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

ANKARA ÜNİVERSİTESİ

ECZACILIK FAKÜLTESİ DERGİSİ

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

Cilt / Vol : 47
Sayı / No : 3
Yıl / Year : 2023

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(*Ankara Ecz. Fak. Derg.*) **eISSN: 2564-6524**
ISSN: 1015-3918 (1971-2010)

Sahibi:

Prof. Dr. Asuman BOZKIR

Alan Editörleri:

Ankara Üniversitesi Eczacılık Fakültesi Dergisi (*Ankara Ecz. Fak. Derg.*) Ankara Üniversitesi Eczacılık Fakültesi'nin resmi bilimsel bir dergisidir. 1971 ve 2010 yılları arasında basılı olarak yayımlanmıştır.

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JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

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

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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 international medium, 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. 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 The manuscript submitted to the journal should not be published previously as a whole or in part and not be submitted elsewhere.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.

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Doi: 10.33483/jfpau.1269790

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND CHARACTERIZATION OF ASCORBIC ACID LOADED POLYELECTROLYTE CHITOSAN-GELATIN HYDROGELS

ASKORBİK ASİT İÇEREN POLİELEKTROLİT KİTOZAN-JELATİN HİDROJELLERİN GELİŞTİRİLMESİ VE KARAKTERİZASYONU

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ABSTRACT

Objective: *Aim of study was to formulate chitosan-gelatin hydrogels containing ascorbic acid, an antioxidant, with/without polyelectrolyte-complex.*
Material and Method: *Effect of formation polyelectrolyte-complex, gelatin concentration (10-20%) and chitosan:gelatin ratio(1:1, 1:2, 2:1w/w) on the rheological properties, in-vitro release, encapsulation efficiency of hydrogels were investigated. Dissolution rates were also compared using area under dissolution curve (AUC), mean dissolution time (MDT), mean residence time (MRT). Also, the potential for topical use of the hydrogel was evaluated by examining the 24-and 72-hours cytotoxic and proliferative effects on L929 cell line using MTT test.*
Result and Discussion: *Polyelectrolyte complex formation led to improved drug release and increased viscosity. Cell viability of the free and drug-loaded polyelectrolyte-hydrogels was over 70% at the end of the 72h in all formulations (except formulations with chitosan:gelatin ratio of 1:2w/w) showed that ascorbic acid and hydrogels did not cause cellular toxicity and could be used safely. It has been demonstrated that the gelatin ratio should be at most 50%, and excess gelatin reduces cell viability. F6-coded-polyelectrolyte-hydrogel (20% gelatin; 2:1 chitosan:gelatin w/w) was ideal formulation as it led to best sustained drug release with high MDT and AUC values, and cell viability >80%. In conclusion, polyelectrolyte-complex formation is more superior, and chitosan:gelatin ratio and gelatin concentration can be manipulated to obtain the desired properties.*
Keywords: *Ascorbic acid (vitamin C), chitosan, gelatin, hydrogels, polyelectrolyte complex chitosan-gelatin*

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Submitted / Gönderilme : 23.03.2023
Accepted / Kabul : 02.05.2023
Published / Yayınlanma : 20.09.2023

ÖZ

Amaç: *Çalışmanın amacı, güçlü bir antioksidan olan askorbik asit içeren, polielektrolit kompleksi olan ve olmayan kitozan-jelatin hidrojelleri formüle etmektir.*

Gereç ve Yöntem: *Polielektrolit kompleksi oluşumunun, jelatin konsantrasyonunun (%10-20) ve kitozan:jelatin oranının (1:1, 1:2, 2:1 a/a) reolojik özellikler, in vitro salım ve enkapsülasyon etkinliği üzerindeki etkisi araştırılmıştır. Salım sonuçları AUC, MDT ve MRT kullanılarak karşılaştırılmıştır. Ayrıca MTT testi kullanılarak L929 hücre hattı üzerindeki 24 ve 72 saatlik sitotoksik ve proliferatif etkileri incelenerek geliştirilen hidrojelin topikal kullanım potansiyeli değerlendirilmiştir*

Sonuç ve Tartışma: *Polielektrolit kompleksi oluşumu, ilaç salımının gelişmesine ve viskozitenin artmasına yol açmıştır. Boş ve ilaç yüklü polielektrolit hidrojellerin hücre canlılığının 72 saat sonunda tüm formülasyonlarda (kitozan:jelatin oranı 1:2 a/a olan formülasyonlar hariç) %70'in üzerinde olması, askorbik asit ve hidrojellerin hücresel toksisiteye neden olmadığını ve güvenle kullanılabilir olduğunu göstermektedir. Jelatin oranının en fazla %50 olması gerektiği ve fazla jelatinin hücre canlılığını azalttığı kanıtlanmıştır. Sonuç olarak F6 kodlu polielektrolit hidrojel (%20 jelatin; 2:1 a/a kitozan:jelatin), yüksek MDT ve AUC değerleri ve >%80 hücre canlılığı ile en uzun kontrollü ilaç salınımına yol açtığı için ideal formülasyondur. Sonuç olarak, polielektrolit kompleks oluşumu daha uygundur ve istenen özellikleri elde etmek için kitozan:jelatin oranı ve jelatin konsantrasyonu manipüle edilebilir.*

Anahtar Kelimeler: *Askorbik asit (vitamin C), hidrojeller, jelatin, kitozan, polielektrolit kompleks kitozan-jelatin*

INTRODUCTION

Ascorbic acid (vitamin C) (AA) is vital for the performance of many important metabolic and physiological functions. There are many studies on topical formulations containing AA and its derivatives to strengthen skin tissues, increase collagen synthesis, reduce pigmentation loss and induce enhanced growth and health activities. AA is a popular antioxidant and due to this effect, it is used as anti-aging and photoprotective agent in cosmetic formulations, as well as being added to various formulations as an antioxidant [1]. Antioxidants can scavenge toxic free radicals and other reactive oxygen species (ROS) formed in cell metabolism. With their talent to avoid the negative effects of free radicals, they are crucial to protect the structural integrity and functions of cells and tissues [2-4]. Although AA has been widely studied, it is still important to prepare different formulations and to investigate its potential contribution to many different aspects of cell metabolism, not only for its antioxidant properties but also for many different purposes, including its anticancer effect [5].

Chitosan is a cationic, hydrophilic, natural copolymer with biocompatibility and biodegradability properties, which are also important for biological devices. Chitosan-based hydrogels have the ability to serve many purposes, including tissue engineering and wound healing. In addition, the fact that chitosan is suitable for use as a controlled release system makes chitosan-based hydrogels suitable drug delivery systems for use in medical and pharmaceutical applications [6,7]. Chitosan is often used with other polymers to improve its biological and mechanical properties. The use of chitosan in combination with gelatin contributes to the enhancement of the biological activity of chitosan by increasing cell adhesion, cell migration and forming a polyelectrolyte complex. Gelatin also contributes to the improvement of the wettability and water absorption abilities of chitosan [8,9].

Gelatin is a partially denatured derivative of collagen, a protein that is the main component of the extracelluler matrix in skin, bone and connective tissue. Gelatin is a suitable polymer for use as a biomaterial due to being biocompatible, biodegradable and non-immunogenic [9,10]. A polyelectrolyte complex is formed by the electrostatic interactions of the negatively charged carboxylic groups of gelatin and the positively charged amino groups of chitosan, and the intermolecular hydrogen bonds [7,11]. Since gelatin is known to have a negative charge above pH 4.7 (isoelectric point) [12], it is essential to raise the pH of chitosan >4.7 to form a polyelectrolyte complex between the positively charged chitosan and gelatin.

There has been numerous studies to show films/scaffolds containing chitosan and/or gelatin are

suitable for tissue engineering [7-10,13]. However, there are limited studies to explain drug-loaded polyelectrolyte complex formed chitosan-gelatin hydrogel/film/scaffold. In a study conducted by Mathew and Arumainathan (2022), dopamine loaded chitosan/gelatin nanocomposite were produced and pH-related cross-linking were confirmed [14]. In addition, to our knowledge, no prior studies have examined AA-loaded chitosan-gelatin drug delivery system. In a study in which chitosan-gelatin sponge was produced for wound healing, AA was used to enhance the mechanical properties of the product by increasing the solubility of chitosan. In the study of Lu et al. (2016), firstly chitosan was dissolved in AA solution and then acetic acid was added [15]. However, AA was not used for drug loading as in present studies and were not evaluated in this sense. Therefore, our study will be first in the literature in terms of producing a polyelectrolyte complex structured chitosan-gelatin hydrogel and producing an AA-loaded chitosan-gelatin drug delivery system.

L929 cells are mouse fibroblast cells and are frequently preferred to figure out cytotoxic concentrations of various samples [16]. They are also designated as reference cells for cytotoxicity testing by international standards [17,18].

The aim of this study was to produce chitosan-gelatin hydrogels containing AA, an antioxidant drug, with and without polyelectrolyte complex between chitosan and gelatin, and to determine the rheological properties, *in vitro* release, encapsulation efficiency and cytotoxicity and proliferation on L929 cell lines. Dissolution rates were also compared using area under the dissolution curve (AUC), mean dissolution time (MDT) and mean residence time of the drug substance molecules in the dosage form (MRT).

MATERIAL AND METHOD

Materials

Chitosan (medium molecular weight, 200-800 cP, 1wt% in 1% acetic acid), 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) for cell culture were purchased from Sigma (USA). Gelatin (bloom 250-270 g) was gifted from Halavet (Turkey). AA was purchased from Riedel-de Haën (Germany). Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) was provided from Biochrom, Germany. The NCTC clone 929 [L cell, L-929, connective mouse tissue) was provided from the American Type Culture Collection (ATCC® CCL-1™), USA. Plates and cell culture flasks were purchased from Corning® Cedex (France). Smart Slides and Trypan Blue solution were purchased from Roche (Switzerland). All other chemicals were analytical grade.

Quantification of AA

Amount of AA was measured using UV-spectrophotometer (Shimadzu 1800, Japan). The maximum spectrum of AA was scanned between 200 nm and 600 nm. AA solutions (5-50 µg/ml) were prepared using a stock solution of AA in the dark. The standard curve was calculated by linear regression, according to the following formula: y=ax+b (x: the concentration of AA as µg/ml, y: the absorbance).

Preparation of Chitosan-gelatin Hydrogels

In our study, polyelectrolyte chitosan-gelatin hydrogels (PCGHs) were produced by making some revisions in the preparation of chitosan-gelatin biomaterials produced by 3D printing by Ng et al. and Fischetti et al. [7,19]. Firstly, chitosan and gelatin solutions were prepared. 1% w/v chitosan was dissolved in acetic acid (2% v/v). 10% and 20% w/v gelatin was dissolved in phosphate buffer saline (pH:7.4) at 40°C. They were stirred on a magnetic stirrer for 6 hours. Since the pH of the chitosan solution needed to be adjusted above 4.7 for the chitosan and gelatin to form a polyelectrolyte complex, the pH was adjusted to 4.8 using 0.5 M sodium hydroxide. AA was added to the gelatin solution at a concentration of 100 µg/ml in each hydrogel and stirred for 30 minutes. AA solutions and formulations containing AA were stored in the dark. Consequently, gelatin and chitosan solutions were added dropwise in different ratios (1:1, 1:2 and 2:1 w/w) during mixing and they were stirred for 2 hours. AA-loaded chitosan-gelatin hydrogels prepared in the study are given in Table 1. In the study, non-pH

adjusted chitosan-gelatin hydrogels (NPCGHs) were also produced without adjusting the pH of chitosan. NPCGHs were designated F1A-F6A.

Table 1. Compositions and characterization parameters of ascorbic acid-loaded chitosan-gelatin hydrogels[a]

	F1	**F2**	**F3**	**F4**	**F5**	**F6**
Chitosan (%) (w/v)	1	1	1	1	1	1
Gelatin (%) (w/v)	10	10	10	20	20	20
Chitosan:Gelatin Ratio (w/w)	1:1	1:2	2:1	1:1	1:2	2:1
Viscosity of PCGHs[b*] (mPa.s)	63.0±0.4	73.4±1.5	42.2±0.1	402.8±1.9	776.9±0.7	281.3±3.5
Viscosity of NPCGHs[c*] (mPa.s)	48.6±0.2	56.1±1.1	48.6±0.4	271.9±0.2	578.1±1.8	97.7±0.2
Encapsulation efficiency of PCGHs (%)	97.4±2.1	94.6±2.0	91.3±1.8	82.4±1.6	81.8±1.2	80.5±1.1

[a]Values are expressed as mean ± standard deviation, $n = 3$.
[b]Polyelectrolyte chitosan-gelatin hydrogels
[c]non-pH adjusted chitosan-gelatin hydrogels
[*]Viscosities at 200 rpm are given.

Characterization of Formulations

Viscosity

The viscosity of the solutions was performed with Brookfield DV3T Rheometer (USA) at 25°C and 37°C at 50-250 rpm (spindle: CP-52). Results were expressed as mean ± standard deviation (n=3).

Encapsulation Efficiency

AA-loaded hydrogels (2 ml) and blank hydrogels were diluted into 40 ml pH 7.4 phosphate buffer. They were centrifuged for 1 h at 25°C (Thermo Scientific SL16R, USA) and supernatants were collected (n=3). The supernatant collected from the blank hydrogels was taken as a blank and the amount of AA in the supernatants was analyzed by UV spectrophotometer at 287 nm and the encapsulation efficiency was calculated [20]. Data were expressed as mean ± SD. The encapsulation efficiency was calculated using the following equation.

$$\text{Encapsulation efficiency (EE\%)} = [(E_{initial} - E_{supernatant}) / E_{initial}] \times 100$$

In Vitro Release

In vitro release study was performed by placing a 12,000 Dalton pore size dialysis membrane between the receptor and donor chambers of Franz diffusion cells for 24 hours at 37°C. One ml of hydrogel was placed in the donor chamber and 2 ml of pH 7.4 phosphate buffer in the receptor chamber and stirred on a magnetic stirrer at 400 rpm during the experiment (n=3). At predetermined time intervals (2^{nd}, 5^{th}, 8^{th} and 24^{th} hours), 2 ml samples were collected and 2 ml of fresh buffer was added. The amount of released AA was determined by UV spectrophotometer. Drug release from the AA solution at the same concentration as the drug in the hydrogel was also determined.

For dissolution rate comparison, AUC, MRT and MDT were calculated by DDSolver Software and equations of dissolution rate parameters were given in Table 2 [21,22].

Cell Culture Studies

L929 cells were grown in an incubator under 5% CO_2 atmosphere at 37ºC with a medium consisted of EMEM containing 25 mM glucose, 1% gentamicin, 5 mM glutamine supplemented with 10% horse serum, and 7.5% sodium bicarbonate. A microscope was used to control the presence of a

confluent monolayer. The medium was replaced with fresh EMEM every 48 hours.

Table 2. Equations of dissolution rate parameters

Dissolution Rate Parameter	Equation
AUC	$AUC = \sum_{i=1}^{n} \frac{(t_i - t_{i-1})(y_{i-1} + y_i)}{2}$
MDT	$MDT = \frac{\sum_{i=1}^{n} \overline{ti} \,.\, \Delta Mi}{\sum_{i=1}^{n} \Delta Mi}$
MRT	$MRT = \frac{\int_0^t t\ (100 - y).dt}{\int_0^t (100 - y)\,.dt}$

n number of sampling points; t*i* *i*th time point; y_i percentage of drug dissolved at time t_i; y percentage of drug dissolved at time t; $\overline{ti}$ time at the midpoint between i and i–1; ΔM_i additional amount of drug dissolved between i and i–1; k order of the moments of dissolution times

Cytotoxicity Assay

MTT test was used for cell viability on L929 cells. L929 cells were seeded (10.000 cells/well) in three different 96-well culture plates [4]. Plates were stored for 24 hours at 37ºC to adherence of cells. Then, the cells were treated with blank and AA-loaded formulations for 24 hours in parallel with *in vitro* release study and for 72 hours to evaluate the proliferation effect. After incubation, the medium was replaced with 13 µl MTT solution (5 mg/ml in phosphate-buffered saline) and 100 µl fresh medium. Then it was incubated for 4 hours at 37°C and 100 µl of DMSO was added to each well to dissolve the formazan precipitate. The colour density was measured at 570 nm with a multi-well ELISA reader (Biotech Synergy HT, USA). The control group, wells containing medium only, were considered 100% cell viability, and the viability of the samples was calculated as a percentage using the control group values.

Statistical Analysis

SPSS 20.0 for Windows (SPSS, Chicago, IL) were used for statistical analysis. The significance was evaluated with one-way ANOVA followed by Tukey's post hoc test (SPSS 20.0). $p < 0.05$ is considered statistically significant.

RESULT AND DISCUSSION

Quantification of AA

The maximum wavelength of AA was determined as 287 nm. The standard curve was determined by linear regression (y=0.0212x+0.014). The calibration curve was linear with a high correlation coefficient (r^2 =0.9998).

Preparation and Characterizations of Chitosan-gelatin Hydrogels

Compositions and characterization parameters of AA-loaded PCGHs were given in Table 1.

Encapsulation Efficiency and Drug Release From Chitosan-gelatin Hydrogels

Encapsulation efficiencies of PCGHs were between 80.5 and 97.4%, while they were between 82.1 and 95.8% for NPCGHs. The increase in gelatin concentration led to a decrease in encapsulation efficiency. However, all hydrogels have been shown to have favorable encapsulation efficiency (≥81%) (Table 1).

It was shown that all hydrogels provide a more sustained release compared to AA solution. While 92.7% of the drug was released from the solution at the 4th hours and completely finished at the 8th hours,

the drug release in the hydrogel could be prolonged for 24 hours (Figure 1 and 2). AA release from its solution and PCGHs was given in Figure 1.

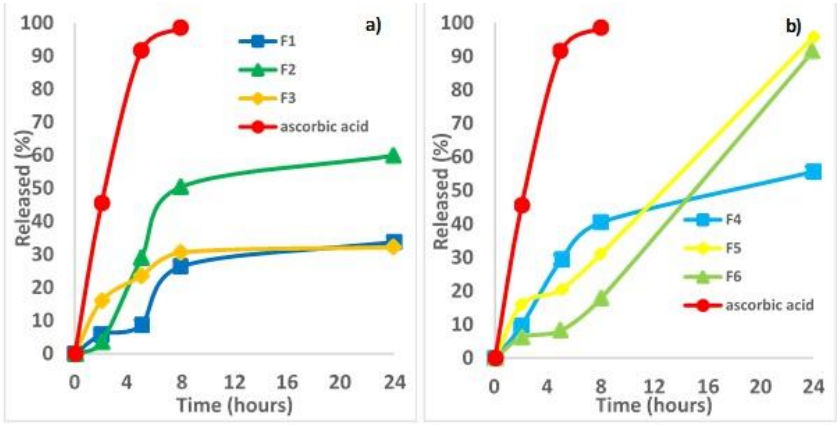

Figure 1. Ascorbic acid release from its solution and polyelectrolyte chitosan-gelatin hydrogels contains a) 10% gelatin and b) 20% gelatin

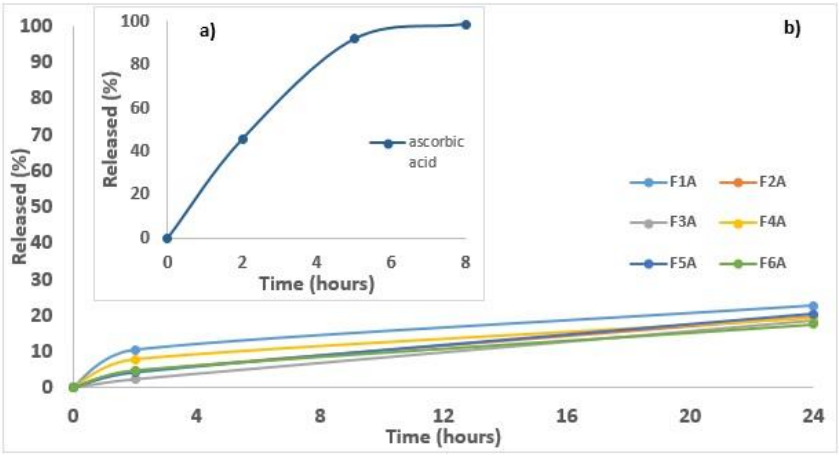

Figure 2. Ascorbic acid release from a) its solution and b) non-pH adjusted chitosan-gelatin hydrogels

AA release from NPCGHs was shown in Figure 2. Drug release was reduced compared to the solution ($p<0.05$). The AA released within 24 hours was only between 18.5% and 22.7% (Figure 2). Drug release in PCGHs was better compared to NPCGHs (Figure 1 and 2) ($p<0.05$). The low cumulative release of AA from NPCGHs was about the anionic character of AA at physiologic pH of 7.4 [23]. In uncomplexed gels (NPCGHs,) the negatively charged chitosan prevented the release of negatively charged AA since chitosan was free in the gel and there was a strong interaction via hydrophobic and/or electrostatic interaction or hydrogen bonding between chitosan and AA at pH 7.4. Sun et al. (2020) claimed that the interaction between the functional group density and the group was the most crucial factor for the drug release from chitosan films [24]. As a result, forming a polyelectrolyte complex had a significant effect on drug release and led to better sustained release profile and improved drug release.

AUC, MRT and MDT results of PCGHs, which were calculated by DDSolver Software, were given in Table 3.

AUC values of F2 and F5, hydrogels with a chitosan:gelatin ratio of 1:2 w/w, were the highest (Table 3). So, hydrogels with higher gelatin content (chitosan:gelatin ratio of 1:2 w/w) showed advanced release characteristics compared to lower gelatin content (chitosan:gelatin ratio of 1:1 and 2:1 w/w)

(Figure 1). This is due to the quicker hydration rate of gelatin and its superior water absorption ability compared to chitosan [8,25].

Table 3. Dissolution rate results (n=3)

Parameter	F1	F2	F3	F4	F5	F6
AUC	561.67	1055.42	659.53	943.49	1164.20	944.34
MRT	10.96	9.67	11.40	9.98	6.18	6.65
MDT	7.37	6.40	3.56	7.05	11.85	13.70

The slower release rate is characterized by high MRT and low MDT. As seen in Table 3, the hydrogel with the slowest drug release rate was the F4-coded hydrogel among the 20% gelatin-containing hydrogels (F4-F6), while the F3-coded hydrogel among the 10% gelatin-containing hydrogels (F1-F3) (Figure 1).

Higher MRT values and lower AUC values at low gelatin concentration for hydrogels containing the same ratio of chitosan:gelatin proved that, the increase in gelatin concentration caused an increase in the amount of AA released in 24 hours (Table 3) (Figure 1) (p<0.05). Therefore, it has proven that higher gelatin concentration led to higher drug release.

As a result, hydrogels coded F5 and F6 were determined as ideal PCGHs as they led to the sustained release with 11.85 and 13.70 MDT values (Table 3), and the cumulative release rate of AA reached 95.8% and 91.7% of AA at 24 h (Figure 2), respectively.

Rheology of Chitosan-gelatin Hydrogels

Viscosities of the hydrogels as a function of shear rate at both room temperature (25°C) and body temperature (37°C) were evaluated. Temperature plays an inhibitory role in the formation of intermolecular attraction forces. According to Figure 3, increasing the temperature from 25°C to 37°C resulted in a decrease in the viscosity of PCGHs ranging from 20-88% (Figure 3) (p<0.05). As the gelatin ratio increased, there was a more significant decrease in the viscosity of the PCGHs with increasing the temperature to 37°C (p<0.05). While PCGHs with a chitosan:gelatin ratio of 1:2 w/w had a viscosity reduction of 62.8% (F2) and 88.1% (F5), they were of 20% (F3) and 74.8% (F6) in PCGHs with a chitosan:gelatin ratio of 2:1 w/w, respectively (Figure 3).

As a result, it was concluded that the increase in concentration and ratio of gelatin led to a higher reduction in the viscosity of the hydrogels as a function of increasing temperature. Because higher temperatures accelerate the destruction of the biopolymer components of gelatin and the formation of low molecular weight fractions [26]. Desbrieres found that the viscosities of chitosan solutions were not dependent on temperature [27]. In addition to literature data, our findings proved the change in viscosity with temperature was dependent on the amount of gelatin, not the amount of chitosan.

The viscosity of PCGHs containing 10% gelatin ranged from 42-89 mPa.s (Figure 3a), while the viscosity of PCGHs containing 20% gelatin ranged from 263-777 mPa.s (Figure 3b). The viscosity of hydrogels containing 20% gelatin was higher than that of containing 10% gelatin, due to the increase in the viscosity of the gelatin solution as the gelatin concentration increased from 10% to 20% (Figure 3 and 4) (p<0.05). The viscosity of PCGHs at 25°C increased 7-9 fold by increasing the gelatin concentration from 10% to 20% (Figure 3).

