ile 6210 µg kg^{-1} arasında bulunmuştur. Doğal olarak DON ile kontamine olmuş buğday örnekleri immünosensör ile analiz edilmiş ve kabul edilebilir geri kazanımlar göstermiştir [67].

Yürütülen bu çalışmada gümüş iyonu (Ag^+)-bağımlı DNAzim yardımlı sinyal amplifikasyonu ve AuNP'ler/MnO_2@GO nanokompozitlerine dayanan ultra hassas T-2 toksin tespiti için elektrokimyasal bir aptasensör oluşturulmuştur. Optimal koşullar altında, 2 fg ml^{-1} ile 20 ng ml^{-1} arasında doğrusal aralık ve 0.107 fg ml^{-1} teşhis sınırı elde edilmiştir [68].

T-2 toksini tespiti için yürütülen bir çalışmada, poli(metil metakrilat) (PMMA) mikroakışkan merkezi kanalda immobilize edilmiş monoklonal anti-T-2 antikorlarına dayalı yöntem kullanılmıştır. Platin tel çalışma elektrodu, iGO - nanoporlu altın (NPA) ile tek adımlı elektrodepozisyon prosedürü ile yerinde modifiye edilmiştir. Elektrokimyasal immünosensör için teşhis sınırı 0.10 µg kg^{-1} olarak bulunmuştur [69].

Zhang ve arkadaşlarının yürütmüş olduğu bir çalışmada, hedef tarafından indüklenen zincir yer değiştirme (HİZY) stratejisine dayanan bir aptasensör, T-2 toksininin tespiti için geliştirilmiştir. AuNP'ler@NH_2-MnO_2 kullanılarak aptamer için daha fazla bağlanma noktası sağlamıştır. DPV sinyal değişimini kaydetmek için kullanılmıştır. Optimal koşullar altında, oluşturulan elektrokimyasal aptasensörün sinyal yanıtı, T-2'nin konsantrasyonu ile iyi bir doğrusal ilişki sergilemiştir. 5×10^{-6} ng ml^{-1} ile 5 ng ml^{-1} arasında doğrusal aralık ve 8.74×10^{-7} ng ml^{-1} teşhis sınırı belirlenmiştir [70].

T-2 toksininin tespiti için yürütülen bir çalışmada, çift sinyal amplifikasyon stratejisine sahip yeni bir sandviç aptasensör geliştirilmiştir. Molibdenum disülfid-poli anilin-kitosan-altın nanopartikülleri (MoS2-PAni-KS-AuNP'ler) modifiye CKE üzerine işlenmiş ve aptasensör platformu olarak kullanılmıştır. Tasarlanan bu sensör ile teşhis sınırı 1.79 fg ml^{-1} olarak belirlenmiş ve 10 fg ml^{-1} ile 100 ng ml^{-1} arasında doğrusal aralık sunulmuştur [71].

T-2/HT-2 toksini impedimetrik ölçüm uygulaması bu çalışmada rapor edilmiştir. DV ve EİS teknikleri ile ölçümler gerçekleştirilmiştir. Anti-T-2 monoklonal antikor (mAb) immobilizasyonu için yüzey fonksiyonelleştirmesi, plkca temizleme ve piranha çözeltisi ile yüzey hidroksilasyonu, ardından (3-Aminopropil) trietoksisilan (APTES) silanizasyonu ve çeşitli konsantrasyonlardaki anti-T-2 toksin mAb ile immünosensör geliştirilmiştir. Doğrusal aralık 0-25 ppb olarak T-2/HT-2 toksinini tespit edebildiği ve tayin alt sınırı ise 4,89 ppb olarak elde edildiği kanıtlanmıştır [72].

Bu çalışmada elektro çekme ve nanofiber ile düzeltilmiş grafit elektrotun üretilmesi, ile sıvı materyaller, süt ve şurupta T-2 mikotoksininin tespiti için kullanımı açısından önemli bir teknik geliştirilmiştir. DV, DPV ve KDV teknikleri kullanılarak incelenmiştir. Düzeltilmiş elektrot, 0.99 korelasyon katsayısına sahip iki farklı konsantrasyon aralığında (30-100 nM) T-2 toksinine karşı doğrusal bir aralık göstermiştir [73].

Lu ve arkadaşlarının 2016 yılında yürütmüş olduğu bir çalışmada, DON espiti için tek kullanımlık PBE kullanıldı ve anti-toksin antikor immobilizasyonu için, geliştirilmiş elektriksel iletkenlik ve biyouyumluluk için AuNP'ler ve PPy ile elektrokimyasal iGO nanokompozit film ile modifiye edildi. Optimize edilmiş test koşulları altında, FB1 için elde edilen teşhis sınırı ve doğrusal aralık sırasıyla 8.6 ppb ve 0.05 ile 1 ppm olarak bulunmuştur. Ayrıca bağıl standart sapma %5.7 olarak tespit edilmiştir. Geliştirilen bu sensör spike edilmiş mısır örneklerine de uygulanmıştır [63].

Bir çalışmada, bizmut oksit modifiye PBE üzerinde DON'un DPV yöntemi ile tespiti için yöntem önerimiştir. Uygun ölçüm koşullarında, teşhis ve tayin alt sınırı değerleri sırasıyla 7,1 µg l^{-1} ve 24 µg l^{-1} seviyelerinde bulunmuştur [74].

Radi ve arkadaşlarının 2019 yılında yapmış olduğu bir çalışmada, DON tayini için altın PBE yüzeyindeki poli o-fenilendiamin (Po-PD) MBP dayalı bir impedimetrik sensör geliştirilmiştir. Uygun koşullarda, 0.3 ng ml^{-1}'lik bir teşhis sınırı ile 5.0 ile 500.0 ng ml^{-1} aralığında DON doğrusal aralık elde edilmiştir. Gıda örneklerinde DON'un belirlenmesi için başarıyla uygulanmıştır [75].

Bu çalışma, DON tespiti için bir impedimetrik etiketsiz immünosensörün geliştirilmesi ve optimizasyonuna odaklanmıştır. DON tespiti için bir monoklonal antikor, sisteamin tabakası ve poliamidoamin (PAMAM) dendrimerleri ile modifiye edilmiş bir altın elektrot üzerine immobilize edilmiştir. DPV tekniği ile 1 ppb'ye eşit düşük bir teşhis sınırına ulaşılmasına izin vermiştir. Makarna örnekleri için kullanılan DON çıkarma prosedürü ve örnekle ilgili matris etkisi dikkate alındığında, önerilen immünosensör, kuru makarnada DON için Avrupa Yönetmeliği tarafından empoze edilen maksimum kalıntı sınırından (750 ppb) daha düşük olan 50 ppb'lik bir tespit sınırı göstermiştir [76].

Subak ve arkadaşlarının 2021 yılında yürütmüş olduğu bir çalışmada, DON mikotoksinini tespit etmek için voltametrik bir aptasensör önerilmiştir. Gelişitirlen bu aptasensör ile doğrusal aralık 5.0–30.0

ng· ml^{-1} aralığında ve teşhis sınırı 3.2 ng· ml^{-1} olarak tespit edilmiştir. DON ile güçlendirilmiş mısır unu numuneleri üzerinde yapılan ön deneyler iyi geri kazanım değerleri vermiştir [77].

SONUÇ VE TARTIŞMA

Bu çalışmada, DNA metilasyonunu etkileyen mikotoksinlerin tespiti için elektroanalitik yöntemlerin etkinliği ve uygulanabilirliği değerlendirilmiştir. AFB1, OTA, FB1 ve Trikotesenlerden T-2 toksin üzerinde odaklanarak, bu mikotoksinlerin gen ifadesi üzerindeki epigenetik etkileri incelenmiştir. Bu mikotoksinler gen ekspresyonunu DNA metilasyonu yoluyla hücre döngüsünü, hücresel proliferasyonu, apoptozu ve diğer önemli biyokimyasal süreçleri etkileyerek kanserojenik, mutajenik ve nörotoksik etkileri epigenetik mekanizmalar aracılığıyla değiştirebilme kapasitesine sahiptir. İlerleyen çalışmalar sayesinde farklı mikotoksinler için genişletilmesi ile mikotoksin teşhis ve tespitinde devrim yaratabilir ve geniş çaplı bir biyogüvenlik ağı oluşturabilir.

Bu mikotoksinlerin tespitinde kullanılan elektrokimyasal yöntemler, özellikle düşük konsantrasyonlarda bile yüksek hassasiyet ve doğruluk sağlayarak, gıda güvenliği ve halk sağlığı açısından önemli bir rol oynamaktadır. Çalışmada ele alınan elektrokimyasal sensörler, çeşitli mikotoksinlerin, özellikle AFB1, OTA ve FB1 gibi mikotoksinlerin, farklı gıda matrislerinde başarılı bir şekilde tespit edilmesini sağlamıştır. Bu sensörlerin doğrusal aralıkları ve teşhis sınırları, mikotoksinlerin düşük seviyelerde tespiti için optimize edilmiş olup, bu da gıda örneklerinde pratik, hızlı, hassas ve düşük maliyetli bir şekilde tespit edilmesi için etkili araçlar olarak ortaya çıkmıştır.

Mikotoksinlerin tespitinde kullanılan elektroanalitik yöntemler, voltametri, potansiyometri ve elektrokimyasal impedans spektroskopisi gibi teknikler, yüksek hassasiyet, hız ve seçicilik gibi avantajlar sunmaktadır. Özellikle, palladyum nanopartiküller, grafen oksit, karbon nanotüpler gibi nanomalzemelerle modifiye edilmiş elektrotlar, mikotoksinlerin tespiti için sensör performansını artırmaktadır. Bu yöntemlerin gelişimi, mikotoksinlerin tespiti ve sağlık üzerindeki olumsuz etkilerinin önlenmesi için önemli bir ilerleme sağlamaktadır. Ayrıca yöntemlerin kullanılması, düşük konsantrasyonlardaki mikotoksinlerin bile başarıyla tespit edilmesini sağlamıştır. Bu çalışmada geliştirilen sensörlerin laboratuvar ortamından saha koşullarına uygun hale getirilmesi, geniş çaplı uygulamalar için önemli bir adım olacaktır. Bu sensörler, çeşitli gıda ve çevresel örneklerde mikotoksin kontaminasyonunun izlenmesinde kullanılabilir, böylece toplum sağlığını korumada ve hastalıkların önlenmesinde kritik bir rol oynayabilir.

Araştırma bulgularımız, bu mikotoksinlerin insan ve hayvan sağlığı üzerindeki olumsuz etkilerinin yanı sıra DNA metilasyon değişiklikleri yoluyla gen ifadesini değiştirebileceğini göstermiştir. Bu etkiler, kanser ve çeşitli genetik hastalıkların oluşum riskini artırabilir. Mikotoksinlerin neden olduğu DNA metilasyonundaki değişiklikler, özellikle uzun süreli maruz kalma durumlarında, hücrelerin epigenetik düzenlenmesini ve dolayısıyla genetik stabilitesini bozabilir. Bu durum, hastalıkların başlaması ve ilerlemesinde önemli bir rol oynayabilir.

Sonuç olarak, mikotoksinlerin DNA metilasyonu üzerindeki etkileri ve elektrokimyasal tespit yöntemleri, mikotoksinlerin biyolojik etkilerini anlamada ve bu toksinlere maruz kalmayı önlemede kritik bir öneme sahiptir. Gıda güvenliği alanında bu tür elektrokimyasal sensörlerin kullanımı, kontaminasyonun erken tespit edilmesi ve önlenmesi açısından büyük bir potansiyel taşımaktadır. Bu çalışmaların devamı, daha yüksek duyarlılık ve spesifiklik sunan yeni sensörlerin geliştirilmesine katkıda bulunarak, mikotoksinlerin sağlık üzerindeki etkilerini daha iyi anlamamızı sağlayacaktır. Bu da, halk sağlığını korumak ve gıda güvenliğini artırmak için önemli bir adım olacaktır.