As can be seen in Table 1 and Figure 3-4, the increase in the gelatin ratio also caused an increase in viscosity (p<0.05). This was probably due to the viscosity of gelatin solutions being more viscous than chitosan solution.

Rheology studies have shown that the viscosities of PCGHs were higher than those of NPCGHs (Figure 4) (p<0.05). This was due to the fact that the polyelectrolyte complex formed by electrostatic interactions and intermolecular hydrogen bonds between chitosan and gelatin resulted in denser zones in the gel network which was described by Nicolay et al. [11]. Ma et al. found that hydrogen bonding increases viscosity and strength [28].

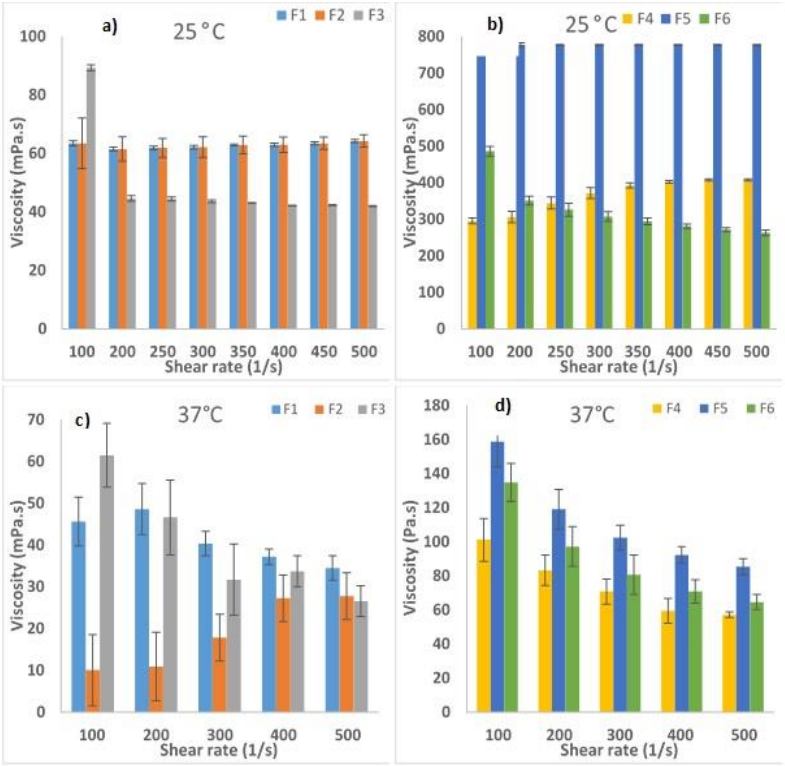

Figure 3. Viscosity as a function of shear rate at different temperatures for PCGHs which containing different ratios of 1% chitosan and a) 10% gelatin at 25°C, b) 20% gelatin at 25°C, c) 10% gelatin at 37°C, d) 20% gelatin at 37°C

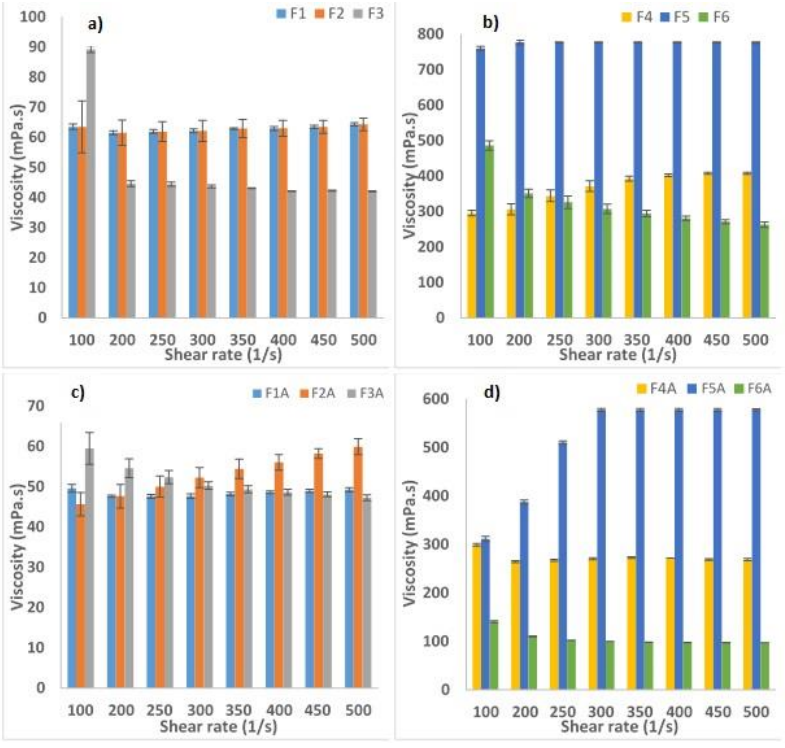

Figure 4. Viscosity as a function of shear rate at 25°C for PCGHs containing a) 10% gelatin, b) 20% gelatin, and for NPCGHs containing c) 10% gelatin and d) 20% gelatin

It is known that pseudo-plastic behaviour is associated with a decrease in viscosity with an increase in shear rate, while dilatant behaviour is associated with an increase in viscosity. In newtonian fluids, the viscosity is constant [29,30]. Hydrogels with high chitosan content (F3, F3A, F6 and F6A) exhibit pseudo-plastic behaviour, while hydrogels with high gelatin content (F2, F2A, F5 and F5A) exhibit dilatant behaviour at 25°C (Figure 3). This may be due to the fact that gelatin exhibits a dilatant and shear-thickening behavior [30], while chitosan exhibits a pseudo-plastic and shear thinning behavior [29].

In addition, newtonian flow was observed in F1, F1A and F4A, while dilatant flow was observed in F4 with a chitosan:gelatin ratio of 1:1 w/w at 25°C (Figure 3). Although the chitosan:gelatin ratio was the same, the PCGH with 20% gelatin (F4) had more gelatin than the PCGH with 10% gelatin (F1). Thus, F4 could exhibit the same flow behaviour as gelatin, while F1 could not exhibit either the gelatin or chitosan flow behaviour. Unlike viscosities at 25°C, all PCGHs had plastic behaviour (except F2) at 37°C, regardless of their chitosan or gelatin ratio.

Cell Culture

The effect of formulation components developed with AA was determined on L929 cell viability. The results are given in Figure 5.

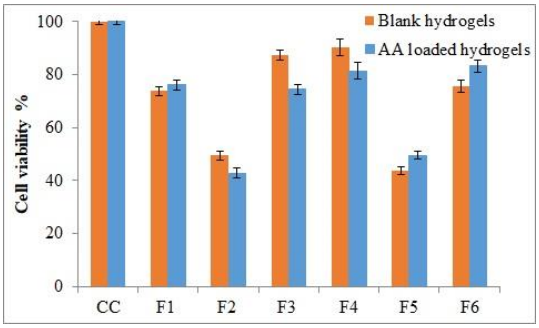

Figure 5. Percentage of cell viability of L929 cells incubated with blank hydrogels and AA-loaded hydrogels for 72 h (CC is cell control, values are expressed as mean ± standard deviation, $n = 6$)

No significant difference in cell viability was observed between the blank hydrogels and the AA-loaded hydrogels (Figure 5) ($p>0.05$).

We measured the effect of formulations on cell viability with the most commonly used method, the MTT test. While the effects of the developed formulations on L929 cell viability were around 50% at 24 h, 70% and above were obtained at the end of the 72 h. The fact that the viability at 72 h was higher than the viability at 24 h showed that the formulations were effective on cell proliferation. According to the literature, the IC_{50} value of AA is 114 µg/ml [31]. In our formulations, 100 µg/ml was used as the initial dose.

According to MTT test results, it was observed that AA and formulations did not cause cellular toxicity. The fact that cell viability was above 70% at the end of the 72 h in all formulations showed that they could be used safely. Also, increasing the gelatin concentration from 10% to 20% enhanced cell viability ($p<0.05$). F4 and F6 coded PCGHs containing 20% gelatin were found to have cell viability over 80%. (Figure 5)

Previous studies have shown that the presence of gelatin increases the cyto-compatibility of chitosan [10]. In our study, while cell viability was over 70% in PCGHs at 72 hours, the viability decreased to 43% (F2) and 50% (F5) with the increase in gelatin concentration to 66.6% (chitosan:gelatin ratio to 1:2 w/w). Adding more gelatin than necessary for complex formation prevented the induction of the cells by electrostatic interaction with the negatively charged L929 cell due to the negative structure of gelatin [32]. In addition, it was found for the first time in our study that the gelatin ratio should be at most 50% for the formation of a suitable chitosan-gelatin polyelectrolyte complex,

and that excess gelatin required for complex formation (>50% gelatin, 1:2 w/w chitosan:gelatin ratio) reduces cell viability.

To sum up, AA-loaded PCGHs with favorable properties such as high encapsulation efficiency, sustained drug release and enhanced cell viability were successfully produced. The effect of gelatin concentration and chitosan:gelatin ratio on the rheological behaviour, *in vitro* release, encapsulation efficiency and cytotoxicity and proliferative effects of chitosan-gelatin hydrogels were determined in detail. AUC, MDT and MRT were also calculated to compare dissolution rates of hydrogels. In the study, NPCGHs were also produced without the polyelectrolyte complex to determine the effect of forming a polyelectrolyte complex on chitosan-gelatin hydrogels. The results of the study confirmed that polyelectrolyte complex formation has proven to be more favorable. It is well known that the addition of gelatin to chitosan has a positive effect on cell viability. However, the study first concludes that there is a limit to the gelatin ratio (≤ 50%) for the formation of a suitable chitosan-gelatin polyelectrolyte complex, and excess gelatin reduces cell viability. As a result of this study, F6 coded hydrogel containing 20% gelatin and a chitosan:gelatin ratio of 2:1 w/w was determined as the ideal formulation as it provided best sustained drug release with high MDT and AUC values. Also, the cell viability of F6 was found to be over 80% (83.2%) at a concentration of 74 µg/ml obtained from the release experiment. In conlusion, it is possible to say that the properties such as drug release, viscosity and cell viability of well-characterized hydrogels that allow topical application of AA and many antioxidants and provide a significant advantage in increasing their effectiveness, can be affected by gelatin concentration and chitosan/gelatin ratio.

AUTHOR CONTRIBUTIONS

Concept: T.E.B., Ç.Y.; Design: T.E.B., Ç.Y.; Control: T.E.B., Ç.Y.; Sources: T.E.B., Ç.Y.; Materials: T.E.B., Ç.Y.; Data Collection and/or Processing: T.E.B., Ç.Y.; Analysis and/or Interpretation: T.E.B., Ç.Y.; Literature Review: T.E.B.; Manuscript Writing: T.E.B., Ç.Y.; Critical Review: T.E.B.; Ç.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

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., 47(3): 731-738, 2023
Doi: 10.33483/jfpau.1112778

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIANGIOGENIC ACTIVITY AND ROS-MEDIATED LUNG CANCER CELL LINE INJURY OF ZERUMBONE

ZERUMBONE'UN ANTİANJİYOJENİK AKTİVİTESİ VE ROS ARACILI AKCİĞER KANSERİ HÜCRE HATTI HASARI

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ABSTRACT

Objective: *Zerumbone (ZER) is a well-known natural compound that has been reported to have anti-cancer effect. Thus, this study investigated the ZER potential to inhibit Thymidine Phosphorylase (TP) and the ability to trigger Reactive oxygen species (ROS)-mediated cytotoxicity in non-small cell lung cancer, NCI-H460, cell line.*
Material and Method: *The antiangiogenic activity for ZER was evaluated by using the thymidine phosphorylase inhibitory test. Reactive oxygen species (ROS) production was determined via DCFDA dye by using flow cytometry.*
Result and Discussion: *ZER was found to be potent TP inhibitory with the IC_{50} value of 50.3±0.31 µg/ml or 230±1.42 µM. NCI-H460 cells upon treatment with ZER produced significant ROS by 55.7%. Consequently, ZER exerts anti-angiogenic properties and modulates ROS production in lung cancer cells, serving as leads for better therapeutic index in cancer drug.*
Keywords: *Flow cytometry, NCI-H460, thymidine phosphorylase*

ÖZ

Amaç: *Zerumbone (ZER), kanser önleyici etkisi olduğu bildirilen, iyi bilinen bir doğal bileşiktir. Bu nedenle, bu çalışma, ZER'in Timidin Fosforilaz'ı (TP) inhibe etme potansiyelini ve küçük hücreli dışı akciğer kanser, NCI-H460, hücre hattında Reaktif oksijen türleri (ROS) aracılı sitotoksisiteyi tetikleme yeteneğini araştırmıştır.*
Gereç ve Yöntem: *ZER'in antianjiyogenik aktivitesi, timidin fosforilaz inhibitör testi kullanılarak değerlendirilmiiştir. Akış sitometrisi kullanılarak DCFDA boyası ile reaktif oksijen türleri (ROS) üretimi belirlenmiştir.*
Sonuç ve Tartışma: *ZER'in 50.3±0.31 µg/ml veya 230±1.42 µM IC_{50} değeri ile güçlü bir TP inhibitörü olduğu bulunmuştur. ZER ile işleme tabi tutulduktan sonra NCI-H460 hücrelerinin, %55.7 gibi yüksek bir oranda ROS ürettiği bulunmuştur. Sonuç olarak, ZER'in, anti-anjiyojenik*

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Submitted / Gönderilme : 05.05.2022
Accepted / Kabul : 09.05.2023
Published / Yayınlanma : 20.09.2023

özellikler sergilediği ve akciğer kanseri hücrelerinde ROS üretimini modüle ederek kanser tedavisinde daha iyi terapötik indeks sunduğu tespit edilmiştir.
Anahtar Kelimeler: *Akış sitometrisi, NCI-H460, timidin fosforilaz*

INTRODUCTION

Angiogenesis is a process of the formation of new blood vessels from pre-existing vasculature. This process is very crucial for tumor growth and progression of cancer [1]. To enhance the process of angiogenesis, thymidine phosphorylase (TP) enzyme plays a vital role as growth factor by promoting endothelial cell migration and the release of different angiogenic factors from malignant and stromal cells in the tumor microenvironment site which play critical roles in the evasion of apoptosis and immune cells [2]. In various cancers, up regulation of angiogenesis plays vital role in the progression of glioblastoma [3], melanoma [4], therefore many compounds are under pre-clinical trial to block angiogenesis pathway. Among different triggering factors, thymidine phosphorylase enzyme has received special attention due to its outstanding ability to trigger vascular growth; therefore this enzyme has been used as a validated target to find antiangiogenic compounds [5].

Reactive oxygen species (ROS) are known potential carcinogens and act as a key player in the progression of mutagenesis by converting normal cells into malignant cells, but its increasing might exploit selective killing leading to ROS-mediated cancer cell injury. The anticancer effect of some chemotherapeutic drugs includes elevation of intracellular levels of ROS which raise the level of oxidative stress in cancer cells and causes ROS mediated cell injury leading to cell death. Most chemotherapeutics often failed because of cytotoxicity and drug resistance [6]. Thus, it is necessary to have safe natural products which can modulate intracellular ROS such that it can prevent cell signaling and cellular functions, thereby preventing the progression and metastasis of cancers [7].

Since ancient time, mankind at different geographical locations had been using different plants, animals, fungal, minerals for treating different diseases [8-11]. Recently, many researches are investigating on natural compounds from different plants for their anti-cancer potential and this approach has led to the discovery of many new therapeutic agents [12,13]. Among various anticancer mechanisms proposed, antiangiogenic and modulating intracellular ROS mechanism has also important role, in which immune systems are given opportunity to identify and respond to the tumor cells [14]. ZER, a natural sesquiterpene from edible ginger *Zingiber zerumbet* Smith, has anti-inflammatory, immunomodulatory, antibacterial [15-17], antioxidant, antinociceptive, and anti-proliferation activities against several cancer cells [18-20]. Even though, the pharmacological studies had shown the anti-cancer effects of ZER through the induction of apoptosis on human lung cancer cells [21], the anti-angiogenic activity of ZER and its possible anticancer capacity by modulating intracellular ROS have not been clearly evidenced yet. In the current study, new mechanism of ZER was explored by targeting against thymidine phosphorylase to inhibit angiogenesis. Together, generation of ROS in NCI-H460 cell was also evaluated after ZER treatment.

MATERIAL AND METHOD

ZER crystals were gifted by Professor Dr. Rasedee Abdullah from Universiti Putra Malaysia (UPM), Malaysia. The crystals were diluted with 0.1% dimethyl sulfoxide (DMSO) to obtain the stock solution.

In vitro Thymidine Phosphorylase Inhibition

The protocol of Iftikhar et al [22] was followed. All the chemicals used in this assay were taken from Molecular Bank of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan.Potassium phosphate buffer with 50 mM and pH 7.0 was prepared and 150 µl of this buffer was kept in tube. In this buffer, 20 µl of TP enzyme (0.058 unit/well) was added. To this solution, 10 µl of test compound (1 mg/ml) was added and whole reaction mixture was incubated at 30^{o}C for 10 min. After incubation, 20 µl thymidine substrate (1.5 mM) was added and continuously monitored at 290 nm to detect any change in the absorbance. This observation was continued for 15

min. Absorbance was taken in 96-well ELISA plate reader (MultiSkan go, ThermoFisher Scientific). For positive control, 7-Deazaxanthine was used as standard drug. Every experiment was run in triplicate.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay of ZER on NCI-H460 Cell Line

The human lung cancer, NCI-H460, cell line was obtained from cell culture bio bank of Dr. Panjwani Center for Molecular Medicine and Drug Research, ICCBS, University of Karachi. The protocol of Albaayit et al [23] was followed in which cells were cultured and passaged using DMEM medium. MTT assay was used to find the cytotoxic effect of the ZER on the NCI-H460 cell lines. After trypsinization, 5×10^5 NCI-H460 cells/well were counted and dispensed in each well in a 96-well plate and incubated at 37°C, 5% CO_2. Next day, cells were properly attached at the bottom of the wells. Cells were then treated with ZER at different concentrations (3.125 to 25 µg/ml), while the solvent control was treated with 0.5% DMSO and incubated for 48 h. On this cell suspension, 20 µl of MTT (0.5 mg/ml) reagent was added and the plate incubated for 4 h at 37°C. MTT dye upon reduction formed the purple formazan crystals at the bottom of the wells. These crystalswere dissolved with 100 µl DMSO and the absorbance measured at 540 nm using an ELISA plate (Multiskan GO, Thermo Scientific). Standard drug doxorubicin at 0.501 µg/ml was used as a positive drug control. The percentage of cytotoxicity of NCI-H460 was determined by using below formula.

$$\%\ \text{cytotoxicity/inhibition} = 100 - \frac{\text{O.D of treated well} - \text{O.D of media control}}{\text{O.D of untreated control} - \text{O.D of media control}}\ \text{X } 100.$$

Reactive Oxygen Species (ROS)

The protocol of Rasul et al [24] was followed to find ROS production by the NCI-H460 cells. Fully grown NCI-H460 cells were trypsinized from culture flask and counted. Approximately 1×10^6 NCI-H460 cells in 500 µl were seeded into each well of 24-well plate. Next day, these cells were treated with 3.05 µg/ml or 14.3 µM of ZER and the plate incubated for 48 h. Cells treated with hydrogen peroxide (50 µM) was considered as positive control. After treatment, the cells were then detached by trypsinization, washed with PBS, and 500 µl of 10 µM 2,7-dichlorofluorescein DCFH-DA dye was added and incubated for 30 min at 37°C in the dark. These cell suspensions were transferred to FACS tube and analyzed the ROS generation by using flow cytometry (FACSCaliburTM Cell Quest Pro Software version 2.0).

RESULT AND DISCUSSION

TP is an important angiogenic enzyme in the malignant progression, which induces migration and angiogenesis in endothelial and tumor cells. It is well known that TP provide pentose for tumor cells from the catalyzing thymidine, augment the expression of several angiogenic growth factors and trigger tumor angiogenesis [25]. There are many drugs that are being used to block angiogenesis and among these are tipiracil, trifluridine. However, these drugs are often compromised with serious side effects including weakness, vomiting, diarrhea, stomatitis, and dysgeusia [26].

Drugs to be used for the treatment for the condition should not only be efficacious but also not to cause significant adverse effects [27-30]. Recently, cancer researchers are focused on the natural sources, especially on traditional medicinal and edible types for the treatment of tumor and for controlling of angiogenesis and metastasis due to their safety issue [31]. In the present study, ZER exhibited TP inhibition with IC_{50} values of 50.3 ± 0.31 µg/ml or 230 ± 1.42 µM. The standard drug (7-deazaxanthine) showed IC_{50} value of 41.0 ± 1.63 µM [23]. Based on the outcome of TP enzyme inhibition study, the potential of ZER to have antiangiogenic effect was observed. This study gave supporting evidence to the previous study by Shamoto et al., 2014 [32] in which zerumbone was reported to inhibit pancreatic cancer through inhibiting angiogenesis process by blocking NF-κB activity.

ZER was reported to have inhibitory effect on several enzymes like pancreatic lipase [33], carbonic anhydrase [33], murA [34], trypsin protease [35], TNF-alpha [36]. From the findings of these similar previous studies, the present study propose that ZER is potential inhibitors of Thymidine

phosphorylase enzyme by interacting to the amino acid residues in the active site of enzyme, and prevent the interaction between enzyme and corresponding substrate and growth of cancer.

Various evidences have shown the role of ROS concentration in the tumor microenvironment and its progression towards malignant state. Numbers of evidences have shown the dual nature of ROS i.e tumor-suppression [37,38] and tumor-promotion effects [39], depending on the level of ROS concentration in the state of tumor cells and its surrounding microenvironment [40]. Overexposure of ROS is toxic to cells and can initiate apoptotic and necrotic pathway [41,42]. The behavior of tumors in response to ROS depends on the threshold concept, in which increase in ROS causes cancer cells to undergo in an adaptive state and later cells will perish upon exceeding the threshold limit [43,44]. The current study showed that ZER was active against NCI-H460 cells and IC_{50} value was found to be 3.05±0.01 µg/ml (Figure 1). NCI-H460 cells, after treatment with 3.05 µg/ml or 16 µM ZER for 24 h, significantly increased ROS by 55.7%. Negative treated cells didn't show increase in ROS whereas, H_2O_2 treated cells showed significant rise in fluorescence due to high ROS production (Figure 2).

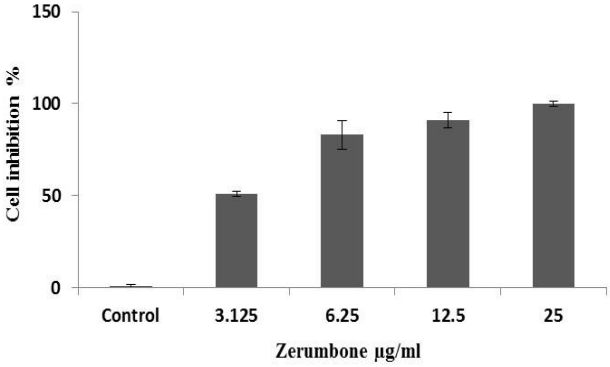

Figure 1. Determination of the cell viability of lung cancer (NCI-H460) cells after treatment with ZER for 48 h exposure

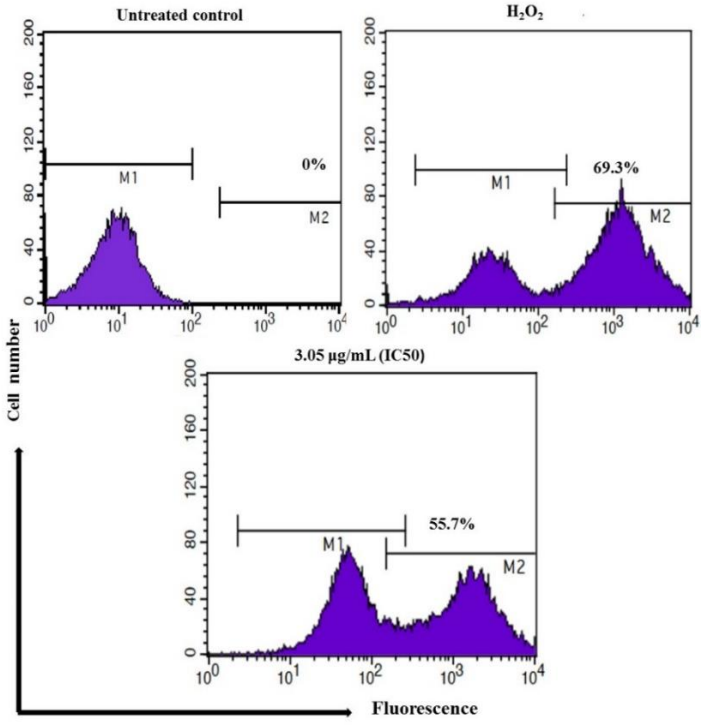

Figure 2. ROS production by NCI-H460 cells after treatment with 3.05 µg/ml of ZER. Cells treated with Hydrogen peroxide (H_2O_2) were the positive control

The present ROS induced cancer cell death result was similar to the previous manuscript in which ZER was reported to produce significant ROS from non-small cell lung cancer line beyond the protective effect of cellular anti-oxidant defense mechanism, resulting in damage to cell membrane and finally cell death [21,45]. ROS kill cancer cells by triggering both intrinsic and extrinsic apoptotic pathways [46]. In addition, zerumbone was reported to have ROS induced cytotoxicity against Glioblastoma multiforme [47], breast cancer [48], colorectal cancer [49], and chronic myelogenous leukemia cells [50,51].

In conclusion, the present study provides supporting evidence for zerumbone to have antiproliferative activity against non-small cell lung cancer through thymidine phosphorylase inhibition and ROS induced cytotoxicity. This support antiangiogenic mechanism of action of zerumbone against many cancers cell lines through suppressing the vascular growth around the tumor.

ACKNOWLEDGEMENTS

The corresponding author is thankful to Prof. Dr. M. Iqbal Choudhary for providing NAM-ICCBS fellowship, 2018 in International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan.

AUTHOR CONTRIBUTIONS

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

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., 47(3): 739-751, 2023
Doi: 10.33483/jfpau.1232868

ORIGINAL ARTICLE / ÖZGÜN MAKALE

CURCUMIN, THE BIOACTIVE COMPOUND OF TURMERIC, MAY IMPROVE THE ANTI-MALIGNANT PROPERTY OF GEMCITABINE IN PROSTATE CANCER CELLS

ZERDEÇALIN BİYOAKTİF BİLEŞİĞİ KURKUMİN, GEMSİTABİNİN PROSTAT KANSERİ HÜCRELERİNDEKİ ANTİ-MALİGNANT ÖZELLİĞİNİ GELİŞTİREBİLİR

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ABSTRACT

Objective: *The aim of this study was to investigate the possible synergistic effect of curcumin on the anticancer features of gemcitabine on prostate cancer cells.*
Material and Method: *The human prostate adenocarcinoma cell line LNCaP was used in the studies. The effect of the co-administration of gemcitabine and curcumin on the viability of LNCaP cells was investigated by the WST-1 assay. Autophagy, ubiquitin-proteasome system (UPS), unfolded protein response (UPR) and cell death-associated proteins, androgenic signaling, proto-oncogenic, angiogenic and epithelial-mesenchymal transition (EMT) associated protein levels were investigated by immunoblotting studies.*
Result and Discussion: *Our results showed that curcumin potentiated the anticancer effects of gemcitabine on LNCaP cells. Co-administration of curcumin and gemcitabine strengthened the suppressive effect of gemcitabine on cell viability. Moreover, co-administration modulated the autophagy, more strongly stimulated UPS and UPR, suppressed androgenic signaling, led to the activation of cell death-related poly [ADP-ribose] polymerase 1 (PARP-1) and caspase-3 and strongly suppressed the expression levels of proto-oncogenic c-Myc and angiogenic vascular*

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Gönderilme / Submitted : 11.01.2023
Kabul / Accepted : 09.05.2023
Yayınlanma / Published : 20.09.2023

endothelial growth factor-A (VEGF-A). In addition, it was determined that co-administration negatively regulated EMT by stimulating E-cadherin expression and suppressing N-cadherin level. These results suggest that the combined usage of gemcitabine and curcumin may offer a potent therapeutic approach for prostate cancer by enhancing the anticancer effects of gemcitabine.
Keywords: *Autophagy, curcumin, gemcitabine, unfolded protein response, prostate cancer*

ÖZ

Amaç: *Bu çalışmanın amacı kurkuminin gemsitabinin prostat kanseri hücreleri üzerindeki antikanser özelliklerine olan olası sinerjistik etkisinin araştırılmasıdır.*
Gereç ve Yöntem: *Çalışmalarda insan prostat adenokarsinoma hücre hattı LNCaP kullanıldı. Gemsitabin ve kurkuminin birlikte uygulanmasının LNCaP hücrelerinin canlılığı üzerindeki etkisi WST-1 yöntemiyle araştırıldı. Otofaji, ubikitin-proteazom sistemi (UPS), katlanmamış protein yanıtı (UPR) ve hücre ölümü ile ilişkili proteinler, androjenik sinyal, proto-onkojenik, anjiyojenik ve epitelyal-mezankimal geçiş (EMT) ile ilişkili protein düzeyleri immünoblotlama çalışmaları ile incelendi.*
Sonuç ve Tartışma: *Sonuçlarımız kurkuminin gemsitabinin LNCaP hücreleri üzerindeki anti kanser etkilerini güçlendirdiğini gösterdi. Kurkumin ve gemsitabinin eş uygulaması gemsitabinin hücre canlılığı üzerindeki baskılayıcı etkisini güçlendirdi. Bununla birlikte eş uygulamanın otofajiyi düzenlediği, UPS ve UPR'yi daha güçlü uyardığı, androjenik sinyali baskıladığı, hücre ölümü ile ilişkili PARP-1 ve kaspaz-3 aktivasyonuna yol açtığı, proto-onkojenik c-Myc, anjiyojenik VEGF-A ifade düzeylerini güçlü şekilde baskıladığını gösterdi. Ayrıca eş uygulamanın E-kaderin ifadesini uyararak ve N-kaderin düzeyini baskılayarak EMT'yi negatif düzenlediği belirlendi. Bu sonuçlar, gemsitabin ve kurkuminin birlikte kullanımının, gemsitabinin antikanser etkilerini geliştirerek prostat kanserine yönelik güçlü bir terapötik yaklaşım sunabileceğini düşündürmektedir.*
Anahtar Kelimeler: *Gemsitabin, katlanmamış protein yanıtı, kurkumin, otofaji, prostat kanseri*

INTRODUCTION

Cancer is a significant health problem and one of the main causative factors for mortality worldwide [1]. Among various cancer types, prostate cancer is the second most frequently diagnosed cancer type in men after skin cancer. According to American Cancer Society (ACS) statistics, 268.490 new cases and 34.500 deaths were recorded in 2022 [2]. Despite androgen deprivation therapy, immunotherapy, radiation and chemotherapy are the primary treatment approaches for prostate cancer, the side effects depending on long-term therapy or acquired resistance limit the efficacy of the treatment [3]. Therefore, to reduce these undesirable effects, natural products offer a good option for many years. The role of natural compounds on cancer progression and their combined application with existing chemotherapeutics, including doxorubicin, docetaxel, paclitaxel and gemcitabine, have been intensively studied around the world [4,5]. The discovery of natural bioactive products with high efficacy and safety and their advanced characterization are being extensively studied worldwide. Curcumin is one of the most studied natural products in terms of its biochemical effects *in vitro* and *in vivo*.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) which is a component of turmeric (*Curcuma longa*) has numerous biological activities, including anti-inflammatory, antioxidant, anti-diabetic, anti-viral and anti-tumor [6-9]. Besides, it has been reported that curcumin has potent therapeutic effects on arthritis, wound healing, Alzheimer's disease and different cancer types, such as pancreatic, colon and prostate [10-12]. In addition, there are many ongoing and completed clinical trials on the use of curcumin in the treatment of various cancers, including breast, colon and prostate [13]. For this reason, it is very valuable to investigate the synergistic effects of curcumin with well-known chemotherapeutics such as gemcitabine which have been used in cancer treatments for many years.

Gemcitabine is a pyrimidine nucleoside analog and has been approved as a chemotherapeutic agent for treating numerous solid tumors, including non-small cell lung cancer (NSCLC), bladder cancer, pancreatic cancer and breast cancer. It exerts a cytotoxic effect on cancer cells by arresting the cells at S and late G1 phases [14-16]. Even though gemcitabine has low toxicity and may be a suitable alternative treatment option for elderly patients, the development of resistance causes limitations in its

clinical use [14]. Therefore, combining traditional chemotherapeutics with natural compounds could prevent developing resistance and reduce the side effects of existing treatments.

In the present study, we aimed to examine the potential booster anticancer effect of curcumin on a conventional chemotherapeutic drug, gemcitabine, in androgen-sensitive human prostate LNCaP cells. In this accordance, we first tested the cytotoxic effect of combining the administration of curcumin with gemcitabine. Moreover, we evaluated the effects of the co-treatment of gemcitabine with curcumin on autophagy, the ubiquitin-proteasome system (UPS), unfolded protein response (UPR) signaling, apoptosis, androgenic signaling, epithelial-mesenchymal transition (EMT), angiogenic and proto-oncogenic factors. Our data suggest that co-administration of gemcitabine and curcumin by determining appropriate dose-response ranges may offer a supportive therapeutic approach in prostate cancer by overcoming the systemic toxic effects of gemcitabine.

MATERIAL AND METHOD

Materials

Fetal bovine serum (FBS), tissue culture media and other supplements were obtained from Capricorn-Scientific (Ebsdorfergrund, Germany). Cell culture plastic materials were purchased from Sarsdeth (Sarsdeth, Ireland). Monoclonal rabbit anti-Hrd1 (#14773)(1:3000) and polyclonal rabbit anti-eIF2α (#9722)(1:2500), anti-phospho-eIF2α (Ser51) (#9721)(1:2500), anti-p62/SQSTM1 (#5114)(1:2000), anti-Beclin-1 (#3495)(1:1500), anti-LC3-I/II (#12741)(1:3000) and anti-caspase-3 (#9692)(1:1000) were provided from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, Massachusetts, USA). Polyclonal rabbit anti-PARP-1 (#13371-1-AP)(1:2000), anti-gp78 (#16675-1-AP)(1:3000), anti-XBP-1s (#24868-1-AP)(1:2000), anti-ubiquitin (#10201-2-AP)(1:1000), anti-PERK (#24390-1-AP)(1:3500), anti-IRE1α (#27528-1-AP)(1:3000), anti-E-cadherin (#20874-1-AP), anti-N-cadherin (#22018-1-AP), anti-AR (#22089-1-AP)(1:2500), anti-c-Myc (#10828-1-AP) and mouse monoclonal anti-PSA (#60338-1-Ig) were obtained from Proteintech (Proteintech Europe, Manchester, UK). Polyclonal rabbit anti-VEGF-A (#E-AB-53277) was purchased from Elabscience (Elabscience, Wuhan, China). Mouse monoclonal anti-beta-actin antibody (#A5316)(1:10000) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). HRP-conjugated goat anti-mouse (#31430)(1:5000) and goat anti-rabbit (#31460)(1:5000) IgG (H+L) were provided from Thermo Scientific (Thermo-Scientific Pierce, Fisher Scientific, Dublin, Ireland). Gemcitabine (G6423) and curcumin (C1386) were provided from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). Staurosporine (#9953) and bafilomycin A1 (#54645) were obtained from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, Massachusetts, USA).

Cell Culture and Treatments

Human androgen-sensitive prostate adenocarcinoma cell line, LNCaP (CRL-1740^{TM}) was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media enriched with 10% FBS, 5 mg ml^{-1} penicillin/streptomycin and 2 mM L-glutamine and were kept in a humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37°C.

Gemcitabine and curcumin were dissolved in sterile cell culture grade H_2O and dimethyl sulfoxide (DMSO), respectively. Compound stocks were prepared at 1000x concentration. DMSO was applied as the vehicle and the final concentration of DMSO applied to the cells did not exceed 0.05%.

Morphological Examination

Morphological alterations were investigated using a phase-contrast inverted microscope (Sunny SopTop ICX41) and a digital camera system (OD400UHW). Cell photographs were taken with 10x or 20x magnifications.

Cell Viability Assay

WST-1 assay (TaKaRa, Mountain View, CA, USA) was performed according to the manufacturer's instructions. Cells were seeded into a 96-well plate (10000 cells/well) and 24 h later

treated with compounds for 48 h. After the incubation time, 10 µl of premixed WST-1 solution was added per well and then the cells were incubated for 2-4 h in conventional cell culture conditions. The absorbance was determined at 450nm with 600nm set as the reference wavelength by microplate spectrophotometer (BioTek, Epoch 2). Results were presented in the graph as a % cell-viability change.

Protein Isolation and Immunoblotting

Cells were seeded in a 6-well plate and incubated for 24 h for protein analysis studies ($2.5x10^5$ cells/well). Cells were then treated with compounds for 24 h and protein isolation studies were carried out. Cells were lysed within radioimmunoprecipitation assay (RIPA) buffer and then centrifugated at 14.000 x rpm for 20 min at 4°C. The supernatant was collected and total protein ingredients were defined by bicinchoninic acid (BCA) protein assay (TaKaRa, Mountain View, CA, USA). 20-30 µg protein content was used in immunoblotting studies. Samples were denatured in 4x Laemmli buffer at 95°C for 5 min and then samples were electrophoretically separated on hand-cast polyacrylamide gels and transferred to an Immobilon®-P polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% nonfat dry milk in Phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-Tween) for 1 h at room temperature and then incubated with primary and secondary antibodies, respectively. Interested proteins were visualized by enhanced chemiluminescence (ECL) solution in ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The densitometric analysis of protein bands was carried out by ImageJ software.

Statistical Analysis

Data were presented as means ± standard deviation (SD). The statistical significance of differences between groups was determined by a two-tailed equal variance Student's *t*-test. The statistical significance of multiple comparisons was analyzed by one-way ANOVA and Tukey's test using GraphPad Prism 7. $p<0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Evaluation of the Co-administration of Curcumin and Gemcitabine on Viability of LNCaP Cells

To evaluate the enhancer effect of curcumin on gemcitabine in human prostate cancer LNCaP cells and WST-1 based cell viability test was carried out. Gemcitabine and curcumin doses used in the study were selected in line with the literature [17-20]. For this aim, we treated the LNCaP cells with 0.1µM gemcitabine and 5, 10 and 20µM curcumin or their combination for 48 h. In this assay system, 0.1µM staurosporine was used as a positive control of programmed cell death induction, which is a well-known potent cell-permeable alkaloid [21]. Microscopic evaluation revealed that as expected staurosporine treatment markedly induced blebbing and separating cellular forms (Figure 1a). Moreover, combined treatments of curcumin and gemcitabine remarkably enhanced the cell-death-related morphological alterations compared to curcumin or gemcitabine-treated alone groups (Figure 1a).

LNCaP cells were treated with 0.1µM gemcitabine and 5, 10 and 20µM curcumin or with their combination for 48 h. **(a)** A morphological examination was carried out by an inverted microscope and photographed. Scale bar: 5 µm. **(b)** Cell viability was analyzed by WST-1 assay. Three independent biological and three technical repeats per experiment were used. Statistical significance among the groups was analyzed by Student's *t*-test or one-way ANOVA and Tukey's test (*$p<0.05$, **$p<0.001$).

WST-1 assay data showed that co-administration of curcumin with gemcitabine more strongly decreased the viability of LNCaP cells compared to gemcitabine or curcumin-treated alone groups in a dose-dependent manner (Figure 1b). Furthermore, 0.1µM gemcitabine administration significantly reduced the viability of LNCaP cells compared to the control group (Figure 1b). Also, the administration of curcumin alone efficiently decreased the viability of LNCaP cells in a dose-dependent manner (Figure 1b). These data suggested that co-administration of gemcitabine with curcumin strongly enhanced the anticancer features of gemcitabine compared to gemcitabine or curcumin administration alone.

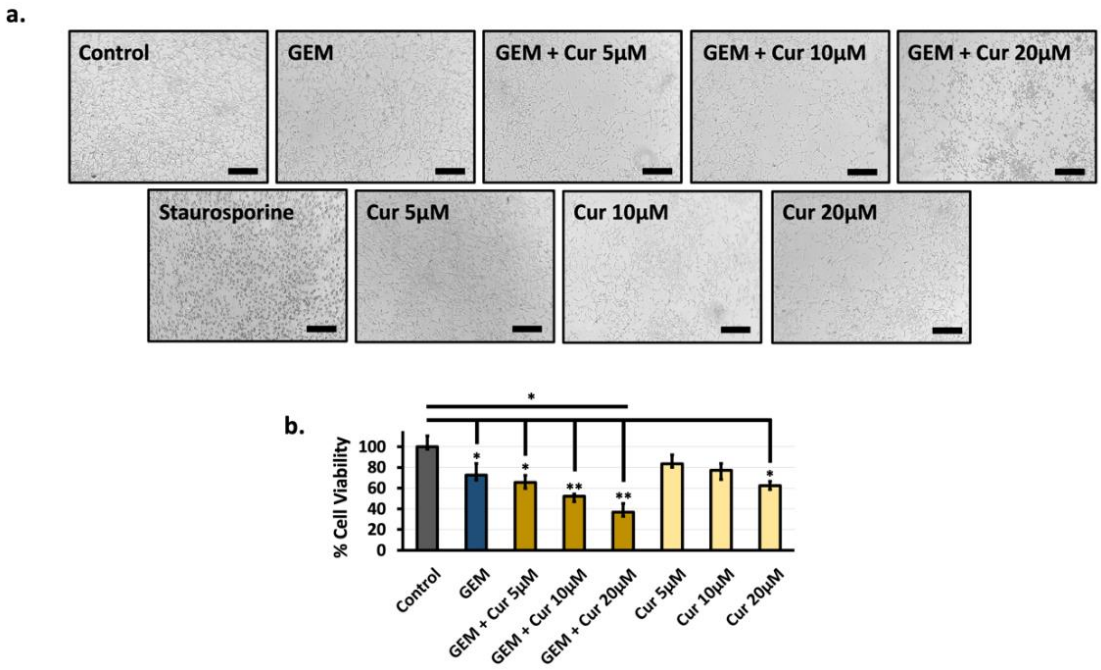

Figure 1. Evaluation of the effect of curcumin and gemcitabine on viability of LNCaP cells

Investigation of the Co-treatment of Curcumin and Gemcitabine on Autophagy

We examined the effect of curcumin, gemcitabine and a combination of curcumin and gemcitabine on changes in cell morphology. 0.1µM gemcitabine treatment did not affect the morphological characteristics of LNCaP cells. Curcumin administration markedly induced the vacuolar structures in cytoplasmic areas of LNCaP cells in a dose-dependent manner. Co-administration of curcumin and gemcitabine more strongly increased the progressively growing vacuolar structures in LNCaP cells. Also, combining the highest dose of curcumin with gemcitabine caused shrinkage and swelling in the morphology of LNCaP cells (Figure 3a). In this experimental system, bafilomycin A1 (BafA1) was used as an autophagic inhibitor and staurosporine was used as a positive control of apoptotic cell death [22]. Compared with the staurosporine and BafA1 treated groups, it was determined that the morphological changes on the LNCaP cells of co-administration were similar to the BafA1 group (Figure 3a).

Cells were treated with vehicle or 0.1µM gemcitabine and 5, 10 and 20µM curcumin or with their combination for 24 h. **(a)** Morphological alterations and vacuolar structures were investigated. BafA1 (1µM) was used as a positive control of autophagic inhibition. Staurosporine (0.1µM) was used as a positive control of apoptotic induction. Arrows are indicated vacuolar structures. Scale bar: 25 µm. **(b)** The expression levels of Beclin1, p62/SQSTM1 and LC3-I/II were analyzed by immunoblotting. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

To understand the impacts of the combined treatment of curcumin and gemcitabine, we tested the levels of some autophagy-related proteins, including Beclin1, p62/SQSTM-1 (sequestosome-1) and microtubule-associated protein 1A/1B-light chain 3 (LC3-I/II) by immunoblotting assay. Our results indicated that curcumin treatment dose-dependently increased the p62/SQSTM1 levels, while decreasing Beclin1 levels (Figure 2b). Moreover, LC3-I levels were not affected by curcumin, whereas the level of LC3-II was increased in a dose-dependent manner. Gemcitabine alone treatment did not prominently affect the levels of Beclin1 and LC3-I or LC3-II, but the expression level of p62/SQSTM1 was markedly decreased by gemcitabine treatment compared to the control group (Figure 2b). Co-treatment of curcumin and gemcitabine caused a gradual decrease in the Beclin1 and p62/SQSTM1 levels and an increase in LC3-I levels (Figure 2b).

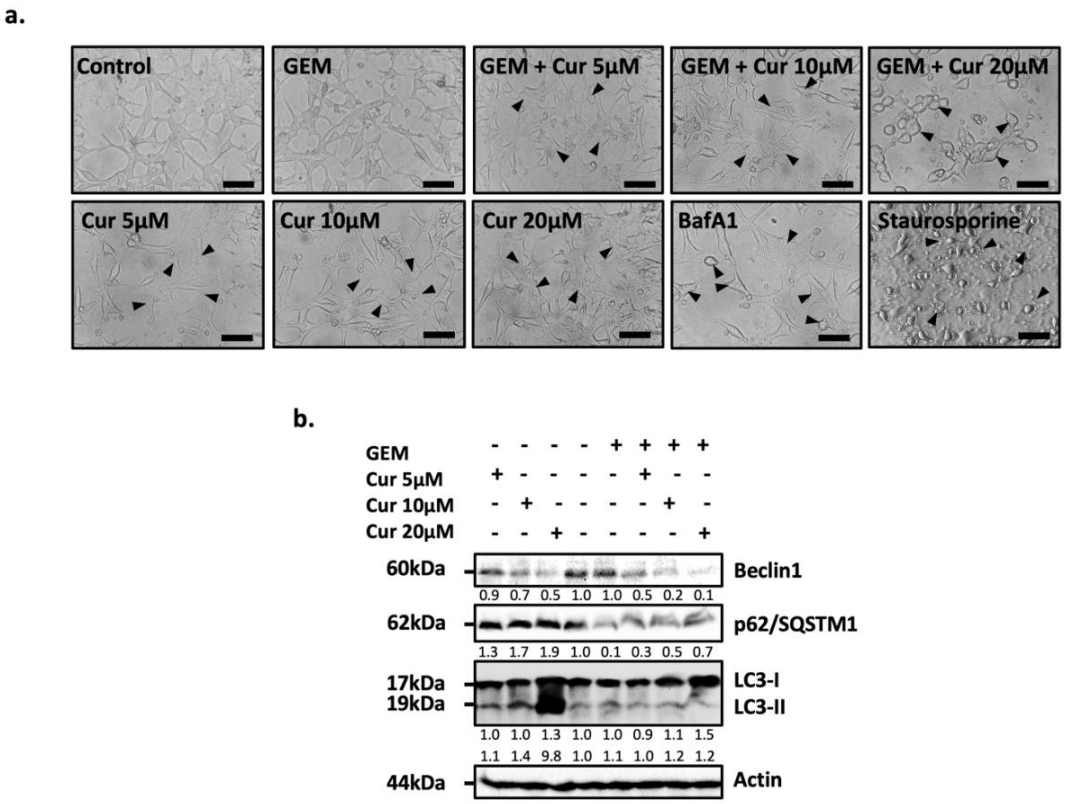

Figure 2. Evaluation of the effect of curcumin and gemcitabine on autophagy proteins in LNCaP cells

Evaluation of the Impacts of Co-treatment of Curcumin and Gemcitabine on UPS and UPR

We examined the effect of the combined treatment of curcumin and gemcitabine on UPS and UPR signaling. Our findings indicated that curcumin treatment increased the hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (Hrd1), glycoprotein 78 (gp78) and poly-ubiquitin levels compared to the control group. Moreover, gemcitabine administration also slightly increased the levels of these proteins. Co-administration more strongly increased the gp78, Hrd1 and poly-ubiquitin levels compared to curcumin or gemcitabine alone in a dose-dependent manner (Figure 3a).