YAZAR KATKILARI

Kavram: M.M.G., T.Ç., B.D.T.; Tasarım: M.M.G., T.Ç.; Denetim: T.Ç., B.D.T.; Kaynaklar: M.M.G.; Malzemeler: M.M.G., T.Ç.; Veri Toplama ve/veya İşleme: M.M.G.; Analiz ve/veya Yorumlama: M.M.G., T.Ç., B.D.T.; Literatür Taraması: M.M.G.; Makalenin Yazılması: T.Ç., B.D.T.; Kritik İnceleme: T.Ç., B.D.T.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(3): 1281-1292, 2024
Doi: 10.33483/jfpau.1504455

REVIEW ARTICLE / DERLEME MAKALE

EVALUATING THE ROLE OF MELATONIN ON THYROID PHYSIOLOGY AND DISEASES

MELATONİNİN TİROİD FİZYOLOJİ VE HASTALIKLARI ÜZERİNDEKİ ROLÜNÜN DEĞERLENDİRİLMESİ

Özlem ÖZTÜRK CEYLAN[1]*, Beyza YILMAZ[1], Sibel SÜZEN[1]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06100, Ankara, Türkiye

ABSTRACT

Objective: *Melatonin (MLT) is largely produced within the pinealocytes from tryptophan, happening during the dark. MLT's binding sites have been established in numerous parts of the brain but also in the cells of the immune system, gonads, kidney, and cardiovascular system. MLT may directly or indirectly affect the thyroid gland and its activities. It can also prevent thyroid cell proliferation and interfere with thyroid hormone synthesis. The underlying mechanisms need to be carefully interpreted.*
Result and Discussion: *In this review the typical inhibitory effects of MLT on the thyroid gland and hormone secretion, as well as its antioxidant effects and relationship with thyroid diseases is investigated.*
Keywords: *Hypothyroidism, melatonin, melatonin receptors, thyroid cancer, thyroid physiology*

ÖZ

Amaç: *Melatonin (MLT) büyük ölçüde triptofandan pinealositler içinde üretilir. Karanlıkta serotonin-N-asetiltransferaz aktivitesinde büyük bir artış olduğunda gerçekleşir ve serotoninin N-asetilserotonine dönüşmesine neden olur. MLT'nin bağlanma bölgeleri beynin birçok yerinde ve aynı zamanda bağışıklık sistemi, gonadlar, böbrek ve kardiyovasküler sistem hücrelerinde tespit edilmiştir. MLT, MT1 ve MT2 membran reseptörleri aracılığıyla birçok farmakolojik ve fizyolojik aktiviteyi etkiler. Ayrıca, reseptör aracılı olmayan etkileri ile antioksidan aktivite gösterir. MLT doğrudan veya dolaylı olarak tiroid bezini ve faaliyetlerini etkileyebilir. Altta yatan mekanizmaların dikkatle yorumlanması gerekmektedir.*
Sonuç ve Tartışma: *Bu derlemede, MLT'nin tiroid bezi ve hormon salgısı üzerindeki tipik inhibitör etkilerinin yanı sıra antioksidan etkileri ve tiroid hastalıkları ile ilişkisi incelenmiştir.*
Anahtar Kelimeler: *Hipotiroidizm, melatonin, melatonin reseptörleri, tiroid kanseri, tiroid fizyolojisi*

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) was first isolated by Lerner and colleagues in 1958 [1]. It is an extensively distributed neurohormone being proficient of entering every cell and all subcellular sections. The melatoninergic system embraces the MLT, an indoleamine, and its molecular targets. It is secreted mainly from the pineal gland, which produces MLT during the nighttime into the

* **Corresponding Author / Sorumlu Yazar:** Özlem Öztürk Ceylan
e-mail / e-posta: ozturkozlemm@gmail.com, **Phone / Tel.:** +905448940252

Submitted / Gönderilme : 24.06.2024
Accepted / Kabul : 14.08.2024
Published / Yayınlanma : 10.09.2024

general circulation or into the cerebrospinal fluid [2,3]. In vertebrates, MLT is synchronizing circadian rhythms, as well as sleep–wake cycle and upregulation of seasonal rhythms. Most of its activities occur via interactions with MLT receptors. Mitochondria are the chief cell organelles that form the MLT that point out that MLT is an "ancient molecule" that chiefly delivered the first cells' defense from the antioxidant species [4,5].

Besides circadian rhythm and sleep-wake cycle, MLT is effected on various biological functions such as gonadal activity, redox homeostasis, neuroprotection, immune-modulation, antioxidant and anticancer activity. Lack of MLT formation is diligently linked to the progress of aging, tumor formation, neurodegenerative diseases etc [6,7,8].

Antioxidant properties of MLT were discovered in 90's [9]. The following information of this development also recognized a MLT metabolite, cyclic-3-hydroxymelatonin (c3OHM), which is made when MLT reacts with free radicals. There have been many studies approving the capacity of MLT to directly react reactive oxygen species (ROS) [10] and to reduce oxidative mutilation to vital cellular macromolecules [11]. Studies revealed that MLT could act as an antioxidant, scavenging free radicals and avoiding cellular injury. MLT may also inhibit thyroid cell proliferation and effect on thyroid hormone production. This action was found advantageous for patients with hyperthyroidism.

Thyroid hormones play a crucial part in growth and metabolic homeostasis in mammals. In the last decade, there has been growing attention on the properties of thyroid hormones through fetal life regarding tissue differentiation and growth. Amongst the structures effected by thyroid activity, the central nervous system seems to be extremely sensitive during the developing phases [12,13]. In the blood stream MLT attaches to MT1 and MT2 receptors, and shows an important part in modifying circadian and seasonal rhythms, and also works as a free radical scavenger, an antioxidant, and an anticancer adjuvant [14]. Additionally, research has revealed that the MLT is effective on immune reactions, and an inequity of the MLT amount may cause to immunity disfunctions [15]. Due to its noticeable immunoregulatory properties, MLT has been widely explored as a potential therapeutic approach for autoimmune diseases.

The purpose of this review is to deliver some information about MLT, thyroid gland and thyroid hormones as well as to investigate the effects of MLT on the growth and activity of the thyroid gland and hyperthyroidism. The role of MLT in the pathogenesis and physiology of thyroid is also discussed.

Melatonin Regulation

MLT is the chief indole ring-containing hormone regulates of the sleep-wake cycle and circadian rhythm, which is a physiological cycle, takes about 25hours. This cycle is also detected in body temperature, feeding, motor activity, and sleep [16]. MLT is released into the circulation and reaches all the cells of the body, constituting the chronobiotic signal that synchronizes rhythms including circadian behavior. Changes in body temperature and locomotor activity rhythms in animal studies are detected during surgical interventions, hypoxic exposure, dietary changes, physical exercise, and numerous forms of stress [17,18]. Research presented that MLT and body temperature collectively contribute to the formation of sleep. An increase in MLT elevates fluctuations in body temperature. It was shown that MLT administration promotes reduction in locomotor activity and earlier sleep onset.

MLT studies now focus on not only sleep problems but also into a mumerous of additional possible applications as our understanding of its physiological activities grows. Aside from the neurodegenerative diseases, research involve cardiovascular disorders, cancer adjuvant treatment, side effects of conventional cancer treatments, treatment of liver diseases and injuries, fertility support, post-surgical recovery, gastrointestinal disorders, and so on [19].

Disruption of circadian rhythms is associated with higher risk of brain disorders. It was observed that chronic shift-workers are vulnerable to various diseases, including psychiatric disorders such as depression [20]. Regular physical activity helps to maintain high-amplitude circadian rhythms, particularly of clock gene expression in the SCN. It promotes their entrainment to external periodicities and improves the internal synchronization of various circadian rhythms [21].

The metabolic pathway of MLT is different in various organisms, and biosynthetic endogenous MLT acts as a molecular signal and antioxidant protection against external stress. A physiologically endogenous MLT is to emphasize the performance correlated to darkness. In mammalians the initial

step in the MLT biosynthesis pathway is the hydroxylation of L-tryptophan's indole ring by the enzyme tryptophan hydroxylase, resulting in the formation of 5-hydroxytryptophan (5-HTP). Subsequently, 5-HTP undergoes decarboxylation, facilitated by pyridoxal phosphate and the enzyme 5-hydroxytryptophan decarboxylase, yielding 5-hydroxytryptamine (5-HT). Serotonin (5-HT) is converted to MLT through the sequential action of two enzymes, serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, or AANAT) and hydroxyindole-O-methyltransferase (HIOMT). While levels of HIOMT activity remain fairly constant, the daily rhythm in MLT synthesis is generated by a concurrent rhythm in AANAT activity. (Figure 1) [22]. Animals have a single source of MLT, mitochondria. Moreover, animals cannot synthesize L-tryptophan themselves, and it must be ingested externally. Therefore, animals have lower MLT metabolism than plants.

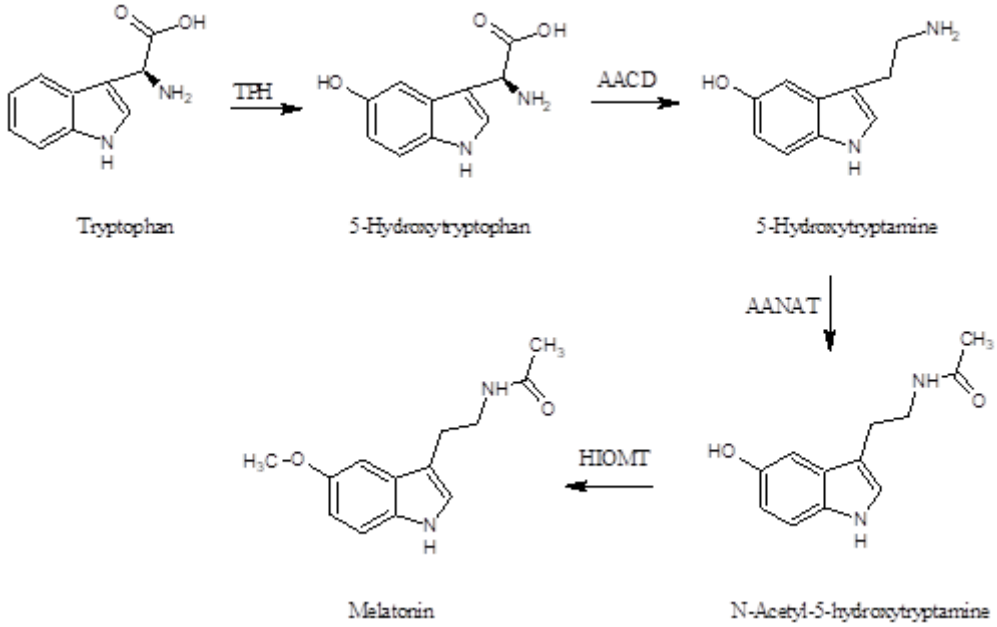

Figure 1. Melatonin biosynthetic pathway from tryptophan
(TPH: tryptophan hydroxylase, AACD: aromatic amino acid decarboxylase, AANAT: arylalkylamine *N*-acetyltransferase, HIOMT: hydroxylindole-O-methyltransferase)

Seasonal alterations in the biological activities of the body is controlled by MLT intermediated functions in the excretion of thyroid-stimulating hormone (TSH) and thyroid hormones. There are seasonal adjustments of TSH and thyroid hormone levels (total triiodothyronine (T3), free triiodothyronine (FT3), thyroxine (T4), free thyroxine (FT4) reliance on gender and age [23].

The concentration and rate of MLT synthesis reduce slowly along with the increase in age that physiopathologically associates to the development of certain neurodegenerative disorders [24]. The antioxidant properties of MLT have been related to its capacity to scavenge ROS and also the stimulus in the formation of anti-oxidative enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPX). Established on these recognized actions and pathological connection in aging-associated diseases of MLT, it is curious to enquire if the MLT administration could provide a protective effect in the aged-associated diseases [25]. MLT rises the defense of cells to the toxic actions of ionizing radiation through motivating DNA damage responses that reduce the risk of genomic instability. In addition, MLT indirectly suppresses autoimmune responses [26].

The task of the endocrine system, including several hormonal-releasing organs, undergoes ageing linked alterations that negatively influence its activity. The thyroid gland, which plays a crucial role in the human body's metabolism, growth and development, also exhibits structural and functional modifications with aging [27].

MLT triggers two high-affinity G protein-coupled receptors, MT1 and MT2, to apply useful activities in sleep and circadian abnormality, mood disorders, learning and memory, neuroprotection, drug abuse, and cancer. It was found that thyroid C-cells produce MLT under TSH regulation, also that MT1 MLT receptors are current in follicular cells and there is an indication for the contribution MLT in thyroid task by modifying the thyroglobulin gene expression in follicular cells [28].

Research have revealed that the oncostatic properties of MLT could be associated to membrane MLT receptors and nuclear RZR/ROR receptors. These activities are facilitated by the contacts of MLT with the two G-protein-coupled receptors MT1 and MT2 [29]. Other activities are proposed to be due to the free-radical-scavenging properties of this MLT. In some cancer cells, MLT interrupts the action of cell death, and inflammation. In addition, administration of MLT can lessen thyroid cancer cells [31]. MLT exhibits a significant part in the regulation of tumor formation. MT1 upregulation may inhibit some protein kinases to prevent cancer cell proliferation [29].

Melatonin on Hypothyroidism

Hypothyroidism is a pathological disorder of thyroid hormone insufficiency that may cause severe adverse health conditions. Hyperthyroidism due to the excess release of thyroid hormones from the thyroid gland manifests various biochemical and clinical findings. It adversely affects the cardiovascular, urogenital, and endocrine systems; electrophysiological functions; and oxidative metabolism in various organs and tissues [30].

There are two pharmacological types of hypothyroidism where the thyroid-stimulating hormone (TSH) amounts are higher than the reference and free thyroxine concentrations lower the regular range and where although the TSH amounts are higher than the reference, free thyroxine concentrations are within the normal range [32]. Hypothyroidism is 10 times more prevalent in women than in men. It is more prevalent in the elderly people, ranging from 2 to 5% of the population [33,34].

MLT is a hormone that shows a controlling role in pregnancy and thyroid physiology. Animal studies showed that, MLT is able to upregulate the estrous cycle and pregnancy [35] and converses symptoms of hypothyroidism in hypothyroid rats with the administration of exogenous MLT [36].

Thyroxin (T4) and triiodothyronine (T3) are produced from thyroid gland, both hormones escalate metabolic rate. The absence of thyroid hormone excretion could lessen body metabolism to 40–50% less than normal [37]. MLT is synthesized from the pineal gland and thyroid c-cells under TSH, therefore it can be effective in thyroid activity. The reduction of thyroid hormone concentration is defined after MLT treatment. MLT effects thyroid hormone concentrations by modulating iodothyronine-deiodinases. MLT has also a prevention activity on cell proliferation and thyroid hormone production as well as a defensive activity against oxidative injury in the thyroid gland [38,39].

Research have proposed the inhibitory action of MLT on the thyroid gland and its functions. Several procedures have been studied including short- and long-term MLT application, creation of darkness to escalate the action of the pineal gland, and pinealectomy. It was observed that MLT could directly or indirectly involve the thyroid gland activities [39,40]. Thyroid hormone is acknowledged to show chief roles in cell proliferation and differentiation, which proposes that seasonal controller of tanycyte proliferation might take part in the photoperiodic synchronization of seasonal rhythms [41].

Hypothyroidism causes to alterations in antioxidant progress [42]. MLT's antioxidative possessions and ability to defend membrane lipids, cytoplasmic proteins, and nuclear DNA. MLT facilitates most of its pharmacological activities such as the upregulation of immune activity via triggering of G-protein coupled MT1 and MT2 cell surface receptors. Additionally, MLT receptors are also placed on several tissues involving the thyroid follicular and parafollicular cells. In an animal study MLT administration of hypothyroid mice increase the thyroid hormones through modifying the neuroendocrine axis and control of hypophyseal TSH. It was observed that MLT indifferently modified the MT1 and MT2 receptor protein expression in the pituitary and thyroid gland. It could be that MLT controls the hypophyseal-thyroid activity in hypothyroid mice via different initiation of MT1 and MT2 receptors in the pituitary and thyroid gland [43].

Graves' disease (GD) and Hashimoto's thyroiditis (HT) are two life-threatening autoimmune thyroid disease (AITD). Thyroid T-lymphocytes are firstly stimulated against a thyroid autoantigen, then stimulation of B cell- or cytotoxic T cell-driven immunity occurs [44]. MLT exerts

immunomodulatory possessions and existing in the thyroid gland. It was established that the single-nucleotide polymorphism of MTNR1A, coding the MT1 protein, was linked with a susceptibility to GD and thyroid autoantibody creation, which supports the idea that the MLT may effect the happening of AITD and clinical features. It is known that MLT treatment elevates plasma thyroid hormone concentrations and enhances thyrocyte destruction and the T-cell proliferation capacity in a TG-immunized thyroiditis mouse model [32].

Thyroid, Ovarian Disorders and Melatonin

Thyroid hormones have been identified in the rat embryos during the second week of growth and in the human fetus before the beginning of functions of the fetal thyroid gland [45]. Thyroid dysfunctions, which are more widespread among females, may inhibit reproduction and fertility. Severe thyroid malfunction could cause menstrual syndromes and infertility through direct and indirect relations with the hypothalamo-pituitary-ovarian axis and the reproductive organs. Nonetheless, the precise occurrence of infertility in women with thyroid dysfunction remains unclear [46]. There is a growing indication for thyroid dysfunctions generally arising in women with ovarian disorders. For this reason, thyroid dysfunctions could be one of the causes of female infertility. Irregular thyroid-stimulating hormone (TSH) concentrations are stated in 6.3% of anovulatory infertile women and there is an indication signifying the connotation of hyperthyroidism with amenorrhea, and hypothyroidism with oligomenorrhea, diminished libido, and anovulation [47]. Accordingly, both hypo- and hyperthyroidism might influence the metabolism of estrogens and androgens eventually halting ovulation [48].

Investigation of the activity of MLT and L-thyroxine (T4) on the expression of several receptors, and some metabolic, reproductive, and gonadotropic hormones in polycystic ovary syndrome (PCOS) in an animal study showed a noteworthy increase in thyroid follicle numbers. MLT and T4 management of PCOS rats occasioned in an important reduction in the circulating amount of T3 and T4 [48].

It is known that one of the reasons of infertility is circadian rhythm conditions. Clock 3111 T/C and Period3 VNTR gene polymorphisms and circadian rhythm hormones in infertile women was investigated. The results revealed that the clock protein concentration of the infertile group was greater than the fertile group. MLT concentration of the fertile group was clearly associated with progesterone amounts and negatively connected with cortisol amounts. Additionally, MLT concentrations of the infertile group were completely related with LH amountss and negatively connected with cortisol amounts [49].

MLT stops lessening of ovarian and thyroid mass, number of pups, follicular diameter and thyroid epithelial proportion of the matrices with hypothyroidism. Animal studies showed that rats without induced hypothyroidism presented less body weight gain, gonad and thyroid weight, and gonad cell proliferation index. MLT inhibits effects of maternal hypothyroidism on the offspring of rats [50]. MLT also assistances to uphold neuronal activity in hypothyroid newborn rats by preventing apoptosis and supporting survival. This highlight the importance of MLT use by the mother in hypothyroid condition during gestation and lactation to preserve the reliability of motor neurons in the newborns [51].

Disorder of the circadian rhythm involving sleeping problems, wakefulness and hormone secretion disorders play an important part in infertility. It was observed that insomnia consequences in lessened MLT production. In females low MLT levels are connected with greater formation of free oxygen radicals (ROS), and therefore poorer quality of oocytes. MLT administration escalates its efficacy by improving the quality of oocytes, rise the percentage of fertilization and the quality of the developing embryos [52]. MLT seems to be a crucial indoleamine associated to female fertility. Its elevated plasma levels have been found in women with infertility due to hypothalamus disorder. MLT is connected not only with fertility, but also with the development of pregnancy. An escalation in MLT amounts during pregnancy and its rapid drop soon after pregnancy ended have been exposed [53].

Melatonin and Thyroid Cancer

The thyroid is an essential endocrine gland situated at the base of the throat anterior to the trachea. The thyroid uses iodine to synthesize hormones that regulate the heart rate, blood pressure, body temperature, and basal metabolic rate. Thyroid cancers are classified as differentiated thyroid cancers

(DTC) which includes papillary, follicular, medullary, and undifferentiated or anaplastic thyroid cancers (ATC). Thyroid cancer happens more often in women than in men, at an estimated ratio of 3:1. It can be seen in any age-group however more so in adults aged 45 to 54 years [54].

Research have established that MLT lessens the pathogenesis of cancer by directly disturbing carcinogenesis and indirectly distracting the circadian cycle. MLT is nontoxic and displays a variety of advantageous properties against cancer through apoptotic, antiangiogenic, antiproliferative, and metastasis-inhibitory pathways [55]. Remarkably, MLT has presented some synergistic activities with ionizing radiation and chemotherapy, which is separate from classical antioxidant compounds that are commonly administered for the lessening of adverse effects of radiotherapy and chemotherapy [26].

Thyroid carcinoma is the most common endocrine malignancy with growing frequency worldwide, so explains the common of deaths from endocrine cancers [56]. MLT has enhanced or induced apoptosis in many different cancer cells. MLT is a powerful antioxidant agent that defends against the toxic effects of radiation and chemotherapy. Additionally, in some cancer cells, MLT assistances alerting cancer cells to healing [31].

MLT and its metabolites have extremely broad antioxidant actions, including the ability to neutralize superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), single oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), nitric oxide (NO), and peroxynitrite anions ($ONOO^-$) [57]. In addition to these well-described actions, melatonin also reportedly chelates transition metals, which are involved in the Fenton/Haber–Weiss reactions; in doing so, melatonin reduces the formation of the devastatingly toxic hydroxyl radical resulting in the reduction of oxidative stress. Furthermore, MLT can increase the expression of antioxidant enzymes (SOD, CAT, and GPx) and scavenge free radicals [58]. Therefore, MLT is proposed to have a potent antioxidant capability and protective properties against oxidative stress [59]. Unlike most small-molecule biological antioxidants such as vitamin C, α-tocopherol and lipoic acid, MLT does not redox-cycle. It undergoes molecular rearrangement, effectively removing the free electron from the system – a so-called suicidal antioxidant [60]. MLT prevents lipid peroxidation by capturing the peroxide radical unlike antioxidants, such as ascorbic acid, alpha-tocopherol, and GSH. It has been revealed that liver, kidney, and brain tissue glutathione peroxidase activity in animal experiments elevated after the administration of MLT. Significant decreases in liver, lung, brain tissue, and glutathione peroxidase activity were reported in rats for which pinealectomy is made [61].

In a study, it was observed that MLT considerably reduced anaplastic thyroid carcinoma (ATC) cells and augmented LDH activity in ATC cells [62]. Investigation of the radioprotective properties of MLT against thyroid gland injury in rats showed that MLT administration before radiotherapy lessened thyroid gland damage caused by irradiation [63]. Papillary thyroid carcinoma (PTC) is the most common subtype of malignant thyroid nodule, and a significant increase in the number of thyroid cancer cases has been attributed to the increase in the incidence of PTC. It was found that, the level of MLT is clearly decreased in PTC, suggesting a potential role for MLT in the treatment of malignant thyroid tumors. [64]. Studies show a protective role for MLT against thyroid oxidative damage caused by carcinogens in thyroid cancer. MLT could be an effective agent in preventing oxidative damage and potentially reducing the risk of thyroid cancer formation caused by iodine compounds used in iodine prophylaxis [65]. Furthermore It was showed that MLT administered before radiotherapy provided significant protective effect against acute rat thyroid gland dysfunction caused by single dose radiotherapy [63].