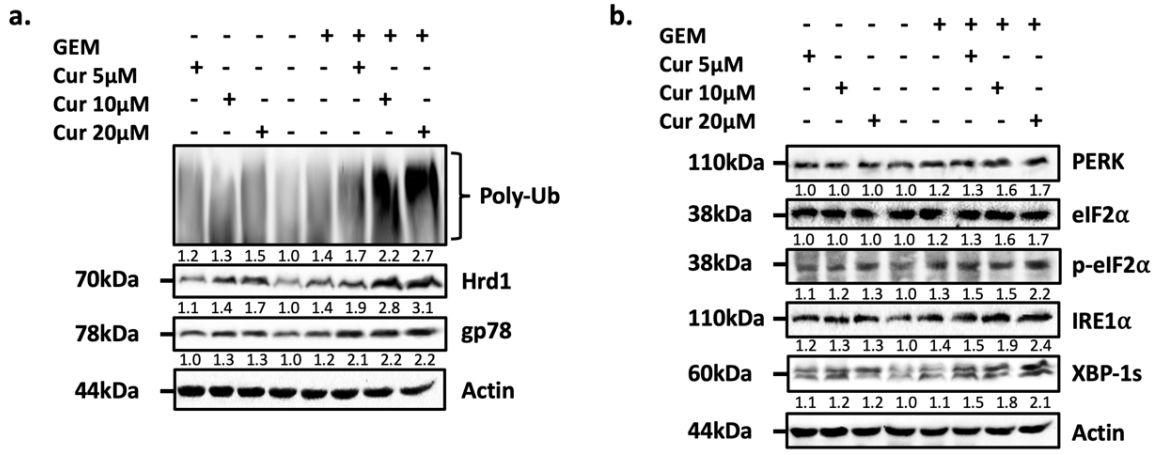

Figure 3. Assessment of the effect of curcumin and gemcitabine on UPS and UPR signaling-related proteins in LNCaP cells

Cells were treated with vehicle or 0.1μM gemcitabine and 5, 10 and 20μM curcumin or with their combination for 24 h. The expression level of **(a)** UPS components, including polyubiquitin, Hrd1 and gp78 levels and **(b)** UPR signaling-related eIF2α, p-eIF2α, PERK, IRE1α and XBP-1s proteins were analyzed by immunoblotting. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

To evaluate the effect of curcumin and gemcitabine on UPR signaling, we examined the protein levels of protein kinase RNA-like ER kinase (PERK), eukaryotic initiation factor 2α (eIF2α), phosphorylated eIF2α at serine 51 position (p(Ser51)-eIF2α), inositol-requiring enzyme-1α (IRE1α) and X-box binding protein-1 spliced (XBP-1s) by immunoblotting studies. Our results revealed that curcumin treatment slightly increased the p(Ser51)-eIF2α, IRE1α and XBP-1s levels. Besides, gemcitabine administration also slightly increased the expression level of p(Ser51)-eIF2α, IRE1α and XBP-1s proteins (Figure 3b). Co-administration of curcumin and gemcitabine more strongly induced the protein levels of PERK, p(Ser51)-eIF2α, IRE1α and XBP-1s in a dose-dependent manner (Figure 3b). Collectively, these results indicated that the combined application of curcumin and gemcitabine remarkably augmented the levels of UPS and UPR signaling-related proteins compared to curcumin or gemcitabine alone.

Determination of the Effect of Co-administration of Curcumin and Gemcitabine on Apoptotic Protein Levels

We tested the possible improving effect of curcumin on gemcitabine-induced programmed cell death. Therefore, we examined the poly(ADP-ribose) polymerase 1 (PARP-1) and caspase-3 levels by immunoblotting. Our data revealed that curcumin treatment alone induced 89 kDa of PARP-1 and 17, 19 kDa of caspase-3 cleavage forms. Gemcitabine administration alone did not lead to the cleavage of PARP-1 or caspase-3 at 0.1μM dose. Co-treatment of gemcitabine and curcumin strongly induced the PARP-1 and caspase-3 fragments compared to curcumin or gemcitabine-treated alone (Figure 4).

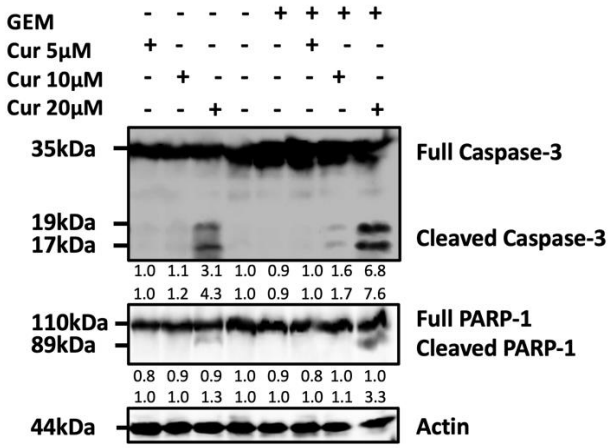

Figure 4. Investigation of the effect of curcumin and gemcitabine on cell death-associated proteins in LNCaP cells

Cells were treated with vehicle or 0.1μM gemcitabine and 5, 10 and 20μM curcumin or with their combination for 24 h. The protein level of full and cleaved caspase-3 and PARP-1 were analyzed by immunoblotting studies. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

Evaluation of the Impacts of Co-treatment of Curcumin and Gemcitabine on the Levels of Several Tumorigenic Proteins in LNCaP Cells

To test the enhancer effect of co-administration of curcumin on gemcitabine in LNCaP cells, we tested the expression level of androgenic signal-related androgen receptor (AR) and prostate-specific

antigen (PSA) proteins, proto-oncogenic protein c-Myc, angiogenic factor vascular endothelial growth factor-A (VEGF-A) and EMT-associated N-cadherin and E-cadherin protein levels by immunoblotting. Our data revealed that gemcitabine treatment slightly reduced the protein levels of PSA, AR, c-Myc, VEGF-A and N-cadherin compared to the control group (Figure 5a). Similar to gemcitabine, alone curcumin treatment decreased the level of PSA, AR, c-Myc, VEGF-A and N-cadherin levels and increased the E-cadherin levels in a dose-dependent manner (Figure 5a, b). Combined treatment of gemcitabine with curcumin more strongly reduced PSA, AR, c-Myc, VEGF-A and N-cadherin levels and increased E-cadherin levels compared to gemcitabine or curcumin treatment alone in a dose-dependent manner (Figure 5a, b).

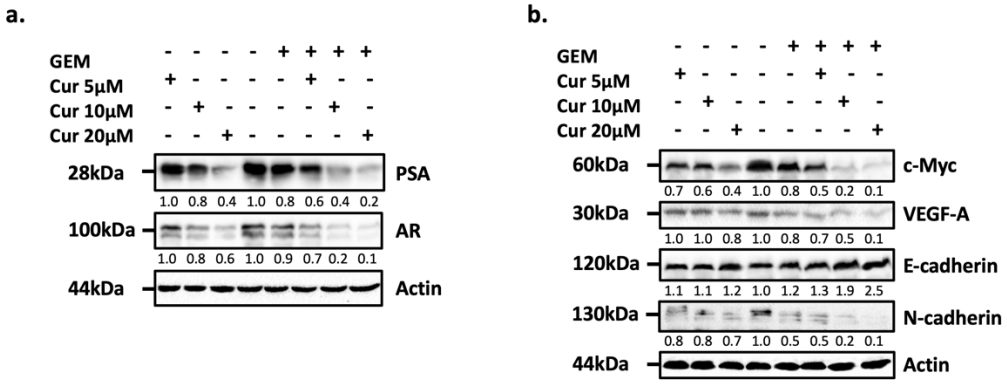

Figure 5. Testing the effect of curcumin and gemcitabine on prostate cancer tumorigenesis-associated protein levels in LNCaP cells

Cells were treated with vehicle or 0.1µM gemcitabine and 5, 10 and 20µM curcumin or with their combination for 24 h. The expression level of **(a)** androgenic signaling proteins PSA and AR and **(b)** tumorigenic proteins c-Myc, E-cadherin, N-cadherin and VEGF-A were analyzed by immunoblotting studies. Beta-actin was used as a loading control. The protein band density of the control group was set as a 1.

Today, potent anticancer drugs with a broad spectrum of action are approved for treatment and commonly used in clinics. Traditional chemotherapeutic agents, including gemcitabine, are still more widely used and are very valuable as it increases the survival rate of patients. However, their systemic side effects and acquired resistance as a result of long-term usage substantially restrict the efficacy of these therapeutics. Therefore, studies have focused on the combinatory administration of natural products and routinely used chemotherapeutics. Numerous *in vitro* and *in vivo* studies, as well as clinical trials, have shown that natural compounds have beneficial effects on the reduction of side effects of chemotherapeutics [15,16].

Research on curcumin, which is one of the most researched natural compounds and is a component of golden spice turmeric (*Curcuma longa*), has been ongoing over the past half-century [23]. Today, its safety and efficacy profile is proven by numerous preclinical and clinical studies. Also, it has been shown that it may be used in numerous diseases such as arthritis, cardiovascular diseases, diabetes and cancer by modulating multiple signaling pathways [24]. Moreover, it also demonstrated that curcumin exhibits potent anticancer properties by inhibiting growth, survival and invasion of cancer cells and inducing apoptotic signaling pathways in malignant cells [25,26]. Possible anti-malignant properties of curcumin have also been reported in prostate tumorigenesis, where abnormal cell proliferation, angiogenesis and metastasis are involved [27].

Herein, we aimed to investigate the booster effect of curcumin on the anti-cancer properties of gemcitabine, a pyrimidine nucleoside antimetabolite widely used in the treatment of various cancer types, including NSCLC, pancreatic cancer and breast cancer. We first tested whether curcumin enhances the anti-proliferative properties of gemcitabine by WST-1 assay. Gemcitabine and curcumin doses were preferred considering the studies performed on LNCaP cells [28,29]. 20µM curcumin and

0.1μM gemcitabine administration significantly decreased the proliferation of LNCaP cells. In contrast, administration of 5 and 10μM curcumin had no significant effect on the proliferation of LNCaP cells (Figure 1b). Similar findings were also observed in microscopic examinations (Figure 1a). Co-administration of curcumin with gemcitabine more strongly decreased the proliferation of LNCaP cells in a dose-dependent manner compared to alone gemcitabine or curcumin-treated groups (Figure 1a, b). These results confirmed the potent enhancer effect of curcumin on the anti-cancer features of gemcitabine.

It is known that many chemotherapeutics and bioactive natural products have inducing or suppressive properties on autophagy [30,31]. Autophagy is a highly conserved and regulated cellular degradation process in eukaryotic cells. Basically, autophagy plays a role in the removal of malformed proteins and damaged organelles through double-membrane vesicles-mediated lysosomal degradation. Complexes formed by many autophagy-related (Atg) proteins are involved in the coordination of autophagy [32]. There are used a variety of methodologies for monitoring autophagy in cells and the most commonly used are the investigation of changes in the levels of Atg proteins, the examination of p62/SQSTM1, which is an autophagic cargo protein and whose levels decrease with increasing autophagic activity and the detection of LC3-I to LC3-II conversion, which is known to be involved in autophagosomal membrane expansion [32]. Since autophagy is a dynamic and cascading mechanism, including induction, nucleation, expansion, fusion and degradation of cargo content through lysosome, many parameters must be examined simultaneously to evaluate the autophagic activity in cells [33].

Present data indicated that curcumin treatment markedly increased the p62/SQSTM1 and LC3-II levels whereas Beclin1, which is an essential mediator of autophagy, decreased in a dose-dependent manner. Additionally, gemcitabine strongly decreased the p62/SQSTM1 levels and had no significant effect on Beclin1 and LC3 levels (Figure 2b). These results showed that curcumin suppresses autophagic flux in prostate cancer cells, while gemcitabine enhances it. In co-administration studies, it was determined that Beclin1 levels decreased in a dose-dependent manner, while p62/SQSTM1 levels decreased due to gemcitabine administration and increased with curcumin administration. However, p62/SQSTM1 levels were still found to be lower compared to the control group. Furthermore, a dose-dependent increase was observed in LC3-I levels due to co-administration (Figure 2b). These results indicated that the increased autophagic activity induced by gemcitabine persisted in LNCaP cells due to the co-administration of gemcitabine and curcumin. In addition, in the results of the microscopic examination, it was observed that there was an increased vacuolization and a change in the cell form in LNCaP cells (Figure 2a). These results supported the immunoblotting findings.

It is known that UPS and autophagy, two main degradation systems in cells, work in interaction [34]. Thus, we examined Hrd1 and gp78 E3 ubiquitin ligase enzyme levels, which are responsible for ubiquitin conjugation to substrate molecules and steady-state level of polyubiquitination [35,36]. It was determined that the application of curcumin or gemcitabine alone increased Hrd1, gp78 and polyubiquitin levels. Also, we found that co-administration more strongly increased the levels of all tested proteins (Figure 3a). These results indicated that the co-administration of gemcitabine and curcumin more strongly induced the UPS. In addition, we examined UPR signaling, which is an essential signaling mechanism in prostate cancer cells and regulates the levels of UPS activity-associated proteins.

To respond to the cell's needs for increased protein synthesis and protein quality control, the UPR generates a series of signals mediated by the three transmembrane proteins IRE1α, PERK and ATF6, which are localized to the ER membrane. Moreover, it is known that cancer cells have increasing UPR activity, thereby UPR signaling plays an adaptive role against the changing metabolic conditions required by cancer cells. It is also well-known that prolonged or excessive UPR stimulation induces programmed cell death [37].

We found that curcumin or gemcitabine alone administration induced IRE1α and PERK branches of UPR signaling. It was determined that eIF2α protein was phosphorylated at the serine 51 position due to PERK activation and XBP-1s levels formed by alternative splicing with IRE1α activation increased. Co-administration of curcumin and gemcitabine more strongly increased the phospho-eIF2α and XBP-1s levels in a dose-dependent manner (Figure 3b). Considering all these results, it seems that the combination of curcumin with gemcitabine more potently stimulates the IRE1α and PERK arms of the UPR. In addition, these results suggest that the effect of the co-administration on the UPS occurs directly

and/or depending on the activation of the UPR.

It is known that caspase-3 and PARP-1, stimulator of programmed cell death, are activated due to the anticancer properties of many chemotherapeutics and natural products [38]. The 89kDa and 17/19kDa cleavage products of activated PARP-1 and caspase-3, respectively, are frequently studied for monitoring programmed cell death at the cellular level [38].

Our data indicated that curcumin administration dose-dependently stimulated caspase-3 and PARP-1 activation. Gemcitabine administration did not cause any response, whereas co-administration strongly activated caspase-3 and PARP-1 in a dose-dependent manner (Figure 4). These results strongly suggest that curcumin efficiently potentiates the anticancer effect of gemcitabine on LNCaP cells.

The androgenic signal and its coordinated oncogenic program as well as EMT are critical in the progression of prostate carcinogenesis [39,40]. It is known that the specialized transcriptional program regulated by the AR arranges numerous molecular signaling pathways [39]. Herein, we found that curcumin or gemcitabine administration reduced the AR levels and also suppressed PSA expression, which is controlled by the transcriptional program of AR. Co-treatment of curcumin and gemcitabine more robustly reduced the AR and PSA levels compared to curcumin or gemcitabine alone (Figure 5a). These results suggest that the co-administration of curcumin and gemcitabine offers a powerful approach to suppressing androgenic signal in prostate cancer cells.

Finally, we examined the levels of proto-oncogenic protein c-Myc, which is known to support tumor progression, angiogenic factor VEGF-A and two key proteins associated with EMT, E-cadherin and N-cadherin. The c-Myc protein is overexpressed in more than 70% of human tumors, including prostate and breast cancers. c-Myc, a transcription factor, has regulatory roles in processes such as cell growth, proliferation, metabolism and differentiation [41,42]. Our results showed that co-administration strongly suppressed c-Myc levels when compared with either curcumin or gemcitabine administration alone (Figure 5b). Additionally, our findings showed that VEGF-A levels, which support the development of tumor-associated blood vessels, provide the way for invasion and are associated with self-renewal and metastasis, were negatively regulated (Figure 5b) [43].

EMT, which is considered a key mechanism for invasion and metastasis, is a fundamental process of the development, progression and spreading of cancer cells. The decrease in E-cadherin levels and the increase in N-cadherin levels act as a stimulating mechanism for invasion and metastasis [44]. Our results showed that co-administration more strongly decreased N-cadherin levels and increased E-cadherin levels compared to curcumin or gemcitabine alone (Figure 5b). These results indicated that curcumin exhibited an enhancer impact on the anti-cancer properties of gemcitabine on prostate cancer cells.

Present data showed that curcumin potentiates the anti-tumorigenic capacity of gemcitabine by enhancing UPS, re-modulating autophagy, activating key regulators associated with programmed cell death, as well as negatively regulating androgenic signaling, suppressing proto-oncogenic and angiogenic protein levels and reducing EMT in prostate cancer cells.

These results indicated that using curcumin, in addition to gemcitabine, might enhance the anticancer properties of gemcitabine in prostate cancer. Present data suggest that curcumin, whose biochemical effects have been widely studied, can be used as a booster in prostate cancer treatments.

ACKNOWLEDGEMENTS

We thank Suleyman Demirel University - Innovative Technologies Application and Research Center for equipmental support. 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.; Data Collection and/or Processing: Y.E., H.K.D., D.C.; Analysis and/or Interpretation: Y.E.; Literature Review: Y.E., H.K.D., D.C.; Manuscript Writing: Y.E.; Critical Review: Y.E., H.K.D., D.C.; 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., 47(3): 752-760, 2023
Doi: 10.33483/jfpau.1240900

ORIGINAL ARTICLE / ÖZGÜN MAKALE

EXTRACTION-FREE SPECTROPHOTOMETRIC DETERMINATION OF MELOXICAM USING BROMOTHYMOL BLUE

BROMOTİMOL MAVİSİ KULLANILARAK EKSTRAKSİYON OLMADAN MELOKSİKAMIN SPEKTROFOTOMETRİK TAYİNİ

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ABSTRACT

Objective: *The purpose of the present work was to develop and validate a fast, simple and sensitive extraction-free spectrophotometric technique for the quantitative determination of meloxicam based on the reaction with bromothymol blue.*
Material and Method: *The reference standard of meloxicam, bromothymol blue and finished dosage forms of meloxicam were used in the study. Absorption measurements were performed on the Analytic Jena UV-visible spectrophotometer model Specord 200.*
Result and Discussion: *The developed method is based on the formation of the colored reaction product between meloxicam and bromothymol blue in acetone medium with absorption maximum at 348 nm. The method meets the requirements of the State Pharmacopoeia of Ukraine for such validation characteristics as specificity, linearity, precision, accuracy, robustness and range of application. The obedience to Beer's law is observed in the range of meloxicam concentrations 0.80-2.40 mg/100 ml, the correlation coefficient is 0.9998. The range of application of the method is 60-140%.*
Keywords: *Meloxicam, bromothymol blue, sulfonephthalein dyes, spectrophotometry, validation studies*

ÖZ

Amaç: *Mevcut çalışmanın amacı, bromotimol mavisi ile reaksiyona dayalı olarak meloksikamın kantitatif tayini için hızlı, basit ve hassas ekstraksiyon gerektirmeyen bir spektrofotometrik teknik geliştirmek ve doğrulamaktır.*
Gereç ve Yöntem: *Çalışmada meloksikamın referans standardı, bromotimol mavisi ve meloksikamın bitmiş dozaj formları kullanılmıştır. Absorpsiyon ölçümleri, Analitik Jena UV-*

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Submitted / Gönderilme : 23.01.2023
Accepted / Kabul : 17.05.2023
Published / Yayınlanma : 20.09.2023

görünür spektrofotometre modeli Specord 200 üzerinde gerçekleştirilmiştir.
Sonuç ve Tartışma: *Geliştirilen yöntem, 348 nm'de maksimum absorpsiyon gözlenerek aseton ortamında meloksikam ve bromotimol mavisi arasındaki reaksiyonun boyalı ürününün oluşmasına dayanmaktadır. Geliştirilen teknik, özgüllük, doğrusallık, kesinlik, doğruluk, sağlamlık ve uygulama aralığı gibi validasyon özellikleri için Ukrayna Devlet Farmakopesi şartlarını karşılamaktadır. Beer yasasına uygunluk 0.80-2.40 mg/100 ml konsantrasyon aralığında gözlemlenir. Korelasyon katsayısı 0.9998'dir. Tekniğin uygulama aralığı %60-140'tır.*
Anahtar Kelimeler: *Meloksikam, bromotimol mavisi, sülfonatalein boyaları, spektrofotometri, validasyon çalışmaları*

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used drug groups in medicine. Their advantage is their complex action (antipyretic, anti-inflammatory, analgesic) and a wide range of indications for use. NSAIDs are used for the symptomatic treatment of pain and inflammation of various etiologies, particularly in lesions of the musculoskeletal system [1,2]. According to statistics, NSAIDs are the most popular medications among doctors and the public for the treatment of musculo-articular pain and take the leading place in the world in terms of consumption [3]. One of the well-known representatives of NSAIDs is meloxicam.

Meloxicam (4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide) refers to the group of oxicams and shows selective inhibition of COX-2 isoenzyme, providing anti-inflammatory and analgesic effects. It is used as a drug for the symptomatic treatment of exacerbations of arthritis, chronic polyarthritis, rheumatoid arthritis. Meloxicam preparations are produced by leading pharmaceutical companies in the form of tablets, capsules and solutions for injection [4,5]. Therefore, the development of reliable and accessible methods of quantitative analysis of the drug is undoubtedly relevant.

The assay of meloxicam is listed in the European Pharmacopoeia, which describes non-aqueous titration with potentiometric fixation of the end-point [6]. The United States Pharmacopeia recommends HPLC method using a UV-Vis absorbance detector for the quantitative determination of the drug [7].

There is a wide choice of various analytical techniques for the assay of meloxicam in pharmaceuticals available in the literature [8-18]. Spectrophotometry is one of the most commonly used methods for the determination of this drug.

Thus, A. Chaplenko et al. proposed spectrophotometric and colorimetric determination of meloxicam, lornoxicam, tenoxicam in drugs using 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD chloride). The techniques are based on alkaline hydrolyses of oxicams with NBD chloride and the subsequent spectrophotometric and colorimetric determination of the colored products of the reaction [19].

Two sensitive and fast methods for determination of meloxicam using direct and indirect flow injection spectrophotometry are described in *Current Pharmaceutical Analysis*. The direct method depended on the coupling of meloxicam with diazotized procaine benzylpenicillin in alkaline medium. On the other hand, the indirect method involved a charge transfer reaction between the alkaline hydrolytic product of meloxicam as n-donor with metol (*p*-methylaminophenol sulfate) as a π- acceptor using sodium periodate as an oxidant [20].

In the literature, there are also data on the quantitative determination of meloxicam using sulfonephthalein dyes. Thus, Sane et al. recommend a method based on the formation of ion-pair complexes of the drug with three acid dyes, namely, bromothymol blue (BTB), bromocresol purple (BCP), bromophenol blue (BPB) in acidic buffer solutions followed by their extraction in organic solvents (chloroform and methylene chloride) [21].

Sulfonephthalein dyes are synthetic substances that are derivatives of triphenylmethane. Their water-soluble sodium salts, as well as acidic forms, are widely used in chemical analysis as pH indicators [22]. Sulfonephthalein dyes are known to form ionic associations when interacting with basic drugs, which are used to determine many pharmaceutical compounds by extraction spectrophotometry [23]. For example, bromothymol blue is used for the extraction spectrophotometric determination of

antifungal [24], antimicrobial [25], cardiovascular [26], and other drugs. Since extraction is a time-consuming procedure, a promising area of research is the development of non-extraction spectrophotometric methods based on ionic pairs in non-aqueous or aqueous solutions.

Therefore, the purpose of the work was to develop and validate a fast, simple and sensitive extraction-free spectrophotometric technique for the quantitative determination of meloxicam based on the reaction with bromothymol blue.

MATERIAL AND METHOD

Analytic Equipment

Absorption measurements were performed on the Analytic Jena UV-visible spectrophotometer model Specord 200 with 1 cm quartz cells. Kern electronic balance ABT-120-5DM was used to weigh the analyzed samples.

Materials and Reagents

All chemicals and reagents used were of analytical or pharmaceutical grade. Bromothymol blue, acetone, chloroform, 1,4-dioxane, ethanol were obtained from commercial sources. The reference standard of meloxicam was supplied by Derivados Quimicos, S.A., Spain (series No. 269001T01D).

Tablets “Meloxicam KV” 15 mg (JSC “Kyiv Vitamin Plant”, Ukraine, series No. UE20820), tablets “Meloxicam” 15 mg (PJSC "Lekhim-Kharkiv", Ukraine, series No. 93029004), tablets “Revmoxicam” 7.5 mg (JSC “Farmak”, Ukraine, series No. 80819), tablets “Meloxicam Teva” 7.5 mg (Merkle GmbH, Germany, series No. V23961A) were purchased from a local pharmacy.

0.15% solution of bromothymol blue was prepared by dissolving 0.15 g of bromothymol blue in 100 ml of acetone.

0.016% working standard solution was prepared by dissolving 0.0160 g of pure meloxicam in 100 ml of acetone.

General Method for the Assay of Meloxicam

The aliquots of the working standard solution containing 0.80-2.40 mg of meloxicam were transferred into 10 ml volumetric flasks. 1 ml of 0.15% bromothymol blue solution was added to each of the above flasks and brought to the mark with acetone. The absorption of the reaction product was measured at a wavelength of 348 nm against the reagent blank.

Method for the Assay of Meloxicam in Tablets

Twenty tablets were weighed and powdered. A portion of the powder containing 4 mg of meloxicam was weighed and transferred into a 25 ml calibrated flask. The volume was brought to the mark with acetone, mixed and filtered using “Blue ribbon” filter. Aliquots of the filtrate were analyzed according to the general method.

RESULT AND DISCUSSION

In order to develop a spectrophotometric method for the quantitative determination of meloxicam by reaction with bromothymol blue, the optimal conditions for the interaction of the components of the reaction were determined.