Research revealed that not only iodine insufficiency, but also its additional presence could be related to thyroid cancer. Potassium iodate (KIO3), which is mostly used in salt iodization, may escalate oxidative damage to membrane lipids. MLT is a strong antioxidant, which defends against lipid peroxidation (LPO) in the thyroid [66]. Showed the defensive effects of MLT in the maximum possible *in vitro* application, against KIO3-induced oxidative damage in several tissues including thyroid. MLT lowered LPO in the thyroid and effective defending action was detected. It was concluded that MLT could be thought to escape the possible harmful effects of iodine derivatives administered in iodine prophylaxis. They also revealed that defending properties of MLT together with indole-3-propionic acid (I3P) were much stronger than those exposed by each molecule used independently. MLT and I3P employ cumulative defensive properties against oxidative damage caused by KIO3 [67]. Studies showed that MLT lessens LPO in various tissues and this defensive action not depend on iron level. This backs

the declaration that MLT is a unique antioxidant, which does not affect biological progressions, whereas it is active under circumstances with additional oxidative stress [78].

Current research showed that a small rise in thyroid cancer mortality was observed in the last two decades [68]. Increasing indication has proposed that circadian disorder is a possible risk factor for thyroid cancer [69]. It was found that extreme insomnia indications were related to a developed thyroid cancer risk in postmenopausal women [70] backup a part of sleep deficits and circadian dysfunction in thyroid cancer growth. Furthermore, a study established that circadian clock gene expression patterns were different in thyroid carcinoma tissue samples than in normal thyroid [71,72].

Dabrafenib has presented potent anticancer actions in patients with thyroid cancer. To prevent the resistance of thyroid cancer cells to dabrafenib, it was used with MLT as a combination on the proliferation, cell cycle arrest, apoptosis, migration and invasion of anaplastic thyroid cancer cells. MLT improved dabrafenib-mediated inhibition of cell proliferation, migration and invasion, and stimulated dabrafenib-induced apoptosis and cell cycle arrest in anaplastic thyroid cancer cells. The outcomes established that MLT synergized the anticancer activity of dabrafenib and delivered new approach for the treatment of anaplastic thyroid cancer [73].

It has been revealed that MLT may increase the effectiveness of anticancer drugs and directly prevent tumorigenesis. Furthermore, while MLT characteristically displays anti-apoptotic activities in normal cells, it shows pro-apoptotic activity in cancer cells. In a study the MDA-T41 thyroid cancer cell line was cultured with changing concentrations of MLT. The outcomes displayed that MLT delivered anti-apoptotic effects to MDA-T41 cells [74].

RESULT AND DISCUSSION

During the last decade, investigations were focused on MLT. It has been recognized not only in the pineal gland, but also in extrapineal tissues. MLT has an exceptional situation between the neurohormones. Research and clinical studies display the direct contribution and dynamic activity of MLT in the pathogenesis of many diseases such as cancer, immune diseases, neurodegenerative conditions, radiation disorders, etc. It was observed that MLT activity could be beneficial for the improvement of the management of numerous diseases.

Since MLT could inhibit thyroid cell proliferation and effect on thyroid hormone synthesis, this properties might be advantageous for hyperthyroidism patients. Earliest studies revealed that MLT treatment improved the triggering effect of TSH on thyroxin secretion. MLT activity is dose-dependent. High doses inhibited thyroid gland reaction to TSH. The control of the female reproductive system is one of the most related activities of thyroid hormones. Sufficient thyroid hormones synthesis is crucial for menstrual task and fertility as well as for the effective maintenance of pregnancy. The association between reproductive dysfunction and thyroid disorders is predominantly significant and draws consideration worldwide.

There are studies indicating a certain connection between MLT and thyroid action. It was proposed that there is a paracrine act for MLT in the regulation of thyroid action. Treatment of MLT is favorable as an antioxidant in thyroid tissues under circumstances of augmented OS, and could be supportive to decrease the oxidative developments involved in thyroid diseases.

Conclusion

MLT is an endogen hormone mainly produced in the pineal gland in response to darkness. Besides from regulating our body’s internal clock, it possesses strong antioxidant properties. MLT reveals promise in preventing and treating various health issues, such as cancer. Melatonin itself has a positive effect on thyroid function. It also affects sleep, general health, and many other relevant symptoms. Within the thyroid gland, thyroid hormone synthesis is mainly regulated by the thyroid-stimulating hormone (TSH). To date, there is data in literature pointing to the relationship between MLT and thyroid activity. There is also reasearch showing MLT activities on the thyroid gland itself, such as the inhibitory effect of MLT on cell proliferation and thyroid hormone synthesis. By supporting the pineal gland, there is also evidence that it can help enhance the body's ability to convert T4 into T3, a key issue for patients with hypothyroidism. The thyroid gland is characterized by a high level of oxidative stress, and the use

of pro-oxidants can lead to miscellaneous damage and diseases of this delicate papillon gland. Research shows that MLT may act as an antioxidant, rounding up excess free radicals and preventing cellular damage. MLT may also prevent thyroid cell proliferation and interfere with thyroid hormone synthesis. This effect may be beneficial for people with hyperthyroidism. Still, it may be problematic for people with normal thyroid function and certainly hypothyroidism. For this reason, people who take MLT regularly may need to check their thyroid levels to be sure they are optimal.

Taken together, MLT potentiates radiation-induced cytotoxicity in thyroid cancer cells via inhibition of NF-κB/p65 phosphorylation and ROS induction. MLT enhances sensitivity of thyroid cancer cells to irradiation *in vitro*. It has been experimentally proven that the MLT molecule has lethal effects on thyroid cancer cells and can be used in the protection of thyroid cancer [75]. It can be concluded that the use of MLT may have beneficial effects on preventing or reducing some thyroid related diseases.

However, the fact that MLT has not yet been approved by the FDA is a factor that limits its clinical use. MLT products are considered dietary supplements.

AUTHOR CONTRIBUTIONS

Concept: Ö.Ö.C., B.Y., S.S., K.B.; Design: Ö.Ö.C., B.Y., S.S.; Control: Ö.Ö.C., B.Y., S.S.; Sources: Ö.Ö.C., B.Y., S.S.; Materials: Ö.Ö.C., B.Y., S.S.; Data Collection and/or Processing: Ö.Ö.C., B.Y., S.S.; Analysis and/or Interpretation: Ö.Ö.C., B.Y., S.S.; Literature Review: Ö.Ö.C., B.Y., S.S.; Manuscript Writing: Ö.Ö.C., B.Y., S.S.; Critical Review: Ö.Ö.C., B.Y., S.S.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

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Yayım Koşulları

1. Ankara Üniversitesi Eczacılık Fakültesi Dergisi (Ankara Ecz. Fak. Derg. – J. Fac. Pharm. Ankara), açık erişimli, hakemli bir dergi olup yılda üç kez (Ocak-Mayıs-Eylül) yayımlanır.

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3. Yayın Komisyonuna gelen makaleler en az 2 danışmana gönderilir. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale değerlendirme süreci çift taraflı kör hakemlik ilkesiyle yürütülür.

4. Makaleler yayına kabul ediliş sırasına göre yayınlanır.

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8. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne aşağıdaki makale türleri kabul edilir:

    a) **Özgün makaleler**: Türkçe veya İngilizce hazırlanmış, şekiller ve tablolar dahil tamamı en çok 25 A4 kağıdı sayfası olan, orjinal araştırmaların bulgu ve sonuçlarını açıklayan makalelerdir. Araştırma makalelerinin yenilikçi ve bilime katkı sağlayan çalışmalar olması beklenir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.
    b) **Derleme makaleler:** Türkçe veya İngilizce hazırlanmış, şekil ve tablolar dahil tamamı en çok 30 A4 kağıdı sayfası olan, yeterli sayıda bilimsel makale taranarak, o güne kadarki gelişmeleri özetleyerek ortaya koyan ve sonuçlarını yorumlayarak değerlendiren makalelerdir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.
    c) **Kısa bildiriler:** Devam etmekte olan bir çalışmanın bulgularını zaman kaybetmeden duyurmak için Türkçe veya İngilizce yazılan en çok 5 A4 kağıdı sayfası olan makalelerdir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.

Yazım Kuralları

1. Metinler, A4 normunda (21 x 29.7 cm) yazılmış olmalıdır.
2. Metinler A4 normundaki sayfanın sağ ve sol tarafından 2.5 cm., üst ve alt kenarlarından 3 cm. boşluk bırakılarak 1 satır aralıkla yazılmalıdır. Yayımı kabul edilen makaleler doğrudan "Microsoft Word" dosyası halinde çevrim içi olarak sisteme yüklenecektir (online submission). Ana metin yazı karakteri "**Times New Roman**" ve **11 punto** olmalıdır.
3. Sayfa numaraları makalede **belirtilmemelidir**.
4. Paragraf başları **1 cm içeriden** başlamalıdır. Paragraflar arası ilave boşluk bırakılmamalıdır.
5. Başlık sayfasında yayın adı, yazar/yazarların adları, ORCID noları ve yazışma yapılacak yazarın açık adresi, telefon ve e-mail adresi belirtilmeli ve ortalı yazılmalıdır. İlk sayfada başlıktan önce yukarıdan 3 satır aralığı bırakılmalıdır. Başlık ile Öz/Abstract arası 1 satır aralıkla yazılmalıdır. Sorumlu yazarın soyadının üstüne (*) işareti konularak belirtilmelidir. Bu kişinin Adı Soyadı, açık adresi, telefon numarası ve e-mail adresi başlık sayfasının en altında belirtilmelidir.
6. **Yazar Adı** (ilk harfi büyük diğerleri **küçük harf**) ve **SOYADI** (tamamı **büyük harf**) **koyu** olarak başlığın altına bir satır aralık verildikten sonra altına unvan belirtmeden yazılmalıdır. Birden çok yazar varsa virgülle ayrılıp bir boşluk bırakılarak yazılmalıdır. Yazarların soyadları üzerine konulacak rakamlarla hemen isimlerin altındaki satıra kurum adları ve posta adresleri (Örneğin: Ankara Üniversitesi Eczacılık Fakültesi, Farmasötik Kimya Anabilim Dalı, 06560, Ankara, Türkiye) açıkça yazılmalıdır.
    - **Tüm yazarlar için ORCID numarası** mutlaka beyan edilmelidir. Yazarların ORCID ID'leri ilgili logoya köprü oluşturularak URL linklerinin eklenmesiyle gerçekleştirilmelidir.
7. Uluslararası kısaltmalar kullanılabilir. Metin içinde mililitre için ml; dakika için dak. olarak belirtilen şekliyle yazılmalıdır.
8. Birimler metrik sistemi kullanılarak ifade edilmelidir.
9. Bütün tablo ve şekiller metin içindeki yerlerine yazım alanından taşmadan yerleştirilmiş olmalıdır.
10. Tablolar üstlerine, şekiller (formül, grafik, şema, spektrum, kromatogram, fotoğraf vb.) de altlarına arabik rakamlarla (**Şekil 1**., **Tablo 2**.,) numaralandırılmalı ve metin içinde yer verilmelidir. "Tablo", "Şekil" sözcükleri ile bunlara ait numaralar koyu yazılmalı ve 11 punto olmalıdır. Şekil/Resim (**JPEG formatında**) makale içinde yerleşmiş ve **resimler 300 dpi veya daha yüksek çözünürlükte** olmalıdır. Üzerinde oynanmış (parlaklık, kontrast, gama ayarı vb.) şekillerde şekil altı metninde yapılan ayarlar belirtilmelidir. **Yazarlar, önceki makalelerinden alıntılanmış olsalar bile, diğer kaynaklardan herhangi bir görüntüyü çoğaltmak için ilgili yayıncılardan yazılı izin almalıdır**.
11. **Tablo** başlıkları Tabloların üstüne ve iki yana yaslı ve bunların genişliğini aşmayacak şekilde 11 punto ve bir satır aralıkta yazılmalıdır. Tabloya ait açıklama varsa tablonun altına 9 punto ile yazılmalıdır. Tablo içindeki metin 8-11 punto arasında yazılabilir. **Şekil** başlıkları ise şekillerin altına birer satır aralıkla ortalı ve 11 punto yazılmalıdır. Şekil başlığı ve şekil arasında 6 nk aralık olmalıdır. Tablo ve Şekiller metin içine yerleştirilirken metin ile aralarında 18 nk aralık olmalıdır.