To establish the optimal reaction medium, the interaction of meloxicam with the reagent in such solvents as chloroform, 1,4-dioxane, acetone, and ethanol was studied (Figure 1). The maximum value of the absorption and satisfactory results when checking the stability of the reaction product in the selected solvent confirmed the feasibility of using acetone for further development of the method.

The effect of reagent concentration on the absorption value was also investigated. For this purpose, the absorption of solutions containing a fixed concentration of meloxicam and different amounts of bromothymol blue was measured. The graph shows that 1 ml of 0.15% reagent is the most acceptable. Further increase in the volume of the reagent does not lead to an increase in the absorption (Figure 2).

The product of the reaction was formed instantly at room temperature and was stable.

Thus, it was found that meloxicam reacts with bromothymol blue in an acetone medium. The maximum absorption of the reaction product is measured at a wavelength of 348 nm (Figure 3).

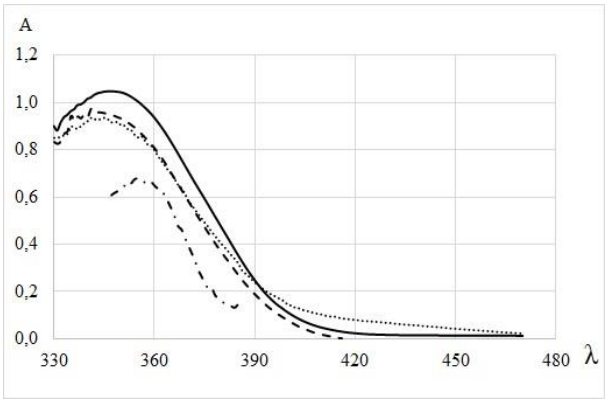

Figure 1. Absorption spectrum of reaction product of meloxicam (0.016%) with bromothymol blue (0.15%) in acetone (──), chloroform (- - - -), 1,4-dioxane (····), ethanol (-·-·-·-)

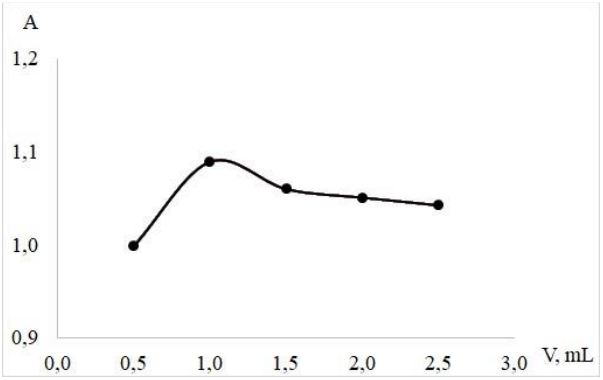

Figure 2. Dependence of the absorption value on the volume of the added reagent (0.15%)

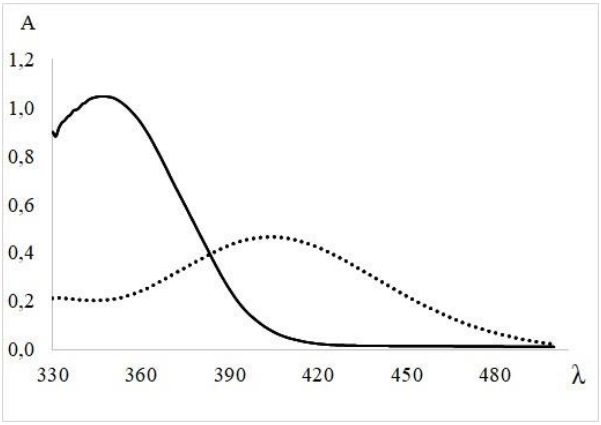

Figure 3. Absorption spectrum of reaction product of meloxicam (0.016%) with bromothymol blue against reagent blank (──); bromothymol blue (0.15%) reagent blank against acetone (····)

It is known that when sulfonephthalein dyes interact with nitrogen-containing compounds in organic solvents, ionic pairs are formed by the proton transfer mechanism [27, 28]. Thus, the secondary

aliphatic amino group of meloxicam attaches a proton to the phenolic group of the dye. The lactoid ring is opened, accompanied by the formation of a complex between protonated meloxicam and the dye anion (Figure 4).

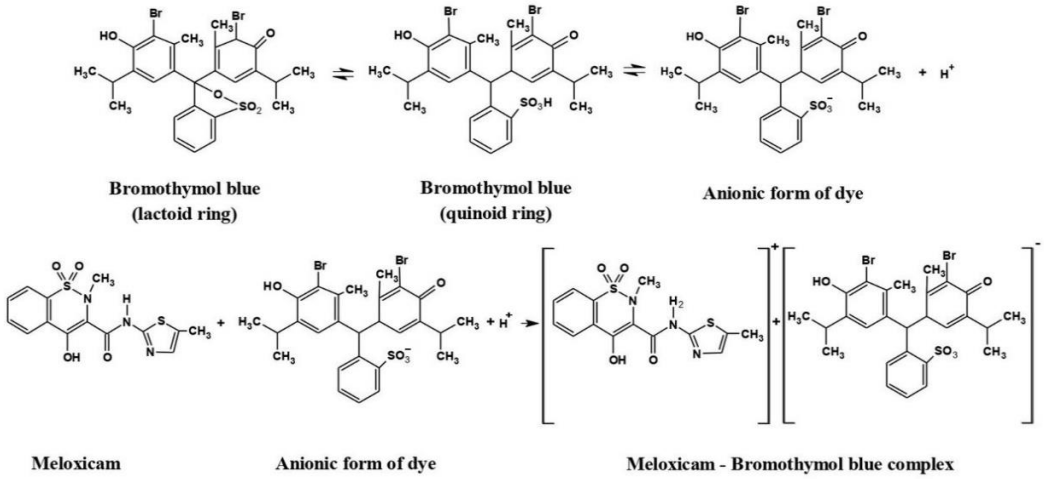

Figure 4. The possible reaction mechanism between meloxicam and bromothymol blue

Definition of Validation Characteristics

Validation was performed to confirm the suitability of the developed method for quality control of meloxicam. The selection and calculation of validation characteristics were performed in accordance with the requirements of the State Pharmacopoeia of Ukraine [29].

Specificity

In order to assess the specificity of the developed method, the contribution of excipients included in the dosage forms to the total absorption of the solution was determined. Tests with a placebo solution were conducted for this study. Model mixtures of excipients were prepared. Meloxicam of the concentration contained in the test drug was added to a part of each model mixture. Then all the stages of sample preparation were reproduced and the absorption of the "placebo" solution and the comparison solution containing the test drug was measured. It was found that contribution of placebo to the total background absorbance is insignificant for the investigated dosage forms (Figure 5).

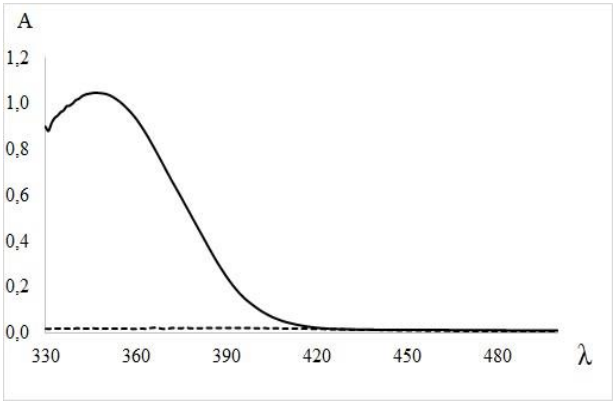

Figure 5. Absorption spectrum of placebo of tablets "Revmoxicam" 7.5 mg (- - -); reaction product of meloxicam (0.016%) with bromothymol blue (──)

Linearity

The linearity of the developed method was studied in the range of meloxicam concentrations of 0.80-2.40 mg/100 ml. A graph of the dependence of absorption (Y_i, %) on the concentration of the test substance (X_i, %) in normalized coordinates was plotted (Figure 6).

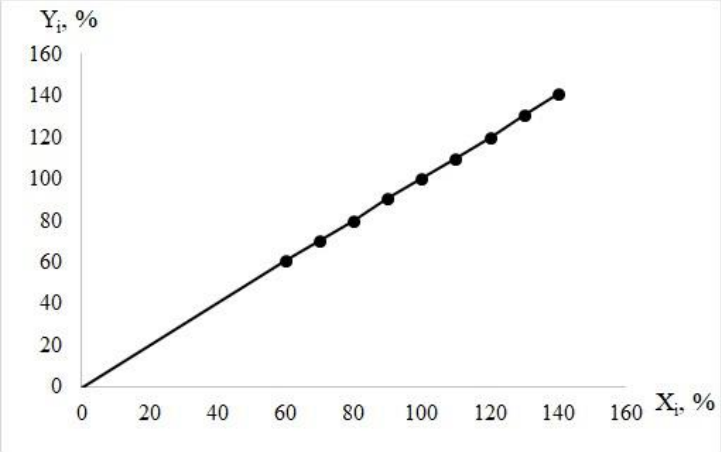

Figure 6. Graph of the absorption dependence on the concentration of meloxicam

The linear dependence parameters were calculated using the least squares method. It was found that the linearity parameters meet the requirements of the State Pharmacopoeia of Ukraine over the entire application range of the method (60-140%). The data are shown in Table 1.

Table 1. Optical characteristics and main parameters of linear dependence

Validation parameter	Results
Wavelength (nm)	348
Beer's law range (mg/100 ml)	0.80-2.40
Molar absorption coefficient, ε	21810
Sendell's coefficient, W_S	0.01611
Limit of detection (LOD), %	2.27
Limit of quantification (LOQ), %	6.89
Equation of linear regression	Y = bX + a
Slope, b±(S_b)	0.9986 ± (0.00667)
Intercept term, a±(S_a)	0.5022 ± (0.6888)
Residual standard deviation, $S_{x,o}$	0.5173
Correlation coefficient, r	0.9998

Precision

Precision was determined at the level of repeatability. Nine samples were analyzed, the concentrations of which are evenly distributed in the studied range of the method (plus a comparison solution, the concentration of which is close to the nominal). According to the requirements for precision of the State Pharmacopoeia of Ukraine, the method is accurate at the level of repeatability if the relative confidence interval (Δ_z) does not exceed the maximum permissible uncertainty of the analysis (Δ_{As}). The data in Table 2 confirm the precision of the developed method.

Table 2. Precision evaluation of the proposed method

Dosage form	Metrological characteristics			
	ΔZ	s_z	Δ_z	Δ_{As}
Tablets "Meloxicam" 15 mg	99.86	0.78	1.45	1.6
Tablets "Meloxicam KV" 15 mg	99.93	0.69	1.28	1.6
Tablets "Meloxicam Teva" 7.5 mg	100.37	0.72	1.34	1.6
Tablets "Revmoxicam" 7.5 mg	100.19	0.79	1.48	1.6

ΔZ - mean, %; S_Z – relative standard deviation; Δ_Z – relative confidence interval; Δ_{As} – maximum permissible uncertainty of the analysis

Accuracy

The accuracy of the developed method was established by the method of standard addition. In the course of the experiment, the absorption of the tested samples and the same samples with the addition of a working standard solution of meloxicam was compared. As shown by the calculations (Table 3), the value of the systematic error does not exceed the confidence interval of the mean value for the ratio "found/injected".

Table 3. Accuracy evaluation of the proposed method

Dosage form	Taken mg/100 ml	Additive mg/100ml	ΔZ	Δ_z	δ	$\Delta_Z/\sqrt{n}$
Tablets "Meloxicam" 15 mg	0.87	0.48	100.44	1.38	0.44	0.84
	0.87	0.64				
	0.87	0.80				
Tablets "Meloxicam KV" 15 mg	1.28	0.32	101.18	5.98	1.18	1.99
	1.28	0.48				
	1.28	0.64				
Tablets "Meloxicam Teva" 7.5 mg	1.52	0.32	99.88	3.92	0.12	1.31
	1.52	0.48				
	1.52	0.64				
Tablets "Revmoxicam" 7.5 mg	0.85	0.48	99.16	2.71	0.84	0.90
	0.85	0.64				
	0.85	0.80				

ΔZ – mean, %; Δ_Z – relative confidence interval; δ – systematic error

Robustness

In order to determine the robustness of the developed method, the stability of the reaction product was investigated. The absorption of the test solutions of the analyzed dosage forms (A_1 – A_4) and the working standard sample (A_0) of meloxicam was measured every 5 minutes for 30 minutes. The relative standard deviation (RSD_t%) and the confidence interval (Δ_t%) of the results obtained were calculated, which should not exceed the permissible systematic error (maxδ). The calculations confirm the stability of the analyzed solutions for at least 30 min (Table 4).

The results of the definition of validation characteristics (specificity, linearity, range of application, precision, accuracy, robustness) confirm the correctness of the proposed method. The simplicity of the experiment, the absence of extraction steps, satisfactory accuracy, and reproducibility make it possible to employ the method for routine pharmaceutical analysis of meloxicam tablets.

Table 4. Evaluation of the stability studies

t, min	0	5	10	15	20	25	30	Mean	RSD,%	Δ_t%	maxδ, %
A_0	1.0501	1.0512	1.0522	1.0528	1.0542	1.0554	1.0562	1.0531	0.210	0.41	0.51
A_1	0.9937	0.9957	0.9965	0.9971	0.9982	0.9987	1.0003	0.9972	0.218	0.42	
A_2	0.9874	0.9882	0.9901	0.9907	0.9925	0.9935	0.9931	0.9908	0.244	0.47	
A_3	1.0016	1.0026	1.0035	1.0038	1.0053	1.0068	1.0057	1.0042	0.183	0.35	
A_4	0.9917	0.9929	0.9941	0.9948	0.9958	0.9973	0.9981	0.9949	0.234	0.45	

RSD,% – relative standard deviation; Δ_t% – confidence interval; maxδ,% – critical value of the systematic error

ACKNOWLEDGEMENTS

The authors are grateful to Zaporizhzhia State Medical and Pharmaceutical University for supporting this study.

AUTHOR CONTRIBUTIONS

Concept: A.D., S.V., N.N.; Design: A.D., S.V., N.N.; Control: A.D., S.V.; Sources: A.D., S.V.; Materials: A.D., S.V.; Data Collection and/or Processing: A.D., S.V.; Analysis and/or Interpretation: A.D., S.V.; Literature Review: A.D., N.N.; Manuscript Writing: A.D., N.N.; Critical Review: S.V.; 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., 47(3): 761-769, 2023
Doi: 10.33483/jfpau.1286359

ORIGINAL ARTICLE / ÖZGÜN MAKALE

COENZYME Q0 INHIBITS CELL PROLIFERATION AND MODULATES MAPK AND AKT SIGNALLING PATHWAYS IN HUMAN CHRONIC MYELOID LEUKEMIA K562 CELLS

KOENZİM Q0 İNSAN KRONİK MYELOİD LÖSEMİ K562 HÜCRELERİNİN PROLİFERASYONUNU ENGELLER VE MAPK VE AKT SİNYAL YOLAKLARINI MODÜLE EDER

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ABSTRACT

Objective: *This study evaluated the antiproliferative and pro-apoptotic effects of coenzyme Q0 (CoQ0) in human chronic myeloid leukemia K562 cell line.*
Material and Method: *The cytotoxic effect of CoQ0 on human chronic myeloid leukemia cell line, K562 was determined by MTT test. The activity of caspase-3, expression of proteins involved in apoptosis, MAPK and AKT signaling pathways were determined with enzymatic assay and western blot analysis, respectively.*
Result and Discussion: *Results showed that CoQ0 inhibited cell viability of K562 cells at 5 µM and higher concentrations and Bax protein expression was significantly decreased at 12.5 µM concentration of CoQ0. However, CoQ0 did not significantly affect caspase 3 activity and Bcl-2 protein expression. p-c-Raf (Ser259) protein expression was significantly decreased at 12.5 µM of CoQ0. Treatment with 10 µM of CoQ0 induced significantly phosphorylation of p38 MAPK and 12.5 µM CoQ0 caused a nonsignificant decrease in p-ERK1/2 protein expression in K562 cell line. Interestingly, in K562 cells, phosphorylation of Akt (Ser473) was diminished at 12.5 µM of CoQ0, with no change observed in p-Akt (Thr308) protein expression among groups. In conclusion, CoQ0 inhibited cell proliferation and suppressed phosphorylation of c-Raf (Ser259), Akt (Ser473), but not ERK1/2 in K562 cells. There is still a need for new insights into the anticancer mechanisms of CoQ0 and develop treatment strategies for chronic myeloid leukemia.*
Keywords: *Chronic myeloid leukemia, coenzyme Q0, K562*

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Submitted / Gönderilme : 21.04.2023
Accepted / Kabul : 17.05.2023
Published / Yayınlanma : 20.09.2023

ÖZ

Amaç: *Bu çalışma, insan kronik miyeloid lösemi K562 hücre hattında koenzim Q0'ın (CoQ0) antiproliferatif ve proapoptotik etkilerini değerlendirmiştir.*

Gereç ve Yöntem: *CoQ0'ın insan kronik miyeloid lösemi K562 hücre hattındaki sitotoksik etkisi, MTT testi ile belirlendi. Kaspaz-3 aktivitesi, apoptozis, MAPK ve AKT sinyal yolağı ile ilişkili proteinlerin ekspresyonu sırasıyla enzimatik analiz ve western blot analizi ile belirlendi.*

Sonuç ve Tartışma: *Sonuçlar, CoQ0'ın K562 hücre canlılığını 5 μM ve daha yüksek konsantrasyonlarda inhibe ettiğini ve Bax protein ekspresyonunu, 12.5 μM konsantrasyonunda önemli ölçüde azalttığını göstermiştir, ancak CoQ0 kaspaz 3 aktivitesini ve Bcl-2 protein ekspresyonunu önemli ölçüde etkilemedi. p-c-Raf (Ser259) protein ekspresyonu, 12.5 μM CoQ0'da önemli ölçüde azaldı. K562 hücre hattında, 10 μM CoQ0, p38 MAPK'nın fosforilasyonunu önemli ölçüde indükledi ve 12,5 μM CoQ0, p-ERK1/2 protein ekspresyonunda anlamlı olmayan bir azalmaya neden oldu. İlginç bir şekilde, 12.5 μM CoQ0 K562 hücrelerinde Akt (Ser473) fosforilasyonu azalttı, ancak p-Akt (Thr308) protein ekspresyonunda gruplar arasında herhangi bir farklılık gözlenmedi. Sonuç olarak, CoQ0, K562 hücrelerinin proliferasyonunu inhibe etti ve c-Raf (Ser259), Akt (Ser473) fosforilasyonunu baskıladı, ancak ERK1/2 fosforilasyonuna etki etmedi. CoQ0'ın antikanser etkisinin altında yatan moleküler mekanizmalara yeni bakış açıları sağlamak ve kronik miyeloid lösemi tedavi stratejilerini geliştirmek için daha fazla araştırmaya hala ihtiyaç bulunmaktadır.*

Anahtar Kelimeler: *Koenzim Q0, kronik miyeloid lösemi, K562*

INTRODUCTION

Chronic myeloid leukemia (CML) is a type of myeloproliferative disorder where an abnormality in chromosome structure, known as t(9;22), leads to the formation of the Philadelphia (Ph) chromosome and Bcr-Abl chimeric oncoprotein [1]. Bcr-Abl oncoprotein enhances cell growth and proliferation of leukemia cells and inhibits apoptosis [2]. The vast majority of individuals diagnosed with CML exhibits a positive response to imatinib, a Bcr/Abl kinase competitive inhibitor. However, there exists a possibility for CML patients to develop clinical resistance to imatinib or experience only temporary remissions despite ongoing treatment [3-5]. Therefore, an alternative therapeutic strategy for CML is required to prevent the progression of the disease to more advanced stages and reduce the risk of death.

Coenzyme Q (CoQ0) is a biomolecule which accumulates predominantly in mitochondria [6,7]. The strong toxicity of CoQ0 has been demonstrated in several cancer cell lines [6,8], and CoQ0 stimulates secretion of insulin by pancreatic islets [9], and has anti-angiogenic and anti-inflammatory properties [10,11]. Researchers have demonstrated that CoQ0 displays potent cytotoxic effects against triple negative breast cancer cell line (MDA-MB-231) by induction of apoptosis and cell cycle arrest, inhibition of metastasis and epithelial-mesenchymal transition [6,12]. CoQ0 significantly induced cell cycle arrest, apoptosis, and inhibited metastasis in melanoma cell lines [13]. In MCF-7 cell line, CoQ0 enhanced ultraviolet B-induced apoptosis [14]. Another CoQ analogs (CoQ2 and CoQ4) induced apoptosis in mutated human acute lymphoblastic leukemia BALL-1 cells [15]. CoQ0 significantly induced cell death in cancerous rat liver MH1C1 cells [16].

Induction of cancer cell apoptosis, the most common form of programmed cell death, is one of the main chemotherapeutic approaches [17]. Activation of caspase-3 is considered to be a main characteristic of apoptosis [18]. Bcl-2 family members (anti-apoptotic proteins Bcl-2 and Bcl-xL and pro-apoptotic proteins Bax, Bak, and Bid) regulate mitochondrial pathway of apoptosis [19,20].

The mitogen-activated protein kinase (MAPK) pathway modulates cell growth and differentiation, apoptosis, migration, survival, and death through the phosphorylation of target proteins [21-23]. MAPKs are comprised of three main subfamilies: the p44/42 (extracellular signal-regulated kinase, ERK1/2), c-Jun N-terminal kinases (JNKs) and p38 MAPKs [24,25]. Raf serine/threonine protein kinase promotes the activation of ERK1/2 which phosphorylates several substrates and modulates different transcription factors and also gene expression [26,27]. Therefore, targeting Raf and ERK1/2 pathway has been considered as potential therapeutic targets in the development of pharmacological agents for anti-cancer treatment [28,29]. Elevated ERK activity in human tumors was

suggested to be a marker of tumor progression [28-30] and decreased ERK1/2 phosphorylation may suppress cancer cell invasion [31].

Enhanced phosphoinositide 3-kinase (PI3K)/Akt activity is associated with poor clinical outcomes in hematological malignancies [32,33], indicating that inhibition of PI3K/Akt activation could be a promising in the management of leukemia [34,35]. Akt inhibits apoptosis by phosphorylation by initiation of multiple additional conversions involved in cell survival or apoptosis [36,37].

The anticancer effect of CoQ0 on chronic leukemia cancer cells was not reported previously. Therefore, we aimed to investigate the cytotoxic and apoptotic effect of CoQ0 on chronic myeloid leukemia K562 cell line through MAPK and AKT signaling pathways modulation.

MATERIAL AND METHOD

Cell Proliferation Assay

K562 cells were seeded in 96 wells plate at a density of $2x10^4$ cells/well and treated with CoQ0 between 1-20 μM during 24 h at 37°C. Then, MTT solution (5 mg/ml) was added to each well, and the plates were incubated for 2 h at 37°C in the dark. After incubation, the formed formazan crystal in each well was dissolved by MTT lysis buffer (20% SDS/50% dimethylformamide) and absorbance value of each well was measured by a spectrophotometer (Molecular Devices Co., CA, USA) at 570 nm. The final DMSO concentration did not exceed 0.25% in the incubation medium.

Caspase-3 Activity Assay

The activity of caspase-3 was determined by colorimetric assay kit (Abcam Inc., UK). K562 cells with and without CoQ0 treatment (10 and 12.5 μM) were lysed in ice-cold lysis buffer and centrifuged at 10000×g for 1 min. The supernatant was collected to obtain total protein of each sample. Firstly, reaction buffer was added to cell lysates which include equal amounts of protein. Next, caspase-3 colorimetric DEVD-pNA substrate was added to the mixture. Following incubation at 37°C for 2 h, the resulting colorimetric product was measured with a microplate reader (Multiskan GO, Thermoscientific, Waltham, MA, USA) at 405 nm.

Western Blot Analysis

K562 cells were harvested and lysed with a lysis buffer. Cell lysates were centrifuged at 8000 rpm at 4°C for 30 min. Protein amount was determined via the Bradford method, and equal amounts of denatured proteins were subsequently subjected to electrophoresis and transfer to a PVDF membrane for 60 min at 100 volts. Blocking of non-spesific binding was performed by incubation with non-fat dry milk (5% w/v) in phosphate buffer saline for 1h. Then, membranes were incubated overnight at 4°C with specific primary antibodies (1:1000), including Bax, Bcl-2, p-c-raf (Ser259), p-p38 MAPK, p-ERK1/2, p-Akt (Ser473), p-Akt (Thr308). The membranes were washed with 1x PBS for 10 min, and then source matched secondary antibodies in PBS-T were added for 1 h at room temperature. Next, the blots were visualized using a chemiluminescence substrate (ECL) and Odyssey Fc system (LI-COR Biosciences, Lincoln).