    Örnek tablolar için bakınız.
    - Tüm satır ve sütun çizgileri yer almalı.
    - Tablo tasarımı tüm makalede tek tip ve düz olmalı, herhangi bir renklendirme/gölgelendirme kullanılmamalıdır.
    - Tablo içinde yer alan başlıklar **bold/koyu** renkte yazılmalıdır. Tablo başlığı ve tablo arasında 6 nk aralık olmalıdır.

Tablo 1. Türlere ait morfolojik özellikler

Bitki kısmı*	***C. nummularia***	***C. integerrimus***
Yaprak	Genişçe eliptik-orbikular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	Orbikulardan ovata kadar farklı şekillerde, 1.2-(4-5) x 0.9-3 cm
Tohum	3.5-4 x 1-2 mm, koyu kahverengi	3-4 x 1.5-2 mm, açık kahverengi

*Açıklama: 9 punto, 1 aralık olmalı.

Tablo 2. Hastaların özellikleri

Demografik bilgiler	**A grubu***	**B grubu**	**C grubu**
Erkek cinsiyet	10 (%30)	20 (%60)	10 (% 30)
Sigara kullanımı	20 (%60)	10 (%30)	20 (%60)

*Açıklama: 9 punto yazılmalıdır.

Örnek şekil;

Şekil 1. *C. nummularia*'nın genel görünüşü (Yazı karakteri "Times New Roman" ve 11 punto, "1" aralık, ortalı)

12. Makalelerin bölümleri **BAŞLIK** (Türkçe ve İngilizce), **ÖZ**, **ABSTRACT**, **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA, TEŞEKKÜR** (varsa eklenmeli), **YAZAR KATKILARI**, **ÇIKAR ÇATIŞMASI**, **ETİK KURUL ONAYI** (varsa eklenmeli) ve **KAYNAKLAR** sırasına uygun olarak hazırlanmalıdır. Bu bölümleri ifade eden başlıklar (Makalenin ilk başlığı hariç) **12 punto ile koyu olarak büyük harflerle ve sayfanın solundan başlanarak** yazılmalıdır. **GİRİŞ**'ten önce ve sonra sırasıyla 18 nk ve 6 nk aralık bırakılmalıdır. Diğer ana başlıklardan önce ve sonra sırasıyla 12 nk ve 6 nk aralık olmalıdır. Bölüm başlıkları ile metin arasında belirtilenin dışında ayrıca aralık **bırakılmamalıdır.**

- **BAŞLIK:** Türkçe ve İngilizce olarak büyük harf ve **ilk başlık** (Türkçe makalelerde Türkçe başlık, İngilizce makalelerde İngilizce başlık ilk başlıktır) **14 punto, koyu** ve ikinci başlık 12 punto, *italik* olarak yazılmalıdır. Başlık metine uygun, kısa, çalışmayı tanıtıcı ve açık ifadeli olmalıdır.

- **ÖZ** ve **ABSTRACT:** Türkçe (**ÖZ**) ve İngilizce (**ABSTRACT**) olarak makalelerin başında **200**'er kelimeyi geçmeyecek şekilde 10 punto ile *italik* olarak yazılmalıdır. Yabancı dilde yazılmış makalelerde önce **ABSTRACT** daha sonra mutlaka Türkçe olarak **ÖZ** bulunmalıdır. **ÖZ** ve **ABSTRACT** başlıkları 12 punto ve koyu yazılıp kendi içlerinde alt başlıklar (aşağıda görüldüğü gibi) halinde makalenin özeti sunulmalıdır. Her bir alt başlık 10 punto, koyu, normal yazılmalıdır. Alt başlıkların içeriğindeki metinler *italik* yazılmalıdır. **ÖZ ve ABSTRACT metni blok halinde sağdan ve soldan 1 cm boşluk bırakılarak yazılmalıdır.**

Özgün makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Gereç ve Yöntem: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Material and Method: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

Derleme makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

- **Anahtar Kelimeler (Keywords):** En az 3 sözcükten oluşmalı, ilgili dilde alfabetik, *italik* olarak, yalnızca ilk anahtar sözcüğün ilk harfi büyük olacak şekilde (büyük harf kullanılarak yapılan kısaltmalar hariç) aralara virgül konularak yazılmalı son anahtar sözcükten sonra ise bir imla işareti **kullanılmamalıdır.**

- **METİN:** Orijinal Türkçe makalede metin kısmı **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA** olmak üzere 3 ana başlıktan oluşmalıdır. Bu ana başlıkların tamamı 12 punto, **büyük harflerle** ve koyu olacak şekilde yazılmalıdır. Derleme makalelerde ise **GİRİŞ** ile **SONUÇ VE TARTIŞMA ana başlıkları olmalı,** diğer başlıklar yazarın belirleyeceği şekilde **her kelimenin ilk harfi büyük diğerleri küçük ve koyu** olacak şekilde yazılmalıdır. Alt başlıklar 11 punto, 1satır aralık, **bold/koyu** yazılmalı ve sola dayalı olmalıdır Alt başlıklarda numaralandırma sistemi **kullanılmamalıdır**. Alt başlıklardan önce ve sonra 6 nk aralık olmalıdır.

- **GİRİŞ:** Araştırmanın amacı ve konuyla ilgili çalışmaların yer aldığı bölüm olmalıdır.

- **GEREÇ VE YÖNTEM**: Kullanılan gereç belirtilerek, uygulanan yöntem hakkında gerekli bilgiler açıkça ifade edilmelidir. **Bileşiklerin karakterizasyonu** ayrı bir paragraf ile gösterilmeli ve yeni bileşiklerin saflıkları ve yapı aydınlatılmaları sağlanmalıdır. Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırıcılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul onayı alındığına dair ifadenin çalışma içinde yer almasını sağlamalıdırlar. Etik Kurul onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem kısmında belirtilmelidir. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiği ve onaylandığı, makalenin gereç ve yöntem bölümüne eklenmelidir. Detaylı bilgi için lütfen http://journal.pharmacy.ankara.edu.tr/en/ethical-principles-and-publication-policy/ web sayfasını ziyaret ediniz.

- **SONUÇ VE TARTIŞMA:** Bulguların verilerek değerlendirildiği bölümdür.
  - Dileyen yazar, RESULT AND DISCUSSION bölümünün son paragrafı olarak "Conclusion" başlığı oluşturabilir. Ancak 11 punto Times New Roman karakterinde İlk harfi büyük diğer harfleri küçük olmalıdır.

- **TEŞEKKÜR:** Varsa araştırmayı destekleyen kuruluşa ve katkısı olan kişilere Yazarların Katkısından önce yer alan bu bölümde kısaca teşekkür edilebilir.

- **YAZAR KATKILARI:** Makalede yer alan yazarların katkısı yazarlar tarafından imzalanan Telif Hakkı Devir Sözleşmesi (*Copyright Transfer Agreement*) uyarınca, çıkar çatışması bildiriminden hemen önce, makalede yer alan isim sırası gözetilerek yazılmalıdır. Lütfen bu bildirim için açık ad ve soyad yerine aşağıdaki örnekte olduğu gibi yazarların baş harflerini kullanınız. Yazar katkısı belirtilmeyecek alanlar için "-" işareti konulmalıdır.

  Örnek:

  YAZAR KATKILARI

  Kavram: İ.Y., M.M.H., C.H., K.B.; Tasarım: İ.Y., C.H., I.Ö.G., Ö.Ü.; Denetim: C.H., I.Ö.G., M.M.H., K.B.; Kaynaklar: Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.; Malzemeler: I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.; Veri Toplama ve/veya İşleme: A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.; Analiz ve/veya Yorumlama: Ö.Ü., B.G., T.C.Ş.T., E.K.S.; Literatür Taraması: B.K., D.Ç.P, B.G., B.E.; Makalenin Yazılması: A.K., İ.A., T.C.Ş.T.; Kritik İnceleme: İ.Y., B.G., Ö.Ü., İ.A.; Diğer: -

- **ÇIKAR ÇATIŞMASI BEYANI**

  Çıkar çatışması varsa ne şekilde olduğu açıkça beyan edilmelidir. Eğer yok ise "Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler." ifadesini kullanmalıdırlar.

- **ETİK KURUL ONAYI**

  Çalışmanın sonunda kaynaklardan önce etik kurul onayı alınmışsa hangi kurumdan ve ne zaman alındığı onay numarası ile mutlaka belirtilmeli ve Etik Kurul Onayını makale gönderim sırasında yüklemelidir. Etik kurul onayına gerek olmayan çalışmalarda aşağıdaki cümle yazılmalıdır.

  "Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir."

- **KAYNAKLAR:** Kaynak yazım stili Amerikan Psikoloji Derneği'ne (APA) göredir. Yazı karakteri "Times New Roman" ve 10 punto, "1" aralık, iki yana yaslı. Metinde, geçiş sırasına göre köşeli parantez içinde, örneğin: [1,6,9], [5-7] gibi numaralandırılmalı ve metin sonunda bu numaralara göre sıralanmalıdır. Alt başlıkların yanına kaynak belirtilmemelidir. Tablo içinde kaynak bildirilmesi gerekiyorsa metin içinde verildiği gibi belirtilmelidir.

  - **Makale için:** Yazarın soyadı, adının baş harfleri (Birden fazla adı olan yazarın her bir isminin baş harfinden sonra nokta konmalı ve arada boşluk bırakılmamalıdır. Birden fazla yazarların arasında virgül yer almalıdır. **Son yazar ile bir önceki yazar arasında "ve" kelimesi veya "&" sembolü <u>kullanılmamalıdır</u>**.), makalenin tam başlığı, derginin adı, cilt no, varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa numarası (veya makale numarası), yıl yazar isimlerinden sonra (parantez içinde) yazılmalıdır. **Birden fazla yazar varsa hepsi yazılmalıdır.** Makalenin adı yazılırken ilk kelimenin ilk harfi büyük diğer kelimelerin ilk harfi küçük yazılmalıdır. Kaynaklarda verilen **dergi adları kısaltma yapılmadan açık olarak yazılmalıdır.**

Her bir referansın sonuna [CrossRef] **ekleyerek aşağıdaki formatta DOI numarasını köprü olarak giriniz. Lütfen https://www.crossref.org/'da yer almayan makaleleri** [CrossRef] şeklinde **belirtmeyiniz.**
https://doi.org/10.1016/0006-2952(89)90403-6

Örnekler:

1. Martinez, M.J.A., Del Olmo, L.M.B., Benito, P.B. (2005). Antiviral activities of polysaccharides from natural sources. Studies in Natural Products Chemistry, 30, 393-418. [CrossRef]
2. Bahiense, J.B., Marques, F.M., Figueira, M.M., Vargasa, T.S., Kondratyuk, T.P., Endringer, D.C., Scherer, R., Fronzaa, M. (2017). Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. Pharmaceutical Biology, 55(1), 991-997. [CrossRef]

- **Elektronik Makale için:**

Örnek:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. British Medical Journal, 317, from http://www.bmj.com/cgi/content/full/317/7150/ Erişim tarihi: 14.03.2021

- **Web sitesi için:**

Örnek:
Clinical Pharmacology Web site. (2001). Erişim adresi http://cpip.gsm.com/ Erişim tarihi: 14.03.2021.

- **Kitap için:** Yazarın soyadı, adının baş harfleri, kitabın adı, cilt no (varsa), kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

- **Kitap bölümü için:** Yazarın soyadı, adının baş harfleri, bölümün başlığı, editör/editörlerin soyadı, adının baş harfleri, (Ed./Eds.) ibaresi, kitabın adı, varsa cilt no, kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Weinberg, E.D. (1979). Antifungal Agents. In: M.E. Wolff and S.E. Smith (Eds.), Burger's Medicinal Chemistry, (pp. 531-537). New York: John Wiley and Sons.

- **Tez için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Ne tür tez olduğu belirtildikten sonra tezin başlığı, nerde yapıldığı yazılmalıdır.

Örnek:
Ahmed, J. (2008). PhD Thesis. Pharmaceutical Botany investigations on *Prangos* Lindl. (Umbelliferae) growing in Konya province. Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, Turkey.

- **Patent için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Patent başlığı ve patent numarası yazılmalıdır.

Örnek:
Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

ETİK İLKELER VE YAYIN POLİTİKASI

Ankara Üniversitesi Eczacılık Fakültesi Dergisi, açık erişimli, hakemli bir dergi olup Türkçe veya İngilizce olarak farmasötik bilimler alanındaki önemli gelişmeleri içeren orijinal araştırmalar, derlemeler ve kısa bildiriler için bir yayım ortamıdır. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale yayın ücreti (APC) veya abonelik ücreti yoktur.

Yayın kurulu olarak dergi kapsamında önemli katkı sağlayan kaliteli yeni çalışmaların yayınlanması amaçlanmaktadır. Bu amaca ulaşmak için gönderilen makaleler, dergide yayınlanmak için bilimsel ve biçimsel gerekli kriterleri karşıladıklarından emin olmak adına baş editör ve/veya editör yardımcıları tarafından ilk değerlendirmeye tabi tutulur. Yalnızca bu ön değerlendirme sürecini geçen çalışmalar, daha ileri değerlendirme için diğer aşamalara devam ettirilir.

Ön Değerlendirme

- Çalışmanın bilimsel kalitesi ve yeniliği dergide yayınlanmak için yeterli olmalıdır.
- Dergiye gönderilen çalışmalar derginin amaç ve kapsamına uygun olmalıdır.
- Metin İngilizce veya Türkçe olarak dilbilgisi kurallarına uygun ve bilimsel olarak iyi yazılmış olmalıdır.
- Dergiye gönderilen çalışmaların benzerlik oranı %20'i geçmemelidir.
- Çalışmalar derginin yazım kurallarına ve şablonuna uygun olacak şekilde düzenlenmelidir.
- Telif hakkı devir formu, etik kurul onay belgesi, yazar katkı formu mutlaka yüklenmeli ve imzalı olmalıdır.
- Çalışmalar elektronik online başvuru sistemi aracılığı ile dergiye gönderilmiş olmalıdır.

Bu yeterlikleri taşımayan çalışmaların ileri değerlendirme süreci başlatılamaz.

Dergi yayınlanma sürecinde dergi editörleri, hakemler ve yazarlara bazı sorumluluklar düşmektedir. Bu sorumluluklar aşağıdaki şekilde açıklanmıştır.

1. Editörün Görevleri ve Etik Sorumlulukları

Editör, dergiye gönderilen makalelerden hangilerinin yayınlanması gerektiğine bağımsız olarak tek başına karar verebileceği gibi editör kurulunun üyelerine veya hakemlere de danışabilir. Derginin etik ilkeleri ve yayın politikası çerçevesinde, çalışmaların ön değerlendirme, hakem değerlendirmesi ve yayınlanma aşamalarının tarafsız, denetlenebilir, adil, çıkar ilişkisinden bağımsız ve gizlilik ilkelerine uygun şekilde yürütülmesinden sorumludur. Yayın politikası ve etik ilkeleri açısından ihlal yoksa derginin amacına ve kapsamına uygun çalışmaları, ön değerlendirme aşamasına almalıdır.

Baş editörün, editör yardımcılarının, alan editörlerinin ve editoryal danışma kurulunun görevleri ve tanımları aşağıdaki gibidir:

Baş Editör: Dergi içeriğinin yayınlanması konusunda tam yetkiye sahip kişidir. Editör yardımcıları, alan editörleri ve editöryal danışma kurulu ile birlikte çalışır.
Editör Yardımcıları: Dergi ilgili sorulara cevap vermek, dergi hakem ve kuruluna önerilerde bulunmak, makale yayın sürecinde baş editöre yardımcı olan kişilerdir.
Alan Editörleri: Çift kör hakem atamalarının gerçekleşmesi ve dergi ile ilgili sorulara cevap vermek konusunda yazarlara yardımcı olan kişilerdir.
Editoryal Danışma Kurulu: Editoryal Danışma Kurulu, Ankara Üniversitesi Eczacılık Fakültesi Dergisinin, amacına uygun ve kaliteli yayın üretilmesine ilişkin konularda Baş Editör ve Editör Yardımcılarına kılavuzluk eder.

1.1. Yayın Politikası

- Baş editör, dergiye gönderilen makalelerden hangilerinin yayımlanması gerektiği kararından tek başına sorumludur. Editörün kararı, derginin editör kurulunun prensipleri doğrultusunda olabileceği gibi, onur kırıcı yayım yapmak, telif hakkı ihlali ve intihal gibi konularla ilgili olarak yürürlükte olan yasal gereklilikler ile sınırlandırılmıştır.
- Baş editör, makale yayımlanmadan önce yazarların yayımcıya makalenin “Copyright Transfer Form” unu, doldurarak telif hakkını gönderdiğinden emin olmaktadır.
- Baş editör, yazarların makale yayımlanmadan önce "Conflict of Interest Form”unu ve “Author Contribution Form” unu doldurduğundan emin olmaktadır.
- Baş Editör, dergiye gönderilen makalelerin biçimsel olarak incelenmesi için editör yardımcılarını görevlendirmektedir. Ankara Üniversitesi Eczacılık Fakültesi Dergisinin kurallarını sağlamayan makaleler kesinlikle değerlendirmeye alınmadan reddedilmektedir.

1.2. Yayın Değerlendirmesi

- Baş editör, yayın değerlendirme sürecinin adil, tarafsız ve zamanına uygun şekilde gerçekleşmesini sağlamaktan sorumludur.
- Editör, tüm makaleleri genel olarak dışardan ve bağımsız en az iki hakem ile değerlendirilmesini sağlamaktadır. Gerek olması durumunda editör üçüncü bir hakemden ek görüş istemektedir.
- Editör, hakem seçimini makale kapsamına uygun olan uzmanları değerlendirerek yapar.
- Editör, olası çıkar çatışmaları için yapılan açıklamaları, hakemler tarafından yapılan “self-citation” önerilerini ve herhangi bir taraflılık olasılığını değerlendirmek ve karar vermek için dikkatli bir şekilde yayın sürecini gözden geçirmektedir.
- Baş editör/editörler, hakem değerlendirmesi veya değerlendirme/yayım sürecinin herhangi bir noktasında bir benzerlik tespit yazılımı (iThenticate) tarafından taratılmasını yazardan istemektedir veya kendisi yapmaktadır. Bu anlamda ifadelerin veya cümlelerin yazarın/yazarların kendileri olsa dahi metin daha önce yayınlanmış verilerle kabul edilemez bir benzerliğe sahip olmamalıdır.
- Baş editör, bir makaledeki hataları yayımlanmadan önce tespit ederse düzeltmektedir. Eğer daha sonra tespit ederse bu durumda düzeltmeleri yayımlamak zorundadır. Tüm düzeltme veya geri çekme bildirimlerini dergide belirgin bir şekilde yayımlamalıdır. Ayrıca içindekiler sayfasında listelemelidir.
- Ankara Üniversitesi Eczacılık Fakültesi Dergisinin editörleri, Yayın Etiği Komitesi (Committee on Publication Ethics (COPE)) tarafından yayınlanan "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" ve "COPE Best Practice Guidelines for Journal Editors" kılavuzlarına uyarak çalışmalarını sürdürür.

1.3. Adil Değerlendirme

- Baş editör/editörler, makaleleri yazarların ırk, cinsiyet, cinsel eğilim, inanç, etnik köken, vatandaşlık ya da politik görüşlerine bakmaksızın bilimsel içeriklerine göre değerlendirmektedir. Derginin editoryal prensipleri şeffaf ve tümüyle dürüst değerlendirmeyi desteklemektedir.
- Editör, hakemlerin ve yazarların kendilerinden bekleneni tam olarak anladıklarından emin olmalıdır.
- Editör, dergi ile ilgili tüm iletişimini derginin elektronik başvuru sisteminden yapar ve kararlarında itirazlar olması halinde şeffaf ve hakkaniyetli bir yol izler.

1.4. Gizlilik İlkesi

- Baş editör/editör, dergiye yapılan başvurudaki tüm materyallerin ve hakemlerle yapılan tüm iletişimin gizliliğini (ilgili yazar ve hakemlerle aksi onaylanmadığı sürece) korumakla yükümlüdür.

- Baş editör/editör, hakemlerin isimlerinin açıklanmasını kabul etmediği sürece, hakemlerin kimliklerini ve haklarını korumakla sorumludur.
- Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan editörün kendi çalışmaları/araştırmaları için kullanılmamalıdır.
- Baş editör/editör, makale değerlendirme sürecinde edinilen tüm bilgileri veya fikirleri gizli tutmalı ve kişisel amaçlar için kullanmamalıdır.

2. Hakemlerin Görevleri ve Etik Sorumlulukları

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale değerlendirme süreci çift taraflı kör hakemlik ilkesiyle yürütülmektedir. Dolayısıyla hakemler yazar/yazarlarla iletişim kuramazlar, değerlendirmeler dergipark yönetim sistemi üzerinden paylaşılır. Değerlendirme sürecinde tam metinlere ilişkin değerlendirme formları hakem yorumları editör aracılığı ile sorumlu yazara iletilir. Hakemler, değerlendirme süreci boyunca tarafsızlık, gizlilik, nesnellik, bilimsel yönden inceleme ilkelerine uygun hareket etmelidir. İlgili alanda uzman ve yetkinliğe sahip olmalıdır. Değerlendirmesine sunulan çalışmaya ilişkin raporunu belirtilen zaman aralığı içinde bitirmelidir. Zamanında sunulamayacak raporlar için gecikmeden editör ile iletişime geçilmelidir. Etik ilkeleri, telif hakkı ihlali, olası çıkar çatışması ve intihal yapıldığının fark edilmesi durumlarında editör kurulunu bilgilendirmelidir.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi için makaleleri değerlendiren hakemlerin aşağıda belirtilen görevlere ve etik sorumluluklara uyması beklenmektedir.

2.1. Editöryal Kararlara Katkı

- Hakemler, yazarların sundukları çalışmaları yapıcı ve uygun şekilde değerlendirmelidirler.
- Hakemler, makalede yer alan araştırmayı değerlendirmeye yetkin olmadığını düşünüyorsa veya yeterli sürede tamamlayamayacaksa editöre durumu bildirmelidirler.
- Hakemler, yazarlara yönelik sert ve kişisel eleştirilerde bulunmamalıdırlar.
- Hakemler, makale değerlendirmesi için davet aldığında eğer kendilerini makalede çalışılan konu hakkında yetersiz hissederlerse makaleyi değerlendirmeyi reddetmelidirler.
- Hakemler, makale değerlendirmesini verilen süre içinde yapmalıdırlar.
- Hakemler, sadece çalışmanın içeriğine ilişkin değerlendirmeyi objektif olarak yapmalıdırlar.