Statistical Analysis

The data were displayed as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's Post Hoc test was performed for statistical analysis via GraphPad Prism 7 (San Diego, CA). $p<0.05$ was considered as the minimum level of significance.

RESULT AND DISCUSSION

CoQ0 exhibits strong toxicity against various cancer cell lines [6,8,13,38]. Owing to its potent anti-cancer properties, we investigated the cytotoxic and apoptotic effects and possible mechanisms of action of CoQ0 in human chronic myeloid leukemia K562 cells.

In the present study, the concentration of CoQ0 between 5-20 μM significantly reduced the cell proliferation in K562 leukemia cells ($p<0.0001$; Figure 1). The IC_{50} value of CoQ0 for 24 h was

calculated as 9.89 µM in K562 cells. CoQ0 has also been reported to decrease the proliferation of various cancer cells [6,8,38]. It has been described that the caspase-3 activation causes fragmentation of PARP which is a key protein involved in cell apoptosis [39-42]. Caspase-3 activity remained unchanged with 10 and 12.5 µM CoQ0 treatment in K562 cells (Figure 2).

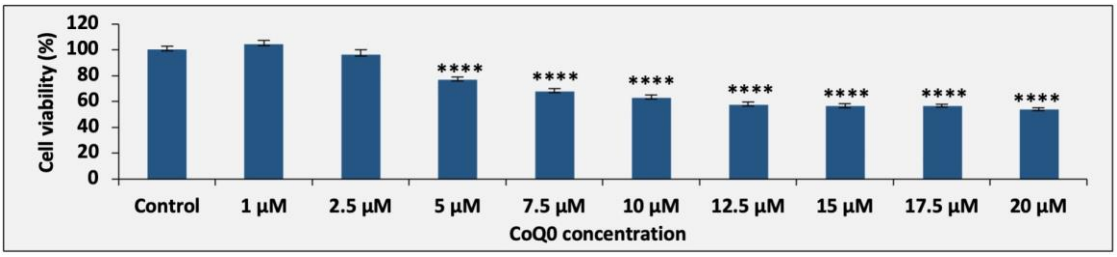

Figure 1. Effect of CoQ0 on the proliferation of K562 chronic leukemia cells $^{****}p<0.0001$ vs. control

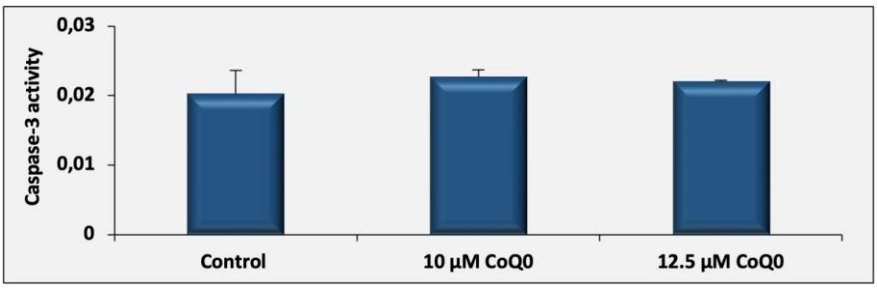

Figure 2. Effect of CoQ0 treatment on caspase-3 activity in K562 chronic leukemia cells

Maintaining a proper balance between the levels of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins is a crucial factor in preserving cellular homeostasis [43,44]. Bax protein expression demonstrated a decrease in 12.5 µM CoQ0 concentration (Figure 3A; $p<0.01$ vs control). Bcl-2 protein expression was decreased in CoQ0-treated groups as compared to the control group; however, this downregulation was not statistically significant at 10 and 12.5 µM CoQ0 concentrations (Figure 3A; p=0.0569, p=0,0666 respectively). Hseu et al. demonstrated a dose-dependent decrease of Bcl-2 and an accompanying increase in Bax expression by CoQ0 in various cancer cells [13,38]. Even if, high Bax expression is considered as a trigger of apoptosis, some apoptosis inducing agents have been shown to reduce Bax expression similar to our findings [45].

In particular, among the major groups of MAPKs, p44/42 (ERK1/2), and p38 MAPK signaling pathways are important targets in the cancer management and the activity of the MAPK is regulated by phosphorylation [21-23,46,47]. MAPK pathways have the potential to either facilitate or hinder the growth of cancerous cells depending on the cellular context [48]. Specifically, ERK may serve as an anti-apoptotic molecule that transduces survival signals, while p38 activation is associated with the induction of apoptosis [49-51]. Interestingly, p38 has been shown to mediate antiapoptotic/pro-growth signals in different systems [52-54]. Furthermore, ERK is a crucial component of the Ras/Raf/MEK/ERK signaling pathway that is regulated by Raf [55]. It has been reported that phosphorylation of the member of the Raf family, c-Raf at Ser259 prevents the activation of c-Raf and dephosphorylation of c-Raf at Ser259 is a pivotal part of the process of c-Raf activation [56]. In K562 cell line, the protein expression of p-c-Raf (Ser259) was decreased by 12.5 µM CoQ0 treatment (Figure 3B). Studies have demonstrated that Akt modulates the Erk pathway through c-Raf phosphorylation at Ser259, which is an inhibitory site [57]. The current investigation has revealed that CoQ0-induced deactivation of Akt leads to c-Raf activity enhancement by means of dephosphorylation in the K562 cell line. The results showed that CoQ0 at concentration of 10 µM also induced expression of p-p38 MAPK (Figure 3B). Moreover, treatment of K562 cells with 12.5 µM CoQ0 resulted in decreased phosphorylation of ERK1/2 which was not statistically significant (Figure 3B). Wang et al.

demonstrated that the induction of apoptosis in human chronic leukemia K562 cells by 2-hydroxy-3-methylanthraquinone is mediated through activation of p-p38 MAPK and downregulation of p-ERK1/2 [59]. In addition, different phosphorylation states of p38 MAPK can be seen in apoptotic cell death, whereas decreased p-p38 MAPK protein expression was shown in the induction of apoptosis in REH leukemia cells [60].

The Akt signaling pathway continues to be an essential pathway of interest for treatment of leukemia [32]. The phosphorylation of Akt is routinely used as a marker of Akt activation [50]. In the present findings, CoQ0 treatment (12.5 µM) decreased p-Akt (Ser473) protein expression, but the phosphorylation of Akt (Thr308) was not affected by treatment (Figure 3C).

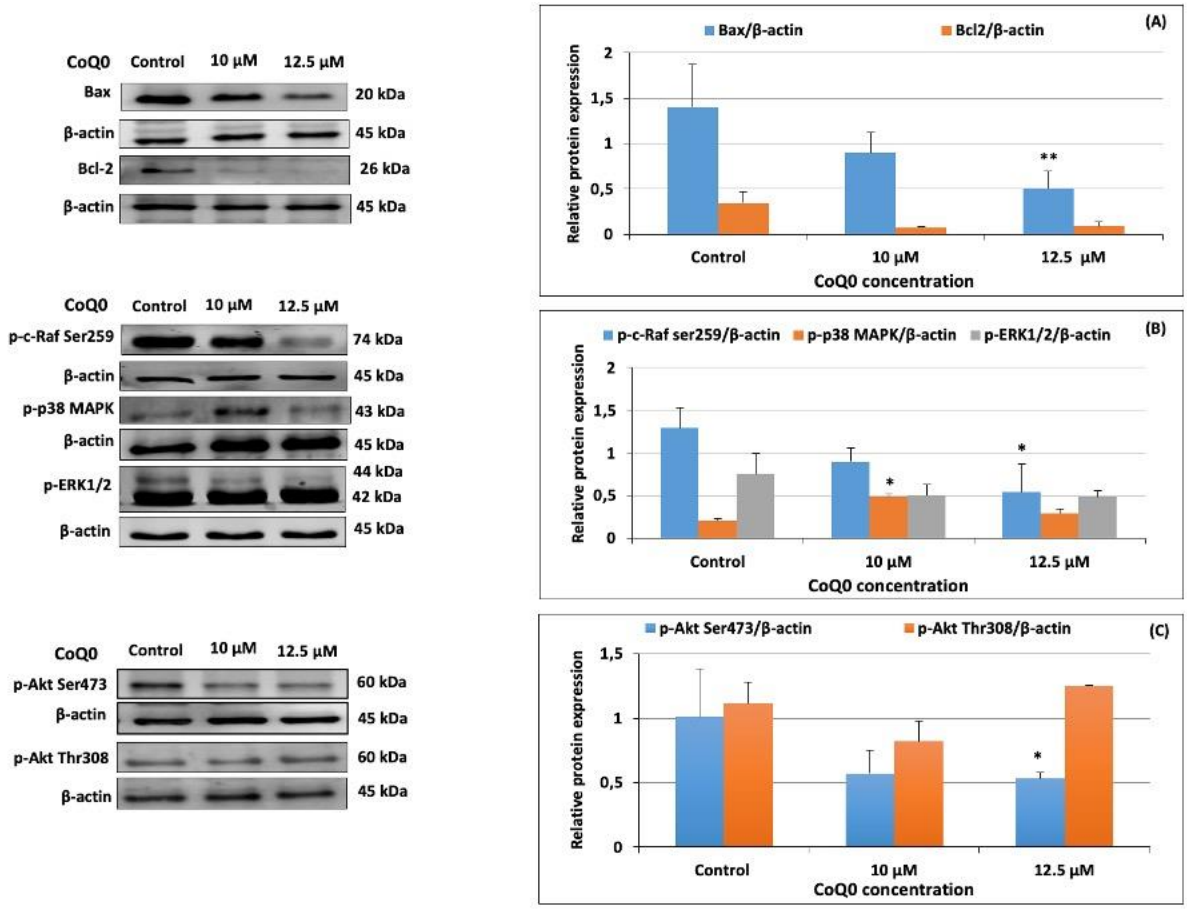

Figure 3. The effects of CoQ0 on the protein expression of apoptosis, MAPK signaling and AKT signaling-related proteins in K562 cells $^{*}p<0.05$ and $^{**}p<0.01$ vs. control

In accordance with these results, we can suggest that CoQ0 may inhibit Akt pathway by reducing Akt phosphorylation at Ser473 at the indicated doses. However, it must be noted that CoQ0 exhibited biphasic effects on p-p38 MAPK in K562 cells.

The results showed that CoQO demonstrated a significant cytotoxicity in chronic myeloid leukemia K562 cells. The antileukemia activity of CoQ0 in K562 cells could be related to the inhibition of phosphorylation of c-Raf at Ser259 and Akt at Ser473. In conclusion, these results contribute to understanding the anticancer activity of CoQ0 in chronic myeloid leukemia K562 cell line. Further studies are required to assess a promising potential of CoQ0 as an anticancer agent in chronic myeloid leukemia treatment.

AUTHOR CONTRIBUTIONS

Concept: A.Z.K.; Design: A.Z.K.; Control: E.K.S., A.Y., A.Z.K.; Sources: A.Z.K.; Materials: E.K.S., A.Y., A.Z.K.; Data Collection and/or Processing: E.K.S., A.Y., A.Z.K.; Analysis and/or Interpretation: E.K.S., A.Y., A.Z.K.; Literature Review: E.K.S., A.Y., A.Z.K.; Manuscript Writing: E.K.S., A.Z.K.; Critical Review: E.K.S., A.Y., A.Z.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., 47(3): 770-783, 2023
Doi: 10.33483/jfpau.1195397

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND CHARACTERIZATION OF CARBOPOL BASED HYDROGELS CONTAINING DEXPANTHENOL

DEKSPANTENOL İÇEREN KARBOPOL ESASLI HİDROJELLERİN HAZIRLANMASI VE KARAKTERİZASYONU

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ABSTRACT

Objective: *The purpose of this study is to create dexpanthenol-loaded hydrogel formulations to alter the release patterns and enhance the physicochemical qualities of the market product.*
Material and Method: *To make hydrogel formulations, Carbopol Ultrez was utilized in concentrations of 1%, 1.5%, and 2% (w/w). The active component dexpanthenol was then added to the formulations at a concentration of 5% (w/w). pH, viscosity, texture profile analysis, spreadability, bioadhesion, and in vitro release characteristics were all assessed for the formulations.*
Result and Discussion: *The formulations were found to be suitable for cutaneous application. TPA analysis revealed that the G1 and G1-DXP formulations had the hardness value 10.185±1.219 and 30.854±1.637 g,* respectively. *That formulations' bioadhesion strength has grown because they are more flexible than previous formulations while having low hardness values. As such, it has been observed that the formulations release more than 50% of DXP in three hours while the market preparation was not even reach the 10% drug release. In the in vitro release kinetics study, it was calculated that all formulations fit the Higuchi model. As a result, a more effective drug delivery*

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Submitted / Gönderilme : 27.10.2022
Accepted / Kabul : 18.05.2023
Published / Yayınlanma : 20.09.2023

system has been developed compared to the market preparation. The currently prepared formulations are also promising formulations in terms of their use in treatment.
Keywords: *Carbopol, characterization, dexpanthenol, hydrogel*

ÖZ

Amaç: *Bu çalışmanın amacı, salım modellerini değiştirmek ve piyasa ürününün fizikokimyasal özelliklerini geliştirmek için dekspantenol yüklü hidrojel formülasyonları oluşturmaktır.*
Gereç ve Yöntem: *Hidrojel formülasyonları yapmak için, %1, %1.5 ve %2 (a/a) konsantrasyonlarında Carbopol Ultrez kullanıldı. Aktif bileşen dekspantenol daha sonra formülasyonlara %5 (a/a) oranında ilave edildi. Formülasyonlar için pH, viskozite, doku profili analizi, yayılabilirlik, biyoadezyon ve in vitro salım özelliklerinin tümü değerlendirildi.*
Sonuç ve Tartışma: *Formülasyonların cilt uygulaması için uygun olduğu bulundu. TPA analizi, G1 ve G1-DXP'nin formülasyonların düşük sertlik değerinin sırası ile 10.185±1.219 ve 30.854±1.637 g sahip olduğunu ortaya koydu. Bu formülasyonların biyoadhezyon mukavemeti, önceki formülasyonlardan daha esnek oldukları ve düşük sertlik değerlerine sahip oldukları için arttı. Geliştirilen formülasyonlar ilk üç saatte %50'nin üzerinde DXP salımı gözlenirken piyasa preparatı %10'un üzerine bile çıkamamıştır. İn vitro salım kinetiği çalışmasında tüm formülasyonların Higuchi modeline uyduğu hesaplanmıştır. Sonuç olarak piyasadaki ürünlere göre daha etkin bir ilaç salım sistemi geliştirilmiştir. Halihazırda hazırlanan formülasyonlar, tedavide kullanımları açısından da umut vadeden formülasyonlardır.*
Anahtar Kelimeler: *Dekspantenol, hidrojel, karakterizasyon, karbopol*

INTRODUCTION

The skin, the largest organ of our body, has many important roles such as creating a barrier between the outside world and our internal organs, providing thermal balance, and taking a role in vitamin synthesis [1]. The skin is basically divided into epidermis, dermis, hypodermis. This multi-layered structure undertakes the task of protecting the body against physical, chemical, radiological, and traumatic damages [2]. Many diseases such as skin urticaria, fungal infections, eczema, psoriasis, skin damage are seen. Wound is one of the most common skin injuries [3]. Wound healing is quite complex and includes 4 different processes. These are: hemostasis, inflammation, profiling, and remodeling [4]. Platelets are activated in order to close the wound opened as a result of the damage to the skin and to prevent blood loss. Monocytes and neutrophils reach the damaged tissue and initiate the inflammation phase. Cytokines are then released that stimulate endothelial cells. Revascularization occurs in the damaged tissue and wound healing is completed [5]. Conditions such as oxidative stress and excessive inflammation slow down wound healing and prevent tissue repair [6]. It is necessary to create a clean and moist environment to accelerate wound healing. For this purpose, ointments, creams, dressings, wound dressings are used. Wound dressings cut off the interaction of the wound with the external environment and protect the tissue that is open to inflammation against microorganisms. It also provides moistening of the scar tissue, thermal insulation, and gas exchange [7].

Hydrogels, known as hydrophilic gels, are 3-dimensional structures. The high amount of water they contain keeps the wound moist. It also helps to relieve pain and pain by keeping the wound cool. Since they do not contain fibers, they do not stick to the damaged tissue and do not cause irritation [8,9]. Hydrogels can be produced with polymers such as chitosan, carbomer, carboxymethyl cellulose. Carbomers are cross-linked polyacrylic acid polymers and are very good drug carriers for transdermal applications [10,11]. Hydrogels are also used in cosmetics. It is especially preferred for moisturizing, anti-aging and cellulite problems [12]. The bioadhesive properties of hydrogels make their use in the cosmetic field widespread. The cooling effect, bioadhesive and non-toxic properties of hydrogels increase their potential for use, especially in skin irritations, laser burns, and sunburns. Since carbomer polymers have several benefits in pharmaceutical applications, including the ability to produce high viscosity gels at relatively low gelling agent concentrations, exhibit bioadhesive, thermostable, and organoleptic properties, and be compatible with a variety of active ingredients, Carbopol Ultrez was used as the gelling agent in this study [13].

Dexpanthenol is an enantiomeric compound with D and L forms. Although both enantiomers have moisturizing properties in topical application, only D-panthenol is a biologically active compound. After D-panthenol is absorbed topically, it is metabolized to pantothenic acid [14]. D-panthenol reduces ROS production and induces tissue regeneration by minimizing tissue damage [15,16]. In a study examining wound healing of D-panthenol, cream containing nebivolol and cream containing dexpanthenol were applied to rats, and as a result of the analyzes, the effects of nebivolol and dexpanthenol on wound healing were found to be comparable [17]. In another study, a multilayered dressing loaded with dexpanthenol was produced. In the *in vitro* study, it was observed that the wound scratches were completely occluded 24 hours after tissue damage was created [18].

In this study, dexpanthenol-loaded hydrogel formulations are made in an effort to change the release patterns and improve the physicochemical properties of the commercial product. As a result, Carbopol Ultrez was used as a gelling agent at various concentrations, and the formulations were assessed for pH, viscosity, textural profile analysis, spreadability, and bioadhesion. Additionally, *in vitro* release investigations were carried out, and the release mechanism was assessed using the mathematical models.

MATERIAL AND METHOD

Materials

Propylene glycol was purchased from Yasin Teknik (Turkey). Glycerin, triethanolamine, Carbopol Ultrez were purchased from Tekkim (Turkey). Propyl paraben was purchased from Doga Ilac (Turkey). Ethanol (EtOH) distilled water and acetonitrile were analytical grade. Acetonitrile and ortho-phosphoric acid were purchased from Sigma (USA). Dexpanthenol (DXP) was a kind gift from BASF (Germany).

Methods

Determination of Dexpanthenol

Dexpanthenol was quantified using an HPLC method. A UV detector, gradient pump, and thermostable column unit are features of the HPLC device (Agilent 1200 Series, USA). The study made use of C18: 4.6x250 mm, 5 micron column (Phenomenex Gemini C18, USA). The HPLC method was modified from previously performed experiments [19]. Briefly, the mobile phase was a mixture of acetonitrile: 0.01 M ortho-phosphoric acid (10:90, v/v). The flow rate, injection volume and UV wavelength were set at 1 ml/ min at 25°C, 10 µl and 205 nm, respectively. Calibration curve was obtained and then the HPLC method was validated in terms of linearity, accuracy and recovery, precision, limit of detection (LOD) and limit of quantification (LOQ).

Preparation of Blank and DXP Loaded Hydrogels

Hydrogels were prepared using 3 different concentrations of Carbopol Ultrez (1%, 1.5%, 2%). Carpobol Ultrez and distilled water were added to the beaker and mixed. Then propylene glycol, ethanol and glycerin were added. Neutralization and cross-linking were achieved by adding 0.5 ml of triethanolamine. Then propyl paraben was added as a preservative. Table 1 shows the formulation components and their amounts.

Preparation of Dexpanthenol Loaded Hydrogels

Dexpanthenol (5%) was dissolved in a 1:1 mixture of distilled water and ethanol. Then, a mixture of distilled water, ethanol, propylene glycol and glycerin were added. Different concentrations (1%, 1.5%, 2%) of Carbopol Ultrez were added to the prepared mixture and mixed. Triethanolamine was added to provide crosslinking.

Characterization of Blank and Panthenol Loaded Hydrogels

pH measurement was performed with a Mettler Toledo S220-K (Switzerland) device. First, pH measurement of the gels without active substance was made. Then, the pH of the active substance loaded

gels was measured. The viscosities of the hydrogels were measured at room temperature (25±2°C) with the Brookfield DV1-LV viscosimeter (UK).

Table 1. Components of the blank and drug loaded formulations

Components	G1 (%)	G1-DXP (%)	G2 (%)	G2-DXP (%)	G3 (%)	G3-DXP (%)
Propylene Glycol	5	5	5	5	5	5
EtOH	2	2	2	2	2	2
Glycerin	2	2	2	2	2	2
Propyl Paraben	0.03	0.03	0.03	0.03	0.03	0.03
Triethanolamine	0.5	0.5	0.5	0.5	0.5	0.5
Distilled Water	89.47	84.47	88.97	83.97	88.47	83.47
Carbopol Ultrez	1	1	1.5	1.5	2	2
Dexpanthenol	-	5	-	5	-	5

Textural Profile Analysis

The mechanical properties such as hardness, compressibility, adhesiveness, cohesiveness, and elasticity of gels were detected using a Texture Analyzer. The test was performed with Perspex probe having 25 mm diameter (P/25P, θ: 25 mm). The pre-test speed was 2.00mm/s, test and post-test speeds were 2 mm/s each with trigger force of 0.001N. The compression depth in each operation was 10.00 mm and the delay period between two compressions was 10 seconds [20,21]. All experiments were performed in triplicates at 25 ± 0.5°C.

Spreadability

The spreadability of blank and loaded gel formulations was determined by using TA-XT Plus Texture Analyzer. Test sample was placed within the female cone. Male cone was moved toward the female cone up to 23 mm at a specified test speed of 3 mm/s and the post-test speed of 10 mm/s. The spreadability of gels was determined in terms of firmness, stickiness, work of shear and work of adhesion.

***Ex vivo* Bioadhesion Experiments**

The mucoadhesion strength was evaluated by using a TA-XT Plus Texture analyzer following the previously described method with some modifications. Dorsal skins of rats sacrificed in previous ethical committee-approved studies were used in this study (Ethical Committee Permission, Kobay Deney Hayvanları Laboratuvarı San. ve Tic. A.Ş., Date: 25.02.2023). Mucoadhesion strength was determined as the detachment force needed to separate the formulation from the skin after applying a force of 0.5 N for 200 seconds with the rate of 0.5 mm/sec. [22]. In summary, 1g of the formulation was placed in the beaker. Rubber has been used to firmly secure animal tissue to the probe. The formulation was then applied to the tissue for the designated amount of time and strength before being removed. Software was used to evaluate the results that were obtained.

***In vitro* Release Studies**

In vitro release studies were performed by using dialysis bag method for the formulations G1, G2, G3 and marketed product (MP, Bepanthol Derma©). Briefly, one gram of accurately weighed gel or MP, containing 5% w/w DXP, was placed inside the dialysis membrane and fixed at both ends. Then, the dialysis bags containing formulations were put in a beaker containing 100 ml of PBS (pH7.4) solution and it was gently stirred on a magnetic stirrer maintained at 32±1°C at 100 rpm. To avoid evaporation, beakers were covered with aluminum foil and parafilm. At each regular interval of time (0.5, 1, 2, 4, 6, 8, 10, 12, 24 h), 0.5 ml of sample was removed and replaced with 0.5 ml of new diffusion

medium. Sink condition was maintained during the experiments [23]. Finally, the samples were analyzed by HPLC-UV method at 205 nm.

Drug Release Kinetic and Mechanism

In this study, to comprehend drug release pattern, *in vitro* drug release data were fitted to various kinetic models, including zero order, first order, Higuchi model, and Hixson-Crowell model. Large value of the coefficient of determination suggested that dissolving behavior and mathematical models were well-matched (r^2) [24].

Similarity and Difference Factors for DXP Release

Fit Factors, which were adapted by the Food and Drug Administration as industry advice for dissolution testing, were used to statistically examine and compare DXP diffusion across the membranes and DXP release patterns from formulations [25,26]. Fit factors are models that are frequently used by researchers to directly assess the variation in drug release percentage per unit time between a reference and a test formulation. Using Equations (1) and (2), the difference factor (*f1*) and the similarity factor (*f2*) were determined.

$$f1 = \left\{\left(\sum_{t=1}^{n}|Rt - Tt| \Big/ \sum_{t=1}^{n} Rt\right) x100 \right. \qquad \textbf{Eq. 1.}$$

$$f2 = 50xlog\left[\left(1 + \left(\frac{1}{n}\right)\sum(Rt - Tt)^{2}\right)^{-0.5} x100\right] \qquad \textbf{Eq. 2.}$$

Where n is the number of dissolution sample periods and Rt and Tt are the percentages of medication released from the reference and test formulations at a certain time point (t), respectively. The relative inaccuracy between the two curves is measured by the difference factor (f_1), which computes the percent difference between the reference and test curves at each time point. The sum of squared errors' logarithmic reciprocal square root translation into the similarity factor (f_2), which measures the similarity in percentage released between curves, is used to calculate the similarity. Arbitrary descriptors of difference and similarity must be selected for data analysis. Curves with $f_1 \geq 10$ and $f_2 \leq 50$ were seen as being distinct.