2.2. Gizlilik

- Hakemler, değerlendirmeyi tarafsızlık ve gizlilik içerisinde yapmalıdırlar.
- Hakemler, makale hakkındaki değerlendirmelerini ya da bilgilerini üçüncü kişilerle paylaşmamalıdırlar.
- Hakemler, makale değerlendirme sürecinde edinilen bilgileri, fikirleri ve basılmamış materyal veya çalışmaları gizli tutmalı ve kişisel amaçlar için kullanmamalıdırlar.
- Hakemler, makalenin bir kopyasını elinde bulundurmamalı veya çoğaltmamalıdırlar.

2.3. Etik Sorunları Fark Etme

- Hakemler, makalede yer alan etik sorunları fark etmeli ve editörün dikkatine sunmalıdırlar.
- Hakemler, makalenin daha önce başka bir yerde basıldığını veya basılmış önceki bir makale ile önemli ölçüde benzerlik ya da örtüşme tespit ederse editöre bildirmelidirler. Daha önce yayımlanmış olan herhangi bir gözlem ve/veya argüman, ilgili referans ile birlikte verilmelidir.

2.4. Tarafsızlık ve Rekabet Standartları

- Hakemler, tarafsız olarak değerlendirmelerini yapmalı ve önyargıdan uzak şekilde değerlendirmelidirler. Yazarın kişi olarak eleştirilmesi uygun değildir. Hakemler, görüşlerini destekleyici argümanlarla ifade etmelidirler.

- Hakemler, makale değerlendirmeyi kabul etmeden önce olası çıkar çatışmasını kontrol etmelidirler. Eğer çıkar çatışmasıyla karşı karşıya olduğunu düşünüyorsa makaleyi incelemeyi reddetmeli ve editörü bilgilendirmelidirler.
- Hakemler, yazar tarafından hakemin (ya da hakemle çalışan kişilerin) çalışmalarının kaynak olarak alındığını ileri sürerse, gerçek bilimsel gerekçeler sunmalılar, bu durumun hakemin kaynak gösterilme sayısını ya da çalışmalarının görünürlüğünü artırmaya yönelik bir girişim olmamasına özen göstermelidirler.
- Hakemler, değerlendirmelerini yaparken bilimsel gerçeklikten uzaklaşmamalı ve gerekirse kaynak gösterme yoluna başvurmalıdırlar.

3. Yazarların Görevleri ve Etik Sorumlulukları

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne gönderilen makaleler, daha önce herhangi bir yayın organında yayımlanmamış olmalıdır veya yayımlanmak üzere aynı zaman diliminde başka bir yayın organına gönderilmiş olmamalıdır. Çalışmalarda yararlanılan araştırmaların ve yayınların, alıntılarının veya atıflarının bilimsel araştırma ilkelerine uygun olarak eksiksiz yapılması ve kaynakların belirtilmesi zorunludur. Çalışmada yer alan yazar sayısı birden fazla ise, yazarların çalışmaya bilimsel ve akademik olarak somut ve yeterli düzeyde katkı sağlaması beklenir. Çalışmaya ait tüm finansal destek kaynakları açıklamalıdır. Olası çıkar çatışması durumlarını yayın kuruluna bildirmelidir.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale gönderen yazar/yazarların aşağıda belirtilen görevlere ve etik sorumluluklara uymalıdır.

3.1. Bildirim Standartları

- Yazar(lar)ın gönderdiği makale (araştırma, derleme veya kısa bildiri) özgün olmalıdır.
- Yazar(lar), çalışmanın önemine ilişkin tarafsız bir tartışma ile gerçekleştirilen araştırmayı net bir şekilde sunmalıdır.
- Yazar(lar), makalede verileri açık bir şekilde sunmalıdır.
- Yazar(lar)ın başka çalışmalardan faydalanması halinde tam ve doğru bir şekilde alıntı yapmalıdır.
- Makale, diğer araştırmacıların çalışmayı tekrar edebilmesine olanak verecek şekilde yeterli detay ve kaynak içermelidir.
- Yazar(lar), etik dışı davranarak yanıltıcı ya da net olmayan ifadeleri makalelerinde kullanmamalıdır.
- Yazar(lar), dergi kurallarına uymadıkları ve belirtilen sürede aksiyon almadıkları sürece makalelerinin dergi tarafından yayımlanmayacağını bilerek hareket etmelidir.

3.2. Veri Ulaşımı ve Saklama

- Yazarlardan editöryal değerlendirme için makalelerini destekleyici araştırma verisi istenebilir.
- Yazarlar, değerlendirme sürecinde makalelerine ilişkin ham verilerin veya makalelerini destekleyecek verilerin talep edilmesi durumunda belirtilen verileri yayın kuruluna sunmaya hazır bulunmalıdırlar.

3.3. Orijinallik, İntihal ve Kaynakların Belirtilmesi

- İntihal, yazarın başka bir makaleyi kendi çalışması olarak göstermesi, kaynak göstermeden başka birine ait çalışmanın belli bölümlerinin kopyalanması ya da başka sözcüklerle anlatılması veya başkaları tarafından yapılan çalışmanın sonuçlarının alınarak sunulması şeklinde olabilir. İntihalin her biçimi etik olmayan davranıştır ve kesinlikle kabul edilmemektedir. Yazarlar intihalden uzak durmalıdır. İntihal tanımı için buraya bakınız.
- Yazarlar çalışmalarının tümüyle orijinal olduğunu garanti etmelidirler. Yazarlar, başkalarının fikirlerini veya metinlerini kullanıyorlarsa mutlaka uygun şekilde kaynak ya da alıntı

göstermeliler ve gerekliyse izin almalıdırlar.

- Yazarlar kendilerine ait olan çalışmayı etkileyen ve çalışmaya ait uygun içeriğin oluşturulmasında katkısı olan tüm yayınları veya eserleri kaynak olarak göstermelidirler. Özel olarak (görüşme, yazışma ya da üçüncü taraflar ile tartışma) ile elde edilen bilgiler kullanılmamalı ya da kullanılacaksa izin alınarak bildirilmelidir.
- Yazarlar, Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderdikleri makalelerini intihal tarama programları (iThenticate) ile taramalı ve dergipark sisteminde çevrim içi makale gönderim sırasında makalelerinin intihal içermediğine dair raporu yüklemek zorundadırlar.

3.4. Çoklu, Gereksiz ve Tekrar Yayınlama

- Aynı makale ile birden fazla dergiye başvuruda bulunmak etik olmayan bir davranıştır ve asla kabul edilmemektedir. Genel olarak, yazar daha önce basılmış bir yayını, özet formunda ya da yayınlanmış bir ders, akademik tez ya da elektronik ön baskının bir parçası olması dışında, değerlendirme için başka bir dergiye göndermemelidir.
- Yazarlar başvuru sırasında makaleyi başka bir dergiye daha aynı anda göndermediklerini garanti etmelidirler.
- Yazarlar, gönderilen yazının değerlendirme aşamasında olmadığını veya başka bir yerde yayımlanmak üzere kabul edilmediğini ve eğer kabul edilirse, aynı biçimde, başka bir dilde, elektronik ortam da dahil olmak üzere, yazarın yazılı izni olmaksızın başka bir yerde yayımlanmayacağını garanti etmelidir.

3.5. Yazar Katkıları

- Yazar katkıları, çalışmanın konseptine, tasarımına, gerçekleştirilmesine ya da yorumlanmasına önemli katkı sağlayan kişiler ile sınırlandırılmalıdır.
- Yazarlar, çalışmaya katkı veren yazarların listesini dikkatli bir şekilde hazırlamalıdır. Bazı durumlar eşyazar (co-author) olmayı bazı durumlar ise çalışmanın "Teşekkür" (Acknowledgement) bölümünde yer almasını hak edebilir.
- Sorumlu yazar, tüm eşyazarların çalışmada uygun şekilde yer aldığına, tüm eşyazarların çalışmayı görüp onayladıklarına ve yayınlanmak üzere başvuru yapılmasına dair verdikleri onaya ilişkin sorumluluğu üstlenmelidir.
- Sorumlu yazar, makaledeki tüm yazarların yazar sıralaması, çalışmanın kesinliği ve bütünlüğü gibi konularda fikir birliğinin sağlanmasından sorumludur ve orijinal başvuru sırasında kesin bir yazar listesi sunmalıdır.
- Çalışmanın başvurusu tamamlandıktan sonra, sadece istisna durumlarda, editör yazar listesinde ekleme, silme ya da yeniden düzenleme yapabilir. Tüm yazarlar bu şekilde yapılacak ekleme, silme ve yeniden düzenleme konusunda fikir birliği içinde olmalıdırlar. Tüm yazarlar çalışmanın ortak sorumluluğunu aldıklarını kabul ederler. Her yazar, uygun şekilde araştırılan ve karara bağlanan çalışmanın kesinliği ve bütünlüğü ile ilişkili sorulardan sorumludur.
- Sorumlu yazar, editör ile iletişime geçen kişi olarak Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale ile birlikte "Yazar Katkı Formu"nun da doldurulup gönderilmesinden sorumludur.

3.6. Çıkar Çatışması Beyanı

- Yazarlar, çalışmalarını uygunsuz bir şekilde etkileyebilecek olarak gördükleri diğer kişi veya organizasyonlarla çıkar çatışması oluşturabilecek her türlü durum ve ilişkileri beyan etmelidirler.
- Sorumlu yazar, editör ile iletişime geçen kişi olarak Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale ile birlikte "Çıkar Çatışması Beyanı Formu"nun da doldurulup gönderilmesinden sorumludur.

- Yazarlar çıkar çatışmalarının olduğu durumları mutlaka açıklamalıdırlar.

3.7. Temel Hataların Bildirimi

- Yazarlar, yayımlanmış, erken görünüm veya değerlendirme sürecinde olan bir çalışmasında önemli bir hata ya da eksiklik fark ettiğinde, acil olarak dergi baş editörüne/yayınevine veya ilgili editöre bildirmek ve editör tarafından gerekli görülmesi durumunda makaleyi geri çekmek veya düzeltmek için editörle işbirliği yapmak ile yükümlüdür.
- Editör/yayınevi yayımlanmış olan makalenin bir hata içerdiğini üçüncü bir taraftan öğrenirse, editör ile işbirliği yapmak ve gerektiğinde destekleyici kanıt sağlamak yazarın yükümlülüğüdür.

3.8. Olası Riskler ve İnsan veya Hayvan Konuları

- Yazarlar, kullanımları sırasında olağan dışı risk yaratan kimyasallar, işlemler ya da malzemeler ile çalışmışlarsa açıkça belirtmelidirler.
- Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırmacılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul Onayı alındığına dair ifadenin makale içinde yer alması sağlamalıdırlar.
- Yazarlar, Etik Kurul Onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarasını, gereç ve yöntem kısmında ve Etik Kurul Onay bölümünde belirtmelidirler. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiğini ve onaylandığını, makalenin gereç ve yöntem bölümüne eklemelidirler.
- Etik kurul raporu alınması gerektiği halde, etik kurul raporu olmayan çalışmalar reddedilecektir.
- İnsanlar veya insandan elde edilen örnekler üzerinde yapılan klinik araştırmalarda bilgilendirilmiş onam formu mutlaka alınmış olmalıdır ve gereç ve yöntem kısmında belirtilmelidir. İnsan gönüllüleri ile yapılan araştırmalar için araştırma protokolüne uygun olarak hazırlanmış yazılı bilgilendirilmiş gönüllü onam formu alınmalıdır.
- Yazarlar, çalışmalarında, hayvan ya da insan örnekleri/gönüllüler kullanmışsa gerekli etik kurul izinlerini aldığından emin olmalıdır. Etik kurul izin ifadesini makalede mutlaka belirtmelidir.
- Bu anlamda yazarlar aşağıda sıralanmış olan kılavuzlara uyarak çalışmalarını gerçekleştirmiş olmalıdırlar:
  İnsanlar üzerinde gerçekleştirilen tüm araştırmalar Helsinki Bildirgesi ilkelerine göre yapılmalıdır (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). İnsan gönüllülerinden bilgilendirilmiş onam formu alınmış olmalıdır. Tüm hayvan çalışmaları ARRIVE kılavuzuna uygun olmalı (Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines) ve "Bilimsel Amaçlı Kullanılan Hayvanların Korunmasına İlişkin Konsey Direktifi"ne (EU Directive 2010/63/EU for animal experiments), "Birleşik Krallık Hayvan Yasası"na (The U.K. Animals (Scientific Procedures) Act 1986) ve/veya "U.S. İnsan Bakımı ve Laboratuvar Hayvanlarının Kullanımına İlişkin Halk Sağlığı Hizmeti Politikası" rehberine (U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals) uygun şekilde yürütülmelidir. Bitkiler ile ilgili tüm deneysel araştırmalar, uluslararası yönergelere uygun olmalıdır.

4. Ücret Politikası

- Hiçbir ad altında yazar veya kurumundan ücret alınmaz.
- Dergi ile işleme ve yayınlama ücretsizdir. Gönderilen veya kabul edilen makaleler için makale işleme ücreti veya gönderim ücreti yoktur.

Publication Terms

1. The Journal of Faculty of Pharmacy of Ankara University (J. Fac. Pharm. Ankara) is an open-access, peer reviewed journal and is published three times (January-May-September) a year.

2. The Journal of Faculty of Pharmacy of Ankara University publishes articles in every field of Pharmaceutical Sciences. The manuscript to the journal should not be published previously as a whole or in part and not be submitted elsewhere. Manuscript should be written in Turkish or in English. The experiments used have to be adhered to the Declaration of Helsinki for humans and European Community Guidlines for animals. In studies where Ethics Committee Approval is mandatory, the name of the institution from which ethics committee approval was obtained and the ethics committee approval number should be stated in the material and method section and the Ethics Committee Approval section, and the relevant document should be uploaded during article submission.

3. All manuscripts will be submitted to a review process by the editors and by qualified at least 2 outside reviewers. The article evaluation process of Journal of Faculty of Pharmacy of Ankara University is carried out on the principle of double-blind refereeing.

4. Manuscripts are published in order of final acceptance after review and revision.

5. If a manuscript returned to the authors for revision is not received back to the editor within 3 months it will be treated as a new article. When the article is published, authors must send the copyright of the article to the Publisher by filling out the "Copyright Transfer Form".

6. Manuscript will be controlled using plagiarism checker. Articles sent to Journal of Faculty of Pharmacy of Ankara University for publication must be scanned with plagiarism scanning programs (iThenticate) and a report stating that the articles do not contain plagiarism must be uploaded during online article submission.

7. Journal of Faculty of Pharmacy of Ankara University does not have an article publication fee (APC) or subscription fee.

8. The following types of articles are accepted in the Journal Faculty of Pharmacy of Ankara University:

    a) **Original articles**: Articles written in English or Turkish in scientific format presenting original research. Articles should be printed on A4 size papers not exceeding 25 pages (including tables and figures). Research articles are expected to be innovative and contributing to science. Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.
    b) **Review articles:** An updated comprehensive review of scientific works on a particular subject. Articles written in English or Turkish should be printed on A4 size papers not exceeding 30 pages (including tables and figures). Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.
    c) **Short communications:** Rapid announcement of the results of a continuing research written in English or Turkish, no longer than 5, A4 size pages. Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.

Preparation of Manuscript

1. Texts must be written in A4 norm (21 x 29.7 cm).
2. Texts should be written with 1 line spacing, with 2.5 cm margins on the left and right sides of the A4 norm page, 3 cm margins each from the top and bottom edges (3 line spacing from the top on the first page). Articles accepted for publication will be directly uploaded to the system as a "Microsoft Word" file (online submission). The main text font should be **"Times New Roman"** and **11 pt**.
3. Page numbers **should not be specified** in the article.
4. Paragraph headings must **begin 1 cm inside**. Additional spaces should not be left between paragraphs.
5. On the title page, the title of the manuscript the name/s, the full address/es and ORCID no of the author/s, and the full address, telephone number, e-mail address of the corresponding author should be written and all should be centered in the text. It should be indicated by placing (*) above the surname of the corresponding author. Name, surname, full address, telephone number and e-mail address of this person should be specified at the bottom of the title page.
6. **Author's Name** (**first letter capital, others lowercase**) and **SURNAME** (**all capital letters**) should be written in bold, three lines spaced under the title, and without a title underneath. If there is more than one author, they should be written by separating them with a comma and leaving a space. The numbers to be placed on the surnames of the authors and the institution names and postal addresses (For example: Ankara University Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06560, Ankara, Turkey) should be clearly written on the line just below the names.
   - **ORCID ID number must be declared for all authors**. ORCID IDs of the authors should be created by creating a hyperlink to the relevant logo and adding URL links.
7. International abbreviations may be used. ml for milliliter in the text; min. for minutes It should be written as specified.
8. Units should be expressed using the metric system.
9. All tables and figures should be placed in their places in the text without exceeding the writing area.
10. Tables should be numbered on the top, figures (formula, graph, chart, spectrum, chromatogram, photograph, etc.) should be numbered below with Arabic numbers (**Figure 1., Table 2.)** and should be included in the text. The words "Table", "Figure" and their numbers should be written in bold and in 11 pt. Figure/Picture (**in JPEG format**) must be placed in the article and pictures must be at least **300 dpi or in higher resolution**. Authors must obtain written permission to reproduce any images from other sources.
11. **Table** titles should be written in 11 font size justified on the top of the tables and not exceeding their width. If there is an explanation for the table, it should be written in 9 font size at the bottom of the table. The text in the table can be written between 8-11 points. **Figure titles** should be written at the bottom of the figures with a line spacing, centered and 11 pt. There must be **6 nk** space between the figure and figure title. There should be **18 nk** space between the text and title of figure and/or table.

    See for below examples for tables:
    - All row and column lines should be included.
    - Table design should be uniform and straight throughout the article, no coloring / shading should be used.
    - Headings in the table should be written in **bold**. There must be **6 nk** space between the table and table title.

Table 1. Morphological characteristics of the species

Plant part*	***C. nummularia***	***C. integerrimus***
Leaf	Broadly elliptical-orbicular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	From orbicular to ovate, 1.2-(4-5) x 0.9-3 cm,
Seed	3.5-4 x 1-2 mm, dark brown	3-4 x 1.5-2 mm, light brown

* Explanation should be 9 font size, 1 range.

Table 2. Patient demographics

Demographics	**Group A***	**Group B**	**Group C**
Male gender	10 (%30)	20 (%60)	10 (% 30)
Cigarette consumption	20 (%60)	10 (%30)	20 (%60)

* Explanation should be 9 font size, 1 range.

Example for figure:

Figure 1. General view of *C. Nummularia* (The font size must be 11 pt with 1 line spacing and "Times New Roman" font, and must be centered in the text)

12. The sections of the articles should be prepared in accordance with the **TITLE** (Turkish and English), **ABSTRACT, INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION, ACKNOWLEDGEMENTS** (if available), **AUTHOR CONTRIBUTIONS, CONFLICT OF INTEREST, ETHICS COMMITTEE APPROVAL** (if available) and **REFERENCES**. Titles expressing these sections (except the first title of the article) should be written in **12 pt**, **bold capital letters and starting from the left of the page**. **There should be 18 nk space before and 6 nk space after the INTRODUCTION.** For, there should be 12 nk space before and 6 nk space after the other titles. Between the chapter titles and the text, a separate space **should not be left** other than the specified in this document.

- **TITLE:** Capital letters and **first title** in Turkish and English (Turkish title is the first title in Turkish articles, English title is the first title in English articles), **14 pt, bold** and the second title should be written in 12 pt, *italic*. The title should be appropriate to the text, short, introducing the work and clearly worded.

- **ABSTRACT** and **ÖZ:** It should be written in English (**ABSTRACT**) and Turkish (**ÖZ**) at the beginning of the articles, not exceeding 200 words, 10 pt, *italic* and within a frame. In articles written in a foreign language, first **ABSTRACT** and then **ÖZ** in Turkish. **ABSTRACT** and **ÖZ** titles should be written in 12 pt. And bold and the summary of the article should be presented as subheadings. Each subtitle should be written in 10 pt, bold, normal and 1 cm indented. **ABSTRACT** and **ÖZ** should be written in blocks with 1 cm margins from the right and left.

For original articles;

Subheadings to be used for **ABSTRACT**:

Objective: *Text should be written in italic.*
Material and Method: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Gereç ve Yöntem: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler: *Text should be written in italic.*

For review articles;

Subheadings to be used for **ABSTRACT**:

Objective: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler:

- **Keywords (Anahtar Kelimeler)**: It should consist of a minimum of 3 words, should be written alphabetically, italic in the relevant language, with only the first letter of the first keyword capitalized (except for abbreviations using capital letters) with commas between them and a spelling mark **should not be** used after the last keyword.

- **TEXT:** The text part of the original Turkish article should consist of 3 main headings: **INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION**. All of these main headings should be written in 12 pt, **capital letters** and bold. In review articles, there should be the main headings of **INTRODUCTION** and **RESULT AND DISCUSSION**, other titles should be written with the first letter of each word capital, the others in lowercase and bold, as determined by the author. Subheadings should be written in 11 font size, 1.5 line spacing, **bold** and aligned to the left. Numbering system **should not be** used in subheadings.

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- **RESULT AND DISCUSSION:** This is the section where findings are given and evaluated.
  - If the author wishes, "Conclusion" can be added as the last paragraph of the RESULT AND DISCUSSION section. The font size must be 11 pt with 1 line spacing and “Times New Roman” font and the first letter must be uppercase and the other letters must be lowercase.

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  Example:

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  Concept: İ.Y., M.M.H., C.H., K.B.; Design: İ.Y., C.H., I.Ö.G., Ö.Ü.; Control: C.H., I.Ö.G., M.M.H., K.B.; Sources: Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.; Materials: I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.; Data Collection and/or Processing: A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.; Analysis and/or Interpretation: Ö.Ü., B.G., T.C.Ş.T., E.K.S.; Literature Review: B.K., D.Ç.P, B.G., B.E.; Manuscript Writing: A.K., İ.A., T.C.Ş.T.; Critical Review: İ.Y., B.G., Ö.Ü., İ.A.; Other: -

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  If the ethics committee approval is obtained before the sources at the end of the study, the approval number must be specified from which institution and when it was obtained. Approval from the ethics committee should be uploaded during the manuscript submission. In studies that do not require ethics committee approval, the following sentence should be written.

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Examples:

1. Martinez, M.J.A., Del Olmo, L.M.B., Benito, P.B. (2005). Antiviral activities of polysaccharides from natural sources. Studies in Natural Products Chemistry, 30, 393-418. [CrossRef]
2. Bahiense, J.B., Marques, F.M., Figueira, M.M., Vargasa, T.S., Kondratyuk, T.P., Endringer, D.C., Scherer, R., Fronzaa, M. (2017). Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. Pharmaceutical Biology, 55(1), 991-997. [CrossRef]

- **Online articles:**

Example:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. British Medical Journal, 317. Retrieved August 12, 2005, from ttp://www.bmj.com/cgi/content/full/317/7150/

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eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

ANKARA ÜNİVERSİTESİ ECZACILIK FAKÜLTESİ DERGİSİ

(Ankara Ecz. Fak. Derg.)

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

(J. Fac. Pharm. Ankara)

Cilt / Vol: 48
Sayı / Issue: 3
Yıl / Year: 2024

Ücretsizdir