RESULT AND DISCUSSION

Preparation of Blank and Drug Loaded Hydrogels

In this study, we have formulated hydrogels that can be easily spread on the skin surface, suitable for use in minor skin wounds and cosmetic purposes. Prepared hydrogels contain 5% dexpanthenol. Hydrogel formulations were prepared in 3 different concentrations of Carbopol Ultrez (1%, 1.5%, 2%). Carbopol Ultrez is regarded as a viable candidate for the development of various polymeric systems, particularly controlled drug-delivery systems, and plays a crucial role in the delivery of drugs to a specified location of the body [27]. Because carbomer polymers are known to have bioadhesive, thermostable, and organoleptic qualities, they may be made into very viscous gels at relatively low concentrations, making these systems appealing from both a pharmacological and patient acceptability perspective. Additionally, Carbopol Ultrez hydrogels' compatibility with a variety of active substances and strong bioadhesiveness are benefits [28]. In our study, transparent, non-irritating, non-sensitizing, and non-gritty hydrogels were produced.

Characterization of Blank and DXP Loaded Hydrogels

Carbomers are acrylic acid homo- and copolymers with high molecular weight that have been cross-linked with a polyalkenyl polyether. They are acidic in their unneutralized condition and anionic in nature, so in order to have the potential to thicken, they must be neutralized with the proper base. Water soluble gel that has been neutralized with inorganic bases is stable. Gels made with triethanolamine may withstand high alcohol concentrations [29]. When our results are examined, it is

observed that in the empty formulation, with the addition of a fixed amount of triethanolamine, the viscosity increases when the concentration of Carbopol Ultrez increases and reaches the plateau level after a concentration. On the other hand, the addition of DXP to the formulation led to a decrease in viscosity, although the concentration of Carbopol Ultrez increased (G2-DXP and G3-DXP). Triethanolamine was used to gel the carboxyl groups of Carbopol Ultrez, which were present in low quantity in the G1-DXP formulation. However, in the G2-DXP and G3-DXP formulations, a fixed quantity of triethanolamine caused some carboxyl groups of Carbopol Ultrez to gel. The alcohol structure of DXP, however, resulted in a decrease in the remaining carboxyl groups' ability to form hydrogen bonds, which in turn reduced viscosity. Because aqueous gels have higher viscosity than hydroalcoholic gels [30].

There is naturally a chemical layer on the skin. This barrier at acid pH is called the acid mantle of the skin [31]. This mantle provides hydration of the stratum corneum. The natural skin pH is between 4.1 and 5.8 in adults. Formulations with this pH range are considered to be compatible with the skin. In our study, the pH values of DXP loaded formulations were between 3.760 ±0.034 and 5.416±0.335. Additionally, the pH values were slightly raised by adding DXP to the formulations, although they are still within the permissible range for dermal applications. The pH ranges of all formulations prepared are shown in Table 2.

Table 2. pH and viscosity measurement results

Formulations	pH	Viscosity (P)
G1	5.043±0.117	14.180±0.288
G1-DXP	5.416±0.335	25.333±1.973
G2	4.560±0.095	40.266±4.004
G2-DXP	5.393±0.379	38.880±22.788
G3	3.760±0.034	37.440±1.499
G3-DXP	5.357±0.054	26.466±12.608
MP	6.767±0.029	22.267±0.115

Rutin-loaded hydrogels were investigated in one research for the treatment of wounds in rats. The pH values of the formulations were 5.7, which was determined to be appropriate for dermal applications because the pH of skin is slightly acidic [32]. Another research examined dexamethasone-loaded nanocapsules incorporated in hydrogels. Carbopol Ultrez was used to create the formulations, which had a pH value of about 5.5. The use of these formulations on the skin's surface was also accepted [33].

Viscosity determines the application time of formulations to the skin and the residence time after application. Although the residence time of the formulations with high viscosity increases, the penetration rate of the active substance into the skin slows down. Table 2 shows the viscosity values of the formulations developed. According to the literature on topical administration, at optimal viscosity, the gel neither flowed immediately after its application to the skin nor resisted application [34]. The viscosity of formulations was determined to be within the permitted range for topical use.

Texture Profile Analysis

The textural parameters of the hydrogel formulations produced by TPA analysis such as hardness, adhesiveness, cohesion, resilience, and springiness were evaluated. Table 4 shows the TPA values of the formulations. Hardness (N or g) is the resistance to product deformation and is measured as the initial compression's maximum force. Gel hardness provides details on how easily gels may be applied to skin, which may be a sign of how long a gel will stay on the application site. The amount of effort needed to separate the probe from the formulation is defined by the adhesiveness value, which is related to adhesive qualities. Greater tissue surface adherence is indicated by a higher adhesiveness rating, which is a desirable property to prolong medication retention. Adhesiveness is determined using the negative force area for the first compression cycle if any attractive force acts between the gel's surface

and the probe. The formulations' response to repeated shearing loads is demonstrated by their cohesiveness. The gel sample distorted during the first compression, and the definition of the gel's reconstruction following its deformation is springiness or elasticity. A product's cohesiveness is determined by comparing how well it stays together following one deformation to how well it kept together following a subsequent deformation [35,36].

Table 3. The mechanical properties of hydrogels

Formulations	Hardness (g)	Adhesiveness (g.sec)	Cohesion	Resilience (%)	Springiness (%)
G1	10.185±1.219	-35.372±8.234	0.885±0.005	14.506±2.581	89.355±0.980
G1-DXP	30.854±1.637	-63.600±0.392	0.810±0.052	9.975±0.945	92.147±2.149
G2	26.819±0.904	-65.927±10.080	0.816±0.035	20.041±0.624	91.816±1.559
G2-DXP	41.292±1.360	-67.759±15.066	0.823±0.016	11.715±1.973	93.811±1.306
G3	24.450±0.180	-62.477±13.674	0.864±0.019	18.370±2.286	90.552±1.662
G3-DXP	43.496±3.824	-53.961±2.062	0.752±0.112	14.218±0.158	93.846±1.248
MP	-2.893±1.033	-125.761±28.063	0.866±0.139	0.026±0.008	0.996±0.001

As was already indicated, different concentrations of Carbopol Ultrez were used as a gelling agent. TPA findings show that increasing concentration has enhanced the gels' hardness as predicted, notably for drug-loaded gels [37]. It is desirable that the hardness value be low for simple administration and spreadability since it is connected to the formulation's application to the skin [36]. In contrast to other formulations, the hardness values of G1 and G1-DXP were found to be lower in our investigation. This outcome may be assessed based on how simple it was to apply the G1 and G1-DXP formulations to the skin's surface. The blank and drug-loaded G2 and G3 formulations approach a plateau at concentrations of 1.5% and 2%, whose values are not statistically different, even if the hardness corresponds with increasing concentration.

Since the formulations attained plateau values for all of the aforementioned properties as well as for adhesiveness, cohesiveness, resilience, and springiness, the results show that all created formulations are appropriate for cutaneous applications.

To correlate data from rheological investigations with textural profile analyses, Carvalho et al. created hydrogels using a variety of gelling agents. Researchers employed various types of polyacrylic polymers for this purpose. The findings showed that formulations' levels of textural analysis plateaued at various concentrations. Results were also assessed in light of how well-suited the formulations' low hardness values are for cutaneous applications [38]. In a different study, Özcan et al. developed chitosan hydrogels loaded with terbinafine hydrochloride to improve topical drug delivery. For this reason, scientists have developed chitosan hydrogels with varying molecular weights. Results indicated that other formulations had textural characteristics comparable to the one we prepared [39].

Spreadability Analysis

In terms of patient compliance, spreadability is one of the key characteristics of the topical formulation. If a product has sufficient spreadability, even applying it to the skin is simpler and more patient acceptable. Additionally, formulations that are easier to distribute might cover more skin upon application, which can enhance the therapeutic impact [40]. Spreadability results of our study are shown in Table 4.

Spreadability study reveals formulation strength (firmness), work of spreading (work of shear), stickiness, and force of extrusion by displaying the fluctuation of force as a function of time (work of adhesion). The formulation's strength may be determined by its firmness, which is the maximal positive force that can distort it. The strength of the formulation is improved by a greater firmness value. The spreadability of the formulation is demonstrated by the area up to the positive force, which is the work

of shear. The weight of the sample raised on the top surface of the male cone during the turn was mostly responsible for the negative section of the graph produced by the probe rotation. This is a result of back motion and indicates if there is resistance to the disc sticking or flowing. The adhesion force for the gel, which symbolizes the force necessary to separate the gel from the tube, is at its maximum negative value. The area of the curve's negative portion was picked to represent the adhesion work [41]**.** Spreadability is inversely related to firmness and work of shear [42]. The values of firmness and work of shear for G2 and G3 formulations were determined to be greater based on the data produced (Table 4, Figure 1). Consequently, they have less spreadability than the G1 formulation.

Table 4. Results of spreadability test

Formulations	Firmness (g)	Work of Shear (g.sec)	Stickiness (g)	Work of Adhesion (g.sec)
G1	360.40±18.39	347.25±15,20	-304.3±13.21	-85.64±7.72
G1-DXP	884.69±14.94	775.3±33,15	-700.91±9.33	-218.18±2.61
G2	917.58±18.48	895.10±43.40	-694.16± 14.06	-205.98±6.76
G2-DXP	1065.09±14.23	1008.09±27.41	-819.00±9.92	-255.52±6.69
G3	862.59±13.67	877.70±36.06	-649.37±13.86	-185.42±9.72
G3-DXP	1237.01±0.88	1256.00±53.66	-911.98±7.47	-305.53±11.49
MP	1159.78±30.67	829.01±8.55	-2122.05±96.64	-332.49±33.21

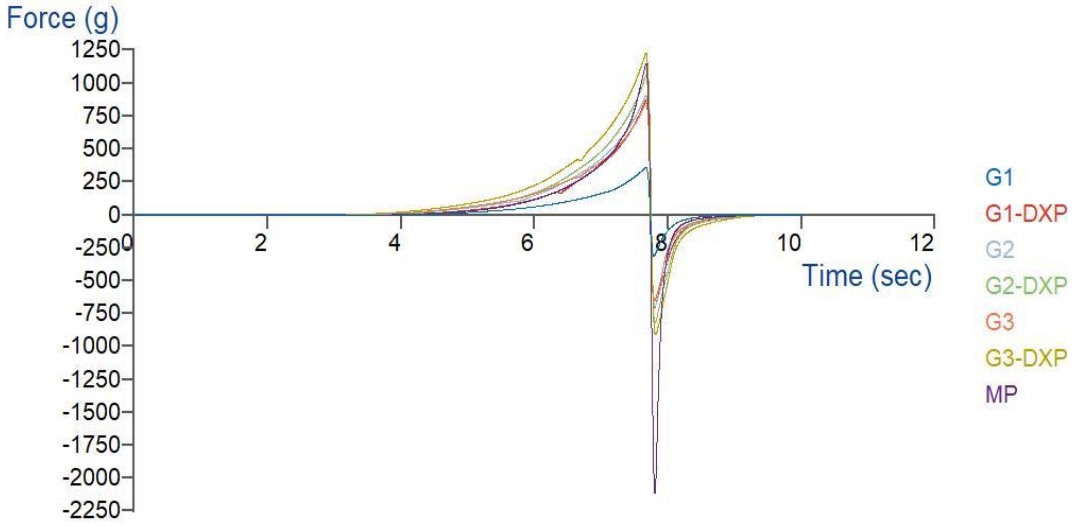

Figure 1. Graphical results of spreadability tests

In one study, scientists made fish-oil-based oleogels for the topical administration of bethametasone dipropionate. As a result, many types of oleogels were produced and described. Spreadability characteristics of produced formulations were measured and evaluated as part of a formulation characterization study. Some oleogels had enhanced firmness levels, according to the results. Results assessed as higher hardness values are associated with lower spreadability behaviors [43]. Another study utilized the loading of capsaicin into lipid-based nano colloidal topical carriers to enhance analgesic potency and lessen cutaneous irritation. Formulations were then added to the Carbopol 934 gel system. Spreadability experiments were carried out to assess the formulation's compatibility with the skin's surface. Results revealed that lower values for firmness, work of shear, and work of adhesion were considered to be more suitable for patient use [44]. Additionally, a correlation between our spreadability test results and the literature was observed. In comparison to G2 and G3

formulations, G1 formulations had lower spreadability values. G1 formulations exhibit good patient compliance because of this.

Ex vivo Bioadhesion Studies

In terms of how well a gel-based formulation adheres to a biological surface (skin), bioadhesion is a crucial component. Its foundation was the idea of measuring the force necessary to rupture the adhesive link between a model membrane and the test formulation (Table 5). Figure 2 shows the textural analysis of prepared hydrogels.

Table 5. Results of *ex vivo* bioadhesion experiments

Formulation	Peak Force (Adhesiveness) (N)	Work of Adhesion (N.sec)	Debonding Distance (mm)
G1	1.13±0.18	0.54±0.00	7.24±0.24
G1-DXP	0.74±0.12	0.65±0.18	5.39±0.06
G2	0.70±0.11	0.79±0.02	14.28±2.97
G2-DXP	0.51±0.05	0.50±0.06	8.20±3.99
G3	0.34±0.00	0.34±0.11	7.88±1.28
G3-DXP	1.05±0.06	0.52±0.02	5.02±0.34

Based on the aforementioned bioadhesion findings, it was found that G1 and G1-DXP hydrogels demonstrated substantial spreadability and bioadhesion, which is ideal for a formulation designed for dermal applications. Additionally, G1-DXP hydrogel had a longer residence time, indicating that the substance stays at the application site for a longer amount of time**.**

The establishment of hydrogen bonds between the functional groups of the bioadhesive polymers and the skin is generally thought to be what produces the adhesion force, not the interpenetration of the Carbopol Ultrez® chains into the skin, which is thought to be what produces the adhesion work. Therefore, the bioadhesiveness should enhance when Carbopol Ultrez content is increased [45]. According to our findings, the formulation with less concentration had better bioadhesive qualities. It is because drug-loaded G2 and G3 formulations and blank samples both displayed greater hardness levels. The formulations' rigidity may reduce how well the polymer interacts with the skin [38].

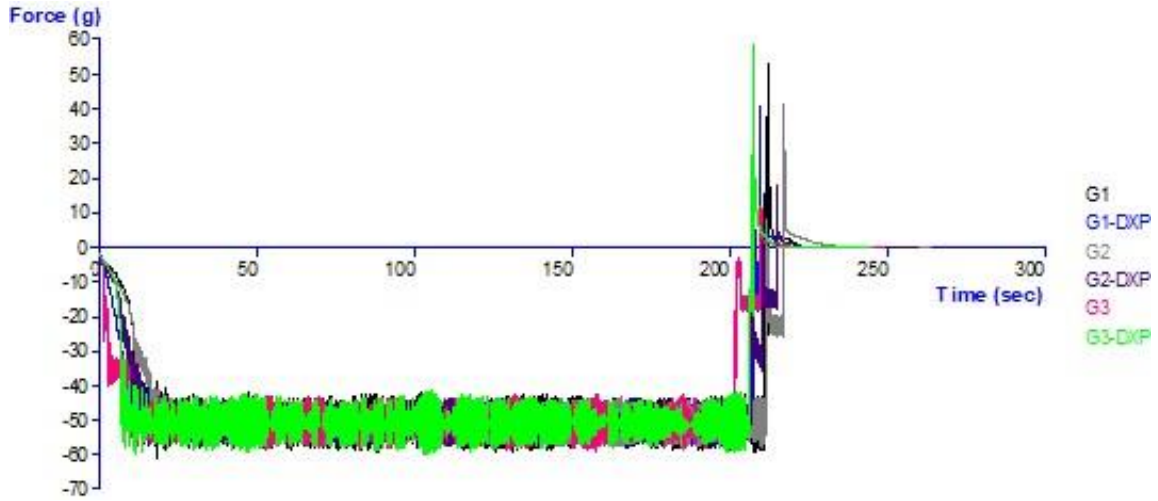

Figure 2. Graphical results of bioadhesion studies

Additionally, the results of the bioadhesion do not correlate with the adhesiveness values found from TPA analysis. It is as a result of the absence of biological membrane (skin).

In vitro Release Studies

DXP hydrogels of G1-DXP, G2-DXP, and G3-DXP as well as a commercial product had their *in vitro* release tested. The investigation was carried out at 32°C using PBS (pH 7.4) as the release medium, and Figure 3 displays the findings. The state of the sink was also preserved.

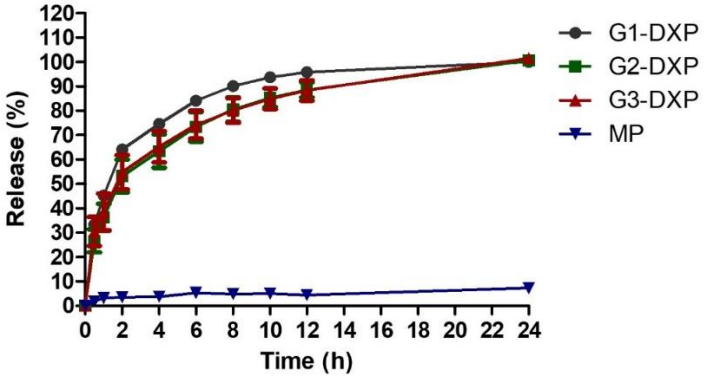

Figure 3. Results of *in vitro* release studies

The drug was released in the following amounts within the first hour: 45.237%, 36.311%, 38.570%, and 3.241% for the formulations G1-DXP, G2-DXP, G3-DXP and MP, respectively. The MP released 7.358% of the drug to the medium at the conclusion of the 24-hour period, while the remaining formulation had reached 100%. MP is a product in the ointment type. Hydrogels demonstrated enhanced release compared to commercial product since they are simple to moisten by the release medium.

To improve *in vivo* absorption characteristics, DXP loaded carboxyvinyl derivatives (Carbopol 980 and Ultrez 10) and poloxamer (Lutrol F 127) were employed as the hydrogel foundation in one research. The *in vitro* release characteristics of DXP from the gel basis were also compared to those of the commercial product (cream). The study's findings, which were consistent with our findings, demonstrated that commercial products released the least amount of DXP over the course of a 24-hour period as compared to gel formulations [46]. Another research looked at how different vehicles affected the pharmaceutical availability of different antirheumatic drugs and tried to create the best technique for cutaneous application. Due to this, the *in vitro* release of indomethacin and diclofenac sodium from several drug carrier systems, including ointment, cream, and gel, was assessed. The study's findings showed that our formulations had a comparable *in vitro* release pattern [47]. Another study looked at the skin's ability to absorb capsaicin and nonivamide from hydrogels both *in vitro* and *in vivo*. Hydrogels and a variety of commercially available capsaicin creams were also contrasted. Results demonstrated that hydrogels released drugs more effectively than cream formulations, which is consistent with our findings [48].

The calculated difference and similarity factor for pair-wise intraformulation comparisons are shown in Table 6. All formulations were found to be similar.

Table 6. Difference (f_1) and similarity (f_2) factors for DXP loaded gel formulations

Release Method	Reference Formulation	Test Formulation	f_1	f_2	*Dissolution Profile*
Dialysis Bag	G2-DXP	G1-DXP	6	59	Similar
	G3-DXP	G1-DXP	6	58	Similar
	G3-DXP	G2-DXP	1	97	Similar

In vitro Release Kinetic and Mechanism

Cojocaru et al. claim that in order to comprehend the release characteristics, the drug release data should be matched with an appropriate mathematical model. In fact, as the r2 grows with the number of included parameters, the modified coefficient of determination should be used when comparing models with numerous parameters [49]. To describe the kinetics of drug release from the test gels and the commercial product, zero-order, first-order, Hixson-Crowell, Higuchi, and Korsmeyer-Peppas models were used. Before doing linear regression analysis for each case, the data were transformed.

Table 7. Fitting various mechanism models to the release kinetics of DXP loaded gels

Formulations	**G1-DXP**			**G2-DXP**			**G3-DXP**			**MP**		
Models	**r^2**	**n**	**m**	**r^2**	**n**	**m**	**r^2**	**n**	**m**	**r^2**	**n**	**m**
Zero-Order	0.5515	45.825	3.3052	0.6752	36.905	3.545	0.6701	38.447	3.4667	0.7002	2.3912	0.2296
First-Order	0.8001	37.682	53.03	0.8696	30.058	53.592	0.8468	32.035	51.913	0.75	2.1233	3.165
Higuchi	0.834	23.586	20.319	0.9174	15.544	20.657	0.9128	17.499	20.228	0.8686	1.1384	1.2782
Hixson-Crowell	0.2908	1.1095	-0.1029	0.3499	1.0464	-0.1077	0.3351	1.3019	-0.1069	0.3363	2.7588	-0.043
Korsmeyer-Peppas	0.9984	0.4704		0.9984	0.4704		0.9866	0.4222		0.8686	0.2884	

The Hixson-Crowell model may generally be used to examine drug formulations with a range of particle surface area and diameter. The zero-order law can be used to find drug delivery methods where the drug dissolves slowly independent of the initial drug concentration and where the medication does not increasingly deteriorate. The first-order rule, on the other hand, works better in systems where the initial drug concentration affects drug release. Finally, the release brought on by drug diffusion from the matrix via pore generation is consistent with Higuchi's hypothesis [50].

The effectiveness of each model in representing the drug release kinetics was evaluated using the r^2 calculation. The outcomes of fitting the *in vitro* release data into several kinetic models are displayed in Table 6. Our results showed that all formulations including commercial product were fitted to Higuchi's model.

In order to identify the release mechanisms for these formulations, the Korsmeyer-Peppas model was once more used in an *in vitro* DXP release behavior analysis. These mechanisms were Fickian (nonsteady) diffusional release when $n \leq 0.5$, case-II transport (zero-order) release when $n \geq 1$, and non-Fickian, "anomalous" release when n is between 0.5 and 1 [51]. As a result, in this work, all formulations including marketed product showed Fickian diffusional release processes since "n" values were smaller than 0.5.

Our project's objective was to develop topical gel formulations with quality and amount of chemicals that produce gel structures with sufficient mechanical strength and stability. According to acceptable rheological/mechanical characteristics and *in vitro* drug release, the G1-DXP formulation was discovered to be a successful topical drug delivery method. To conclude, G1-DXP may be a successful substitute for formulation for cutaneous applications. To corroborate the findings of this study, more *ex vivo* and *in vivo* research is needed.

AUTHOR CONTRIBUTIONS

Concept: E.Ş.Ç., G.K.G., N.Ü.O.; Design: E.Ş.G., N.Ü.O.; Control: E.Ş.Ç, G.K.G., N.Ü.O.; Sources: E.Ş.Ç, G.K.G., N.Ü.O.; Materials: E.Ş.Ç., G.K.G., N.Ü.O.; Data Collection and/or Processing: E.Ş.Ç., G.K.G., N.Ü.O.; Analysis and/or Interpretation: E.Ş.Ç, G.K.G., N.Ü.O.; Literature Review: E.Ş.Ç., G.K.G., N.Ü.O.; Manuscript Writing: E.Ş.Ç., G.K.G., N.Ü.O.; Critical Review: N.Ü.O.; 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., 47(3): 784-791, 2023
Doi: 10.33483/jfpau.1161440

ORIGINAL ARTICLE / ÖZGÜN MAKALE

TOTAL ANTIOXIDANT STATUS, ANTIMICROBIAL AND ANTIPROLIFERATIVE POTENTIALS OF *VIOLA ODORATA* (FRAGRANT VIOLET)

VIOLA ODORATA'NIN (KOKULU MENEKŞE) TOPLAM ANTİOKSİDAN DURUMU, ANTİMİKROBİYAL VE ANTİPROLİFERATİF POTANSİYELLERİ

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ABSTRACT

Objective: *Plants are preferred for biological effect. It is a natural resource used in the field of alternative medicine due to its biological effect. In our study, the total oxidant status (TOS) and oxidative stress index (OSI) and total antioxidant status (TAS) of Viola odorata L. species were detected. In addition, antimicrobial and antiproliferative effect of species was detected.*
Material and Method: *The some parts of the species were used with the help of a soxhlet equipment, and ethanol was preferred as a solvent. TOS, OSI and TAS capacitiy were detected using Rel Assay kits. Agar dilution method was preferred to determine antimicrobial effect against bacteria and fungi. Lung cancer cell line (A549) was used to find out the antiproliferative effect by MTT assay.*

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Submitted / Gönderilme : 12.08.2022
Accepted / Kabul : 23.05.2023
Published / Yayınlanma : 20.09.2023

Result and Discussion: *Consequently, the studies, the TAS capacitiy of V. odorata extract was detected as 6.752±0.139, the TOS capacitiy as 7.886±0.224 and the OSI capacitiy as 0.117±0.001. V. odorata extracts were determined to be influential against standard bacteria at 25-100 µg/ml intensiy and against fungi at 100-200 µg/ml intensiy. It was detected that the antiproliferative effect of V. odorata extract increased depending on the extract intensiy and showed strong effects. Consequently, it has been detected that V. odorata has important biological effects and in the pharmaceutical industry, it can be preferred after certain stages.*
Keywords: *Antioxidant, fragrant violet, medicinal plants, oxidant, Viola odorata*

ÖZ

Amaç: *Bitkiler birçok biyolojik aktiviteden sorumludur. Bu kapsamda tamamlayıcı tıpta önemli doğal materyallerdir. Bu çalışmada Viola odorata L. bitkisinin toplam antioksidan durumu (TAS) ve toplam oksidan durumu (TOS) ve oksidatif stress indeksi (OSI) belirlenmiştir. Ayrıca bitkinin antimikrobiyal ve antiproliferatif aktivitesi tespit edilmiştir.*
Gereç ve Yöntem: *Bitkinin toprak üstü kısımlarının etanol ile soxhlet cihazından ekstraksiyon işlemi yapılmıştır. TAS, TOS ve OSI değerleri Rel Assay kitleri kullanılarak belirlendi. Antimikrobiyal aktivite agar dilisyon metodu ile bakteri ve fungus suşlarına karşı test edilmiştir. Antiproliferatif aktivite A549 akciger kanser hücre hattına karşı MTT testi ile test edilmiştir.*
Sonuç ve Tartışma: *Yapılan çalışmalar sonucunda bitki ekstraktının TAS değeri 6.752±0.139, TOS değeri 7.886±0.224 ve OSI değeri 0.117±0.001 olarak belirlenmiştir. Bitki özütleri standart bakterilere karşı 25-100 µg/ml, funguslara karşı 100-200 µg/ml konsantrasyonlarda etkili olduğu görülmüştür. Bitki özütünün antiproliferative aktivitesi özüt konsantrasyonuna bağlı olarak arttığı ve güçlü etkiler gösterdiği belirlenmiştir. Sonuç olarak V. odorata'nın önemli biyolojik aktivitelere sahip olduğu bu kapsamda farmakolojik ilaç dizaynlarında doğal kaynak olarak kullanılabileceği belirlenmiştir.*
Anahtar Kelimeler: *Antioksidan, kokulu menekşe, oksidan, şifalı bitkiler, Viola odorata*

INTRODUCTION

People have used many natural materials for different purposes since ancient times. The environment we live in is the habitat of fungi, plants and animals that contain beneficial compounds for humans. These living organisms, which have an important place in the ecosystem, contain nutrients and metabolic products that are very important in human health [1]. Plants are the living groups that contain the largest number of organisms among these organisms. They have been used to meet basic human needs such as shelter, medicine and food [2]. Medicinal features of plants with important nutritional features have been emphasized by many researchers [3]. Some trials have detected that some plant species preferred in experiments have biological effect such as antiaging, anticancer, antitumor, antiproliferative, DNA damage protective, antimicrobial, antioxidant, antialergic, hepatoprotective and anti-inflammatory [4-7]. Consequently, it is very important to research plants in the discovery of new effects and new natural products.

V. odorata L. is a dwarf, herbaceous and perennial flowering plant of European and Asian origin in the Violaceae family. Common names include tree violet, English violet, garden violet, sweet violet and common violet. It has been widely used in perfumes and cosmetics because of the sweet scent of the flowers of *V. odorata* [8]. *V. odorata*, which is used for many purposes in different region of the world, was used by the French to make violet syrup, in the USA it was used to make syrup, donuts and confectionery. In addition to these features, it is used against respiratory disorders, insomnia and skin disorders [9]. The antimicrobial, antioxidant, oxidant, and antiproliferative effects of the plant used in our study were analyzed.

MATERIAL AND METHOD

V. odorata used in the study was obtained from Duhok (Iraq). Soil and soil-like materials in the sample we used were cleaned. *V. odorata* was ground into powder with the help of some grinding materials. After pulverization, approximately 30 g was weighed. This powdered sample was treated at 50°C for approximately 6 hours. Crude extracts were obtained from the extracts formed consequently

the process with the support of a rotary evaporator device.

Total Oxidant and Antioxidant Tests

The extract kits obtained from the plant were celebrated and their antioxidant and oxidant capacitiy were determined. Total oxidant was detected with TOS kits. Trolox and hydrogen peroxide were prefered as calibrators for TAS and TOS tests. The manufacturer's protocol was followed for the tests [10,11]. The oxidative stress index is formed by the ratio of total oxidant to total antioxidant [12].

Antimicrobial Test

Antimicrobial effect with ethanol extract of *V. odorata* was detected by the agar dilution method. Bacteria used in the experiment were cultured using Mueller Hinton Broth medium. The fungi used in the experiment were added to the culture medium using RPMI 1640 Broth medium. All planted plates were evaluated after they were kept in an oven at 35°C for 16-20 hours for bacteria and 48 hours for fungi.

Test bacteria: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* MRSA ATCC 43300, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212

Test fungi: *Candida krusei* ATCC 34135, *Candida glabrata* ATCC 90030 and *Candida albicans* ATCC 10231

All extracts were tested at concentrations of 800-12.5 µg/ml and all dilutions were made with distilled water. Fluconazole, amphotericin B (Fungi) and amikacin, ampicillin and ciprofloxacin (Bacteria) were used as reference drugs. The lowest intensiy that inhibited the position of fungi and bacterial strains was detected. The data obtained were expressed as µg/ml [12-15].

MTT Test

Consequently, the extract obtained from *V. odorata* on the lung cancer cell (A549) were examined using the MTT test. These preferred cells were separated from each other using 3.0 ml of Trypsin-EDTA solution (Sigma-Aldrich, MO, USA) after 70-80% confluence. These separated structures were then incubated for approximately 24 hours. Controls were cultured in growth medium without FCS treatment. After 48 hours of incubation, the supernatants were thawed in growth medium. MTT (Sigma) (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added to the supernatant at a intensiy of 1 mg/ml. Incubation was carried out at 37°C until a purple precipitate formed was observed and then removed. Dimethyl sulfoxide (DMSO) was added to cell-penetrating MTT (Sigma-Aldrich, MO, USA) to dissolve it. After these procedures, the results were read at 570 nm with the help of Epoch spectrophotometer (BioTek Instruments, Winooska, VT) [16].

RESULT AND DISCUSSION

Total Antioxidant and Oxidant Effect

Plants are natural products responsible for many biological effects. These natural products attract attention especially with their antioxidant features [17]. Antioxidants are attached to combat reactive oxygens. And it usually suppresses [18]. In cases where antioxidant compounds are insufficient to suppress oxidant compounds, oxidative stress occurs. In addition, antioxidants used are important in both reducing and suppressing oxidative stress [19]. Consequently, examination of antioxidant status in plants is important for new studies. In this study, antioxidant and oxidant results of *V. odorata* were found. The results are presented in Table 1.

Table 1. Antioxidant and oxidant effect of *V. odorata* extract

Sample	TAS (mmol/l)	TOS (µmol/l)	OSI
V. odorata	6.752±0.139	7.886±0.224	0.117±0.001

Capacitiy are presented as mean±S.D.

No data on TAS, TOS and OSI capacitiy of *V. odorata* have been found in the literature before. It has been indicated that V. odorata has antioxidant potential using different methods [20-23]. In our study, it was observed that the antioxidant status of *V. odorata* was high. The data we obtained Consequently are in agreement with the literature data. In our study, the total antioxidant capacitiy of *V. odorata* were detected for the first time. In studies on different plant species using this method, TAS capacitiy as 3.628, TOS capacitiy as 4.046 and OSI capacitiy as 0.112 of *Mentha longifolia* ssp. *longifolia*, TAS capacitiy as 7.342, TOS capacitiy as 5.170 and OSI capacitiy as 0.071 of *Rhus coriaria* var. *zebaria*, TAS capacitiy as 5.853, TOS capacitiy as 16.288 and OSI capacitiy as 0.278 of *Allium calocephalum*, TAS capacitiy as 6.328, TOS capacitiy as 11.525 and OSI capacitiy as 0.182 of *Scorzonera papposa*, TAS capacitiy as 8.656, TOS capacitiy as 4.951 and OSI capacitiy as 0.057 of *Rumex scutatus*, TAS capacitiy as 9.490, TOS capacitiy as 14.839 and OSI capacitiy as 0.157 of *Helianthemum salicifolium*, TAS capacitiy as 6.831, TOS capacitiy as 3.712 and OSI capacitiy as 0.054 of *Gundellia tournefortii* have been indicated [24-30]. The TAS capacitiy of *V. odorata* was detected to be higher than *M. longifolia* ssp. *longifolia*, *A. calocephalum*, *S. papposa*, and lower than *R. coriaria* var. *zebaria*, *R. scutatus*, *H. salicifolium* and *G. tournefortii*. Plants contain phenolic compounds with antioxidant effect and electron source feature in many different features and structures. In addition, it is abundance in antioxidant vitamins A, C and E. With these features, they have potentially powerful antioxidant characters [31,32]. TAS capacitiy is an indicator of antioxidant compounds used to reduce the effect of oxidant compounds for living things [33]. Consequently, the determination of TAS capacitiy of plants is important for the detection of new antioxidant sources. In our study, TAS capacitiy of *V. odorata* were detected and it was detected that it has an important antioxidant potential.

The TOS capacitiy of *V. odorata* was detected to be higher than *M. longifolia* ssp. *longifolia*, *R. coriaria* var. *zebaria*, *R. scutatus* and *G. tournefortii*, and lower than *A. calocephalum*, *S. papposa* and *H. salicifolium*. TOS value is an indicator of all oxidant compounds produced in living things [33]. In the literature, the differences in TOS capacitiy attract attention in the studies of different researchers on different plant species. It is thought that the main reason for this is the differences in the regions where the plants are collected, the differences in the plant species and the potential to produce and accumulate oxidant compounds consequently metabolic processes. The OSI capacitiy of *V. odorata* was detected to be higher than *M. longifolia* ssp. *longifolia*, *R. coriaria* var. *zebaria*, *R. scutatus* and *G. tournefortii*, and lower than *A. calocephalum*, *S. papposa* and *H. salicifolium*. The OSI value shows how much the oxidant compounds produced in living organisms are suppressed by endogenous antioxidant compounds [33]. The TOS capacitiy of *V. odorata* detected in our study resulted in lower OSI capacitiy consequently the plant's total antioxidant system being more potent and influential. Consequently, oxidative stress triggered by oxidant molecules could be prevented by being eliminated by antioxidant compounds, which are a reflection of enzymatic and nonenzymatic systems, and consequently, OSI capacitiy were detected at low levels.

Antimicrobial Effect

The study and use of herbal medicine has gained importance in last period. The number of diseases caused by microorganisms has been rising in last years [34]. In the fight against diseases caused by microorganisms, drugs of synthetic origin take the lead. Due to the possible side effects of synthetic drugs and unconsciously used antibiotics, the number of resistant microorganisms has been increasing in last years [35]. Consequently, the research of timely antimicrobial sources has become inevitable in the fight against microbial diseases. The antimicrobial agent status of *V. odorata* ethanol extract against some bacterial and fungal strains was investigated. The analyzed findings are presented in Table 2.

It has been indicated that petroleum ether, dichloromethane, ethyl acetate and aqueous extracts of *V. odorata* are influential against *Klebsiella pneumoniae* and *Escherichia coli* at different intensiy [36]. In another study, it was indicated that petroleum ether, acetone, methanol and aqueous extracts of *V. odorata* had effects against *Haemophilus influenzae*, *Staphylococcus aureus*, *S. pyogenes*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* at different intensiy [37]. It has been indicated that the aqueous extract of *V. odorata* is influential against *Shigella flexneri*, *K. pneumoniae*, *Salmonella typhi*, *P. aeruginosa*, *E. coli* and *S. aureus* [38]. In our research, the ethanol extract of *V. odorata* was preferred. It was detected that the plant extract was influential against *A. baumannii* as 25 µg/ml extract intensiy.

The plant extract was influential against *P. aeruginosa* and *E. faecalis* as 50 µg/ml, against *S. aureus*, *E. coli*, *S. aureus* MRSA, *C. krusei* and *C. glabrata* as 100 µg/ml, against *C. albicans* as 200 µg/ml extract intensiy. Consequently, it has been detected that the antimicrobial effect of the plant extract is high. Consequently, it is thought that the plant can be used as antimicrobial agent.

Table 2. MIC values of *V. odorata* extract

Sample	A	B	C	D	E	F	G	H	J
***V. odorata* Extract (µg/ml)**	100	100	50	100	50	25	100	200	100
Ampicillin	1.56	3.12	1.56	3.12	3.12	-	-	-	-
Amikacin	-	-	-	1.56	3.12	3.12	-	-	-
Ciprofloxacin	1.56	3.12	1.56	1.56	3.12	3.12	-	-	-
Fluconazole	-	-	-	-	-	-	3.12	3.12	-
Amphotericin B	-	-	-	-	-	-	3.12	3.12	3.12

(A) *S. aureus*, (B) *S. aureus* MRSA, (C) *E. faecalis*, (D) *E. coli*, (E) *P. aeruginosa*, (F) *A. baumannii*, (G) *C. glabrata*, (H) *C. albicans*, (J) *C. krusei*.

Antiproliferative Effect Against Lung Cancer Cell

In last years, efforts to minimize the possible side effects of many drugs used in cancer treatments, to prevent tissue damage, to increase drug efficacy and to design new drugs have been increasing [39]. Many methods are used in cancer treatments. In addition, different methods can be used together in complexity. These methods often have serious side effects [40]. Many supplements are used to cope with these side effects and accelerate the healing process [41]. Consequently, the discovery of new natural resources to be used in cancer treatments is inevitable. In this study, the antiproliferative effect of the plant species we used against the condition known as the lung cancer cell (A549) was examined. The data found are expressed in Figure 1.

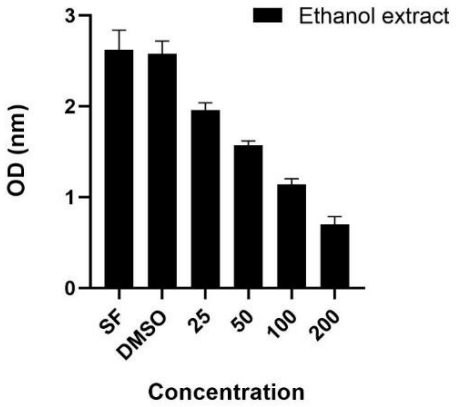

Figure 1. Antiproliferative effect of *V. odorata* extract

In our study, it was detected that the effects of ethanol extract of *V. odorota* against Lung Carcinoma cell (A549) increased with increasing intensiy. Strong cytotoxic effects were observed at 200 µg/ml test intensiy. It has been previously indicated that *V. odorota* has significant effects against prostate (PC-3), breast (MDA-MB-231), and ovarian cancer cell (OVCAR3) [42]. In another study, it was indicated that *V. odorota* was influential at different intensiy against B16F10 murine melanoma cells [43]. In addition to these studies, in our study, it was detected that *V. odorota* has strong effects against Lung Carcinoma cell (A549).

In our research, the antioxidant, antimicrobial and antiproliferative effects of the used parts of *V.*

odorota in the extract formed with the thanks to ethanol, which is preferred as a solvent, were investigated. According to the datas obtained, it was detected that *V. odorota* extract can be used as anticancer agent, antioxidant and antimicrobial.

AUTHOR CONTRIBUTIONS

Concept: M.D., F.S.M., I.U., E.K., M.S.; Design: M.D., F.S.M., I.U., M.P., M.S.; Control: M.D., F.S.M., I.U., M., M.S.; Sources: F.S.M.; Materials: M.D., F.S.M., I.U., K.M., M.S.; Data Collection and/or Processing: M.D., F.S.M., I.U., K.M., M.S.; Analysis and/or Interpretation: M.D., F.S.M., I.U., K.M., E.K., M.P., M.S.; Literature Review: M.D., F.S.M., I.U., K.M., E.K., M.P., M.S.; Manuscript Writing: M.D., F.S.M., I.U., K.M., E.K., M.P., M.S.; Critical Review: M.D., F.S.M., I.U., K.M., E.K., M.P., M.S.; Other: M.D., F.S.M., I.U., K.M., E.K., M.P., M.S.

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., 47(3): 792-799, 2023
Doi: 10.33483/jfpau.1263375

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ASSESSMENT OF PHARMACY STUDENTS' KNOWLEDGE OF HANDLING HIGH ALERT MEDICATIONS

ECZACILIK ÖĞRENCİLERİNİN YÜKSEK RİSKLİ İLAÇLARIN KULLANIMI HAKKINDA BİLGİSİNİN DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *High alert medications (HAM) are medications commonly used in health care settings that are associated with significant harm when used in error. Improvement in the knowledge and practice of pharmacists is crucial to prevent and solve medication errors associated with HAM. This study aimed to assess the knowledge of pharmacy students about HAM.*

Material and Method: *A cross-sectional study was conducted among pharmacy students (3rd, 4th and 5th-year students) through an online survey in Ankara, Türkiye between 1 May 2021 and 31 October 2021. A Turkish translated and validated version of the High Alert Medications Knowledge Questionnaire was used. Only a correct answer was given score of 1 among the knowledge questions. The common resources for HAM use in the practice were assessed.*

Result and Discussion: *Among 124 students, 80.6% were female. The mean age (standard derivation ± SD) of the students was 23.3 ± 1.37 years. The percentages of the 3rd, 4th and 5th-year students were 51.6%, 16.1% and 32.3%, respectively. The mean ± SD score of the students was 6.8 ± 3.28 out of 20. The 5th-year students (8.00 ± 2.90) were more likely to get higher scores compared to 3rd-years (5.89 ± 3.15) (p = 0.003). RxMediaPharma® (local online drug information database) was the most (75.0%) commonly used resource by pharmacy students. The pharmacy students' knowledge about HAM was poor. Educational interventions are necessary early in the clinical pharmacy curriculum to improve pharmacy students' knowledge and preparedness to handle HAM.*

Keywords: *High alert medication, clinical pharmacy education, knowledge, questionnaire*

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Submitted / Gönderilme : 10.03.2023
Accepted / Kabul : 26.05.2023
Published / Yayınlanma : 20.09.2023

ÖZ

Amaç: *Yüksek riskli ilaçlar (HAM), sağlık hizmeti sunulan ortamlarında yaygın olarak kullanılan ve yanlışlıkla kullanıldığında önemli zararlarla ilişkilendirilen ilaçlardır. Eczacıların bilgi ve uygulamalarındaki gelişme, HAM ile ilişkili ilaç hatalarını önlemek ve çözmek için çok önemlidir. Bu çalışma, eczacılık öğrencilerinin HAM hakkındaki bilgilerini değerlendirmeyi amaçlamıştır.*

Gereç ve Yöntem: *Eczacılık öğrencileri (3., 4. ve 5. sınıf öğrencileri) arasında çevrimiçi anket yoluyla kesitsel bir çalışma Ankara, Türkiye'de yapılmıştır. Türkçe validasyonu yapılan Yüksek Riskli İlaçlar Hakkında Bilgi Anketi kullanılmıştır. Bilgi sorularından sadece doğru cevaba 1 puan verildi. Uygulamaları sırasında HAM kullanımı için kullandıkları yaygın kaynaklar değerlendirildi.*

Sonuç ve Tartışma: *Ankete katılan 124 öğrencinin %80.6'sı kızdır. Öğrencilerin yaş ortalaması [standart sapma (SSD)] 23.3 ± 1.37 olarak bulunumuştur. Eczacılık 3., 4. ve 5. sınıf öğrencilerinin oranı sırasıyla %51.6, %16.1 ve %32.3'tür. Öğrencilerin ortalama ± SD puanı 20 üzerinden 6.8 ± 3.28'dir. 5. sınıf öğrencilerinin (8.00 ± 2.90) 3. sınıf öğrencilerine göre (5.89 ± 3.15) daha yüksek puan alma olasılığı daha fazla bulunumuştur (p = 0.003). RxMediaPharma® (yerel çevrimiçi ilaç bilgi veritabanı), eczacılık öğrencileri tarafından en çok (%75,0) kullanılan kaynaktı. Eczacılık öğrencilerinin HAM hakkındaki bilgileri yeterli bulunamamıştır. Eczacılık öğrencilerinin HAM'ı yönetme konusundaki bilgilerini ve hazırlıklarını geliştirmek için klinik eczacılık müfredatının başlarında eğitimsel müdahaleler gereklidir.*

Anahtar Kelimeler: *Anket, bilgi, klinik eczacılık eğitimi, yüksek riskli ilaçlar*

INTRODUCTION

Medication error is defined as any avoidable event that may lead to or cause inappropriate medication use or harm to patients [1]. It can cause injury and avoidable harm in health care systems [2]. It can occur when medications are being prescribed, prepared, dispensed, or administered [1,3]. Not all medication errors cause harm to the patients [3]. Harm due to medication errors are commonly associated with high alert medications (HAM). The Institute for Safe Medication Practices described HAM as a group of medications causing serious harm to the patients when they are used in error [3]. These medications involve groups of narcotics, electrolytes, opiates, anticoagulants, benzodiazepines, cardiovascular, chemotherapeutic, and neuromuscular blocking drugs [4]. The top HAM is insulin, intravenous anticoagulants, opiates and narcotics, injectable potassium chloride or phosphate concentrate, and concentrated sodium chloride [5].

According to the World Health Organization (WHO)'s 2017 "Medications without Harm" Report, the overall WHO global aim is to reduce medication errors by 50% over 5 years [6]. To achieve this aim, improvements in medication prescribing, dispensing, administering, monitoring and use are necessary [6]. However, there could be knowledge gap among the health care professionals to identify HAM. For example, a survey showed that less than 70% of pharmacists, nurses and physicians were able to define HAM [7]. It was improved by the implementation of medication safety-related HAM interventions within the health care setting [7]. One approach is to educate and train health care professionals about the use of HAM. A HAM program was introduced by the leaders, physicians, nurses, pharmacists, quality leaders, and labor unions which worked on medication safety issues so that medication harms would be reduced [8]. The program included a standardized process for handling HAM, education about HAM and monitoring of its sustainability [8]. A statistically significant reduction in medication errors was determined for 23 months after the program initiation [8]. Therefore, developing standardized processes, education and training of health care professionals while handling HAM was useful.

Pharmacists play an important role in medication safety. This role covers leading, planning and monitoring of safe medication use [9]. Specifically, developing risk-specific protocols for HAM, determining and assessing high-risk processes that need attention, protocols, and training as well as tracking and monitoring medication errors can be included in the list of pharmacists' responsibilities [9]. Therefore, education and training about medication errors especially for HAM must be improved in the current pharmacy curriculum to make students ready for pharmacy practice where they will routinely review prescribing, dispensing, and administering practices of HAM. However, little is known about

pharmacy students' knowledge and practice about HAM in Türkiye. The present study aimed to assess the knowledge of pharmacy students about HAM to consider whether it is sufficient enough in the pharmacy curriculum.

MATERIAL AND METHOD

A cross-sectional study was conducted among pharmacy students at a state university faculty of pharmacy located in Ankara, Türkiye. An online survey was created using Google Forms. All 3rd, 4th and 5th-year students were invited to participate. During the study period, a total of 587 pharmacy students were eligible. Students were eligible if they were 18 years or older and either 3rd, 4th or 5th-year. The 1st and 2nd-year students were not involved because the questions were associated with the lectures taught at senior levels. The responses were collected from 1 May 2021 to 31 October 2021. Electronic informed consent was taken from all the students who accepted to participate. It was mandatory to answer all the questions anonymously. The study was approved by the Ankara University Ethics Committee (Date: April 26, 2021; No: 07-87).

The High Alert Medications Knowledge Questionnaire was developed by Hsaio and his team [10]. Turkish reliability and validation were performed by Ozturk and her team [11]. The approval to use the Turkish version of the questionnaire was taken from the author. Turkish version of the survey applied to students. The questionnaire includes a total of 20 items about important knowledge of HAM such as its use, prescribing, dosage, storage, delivery route, administration and regulation. Items are to be ranked as true, false or unknown by survey participants. The scale ranged from 0-20, a correct answer is given 1, and the wrong answer/ not knowing was evaluated as 0. A higher score means a better knowledge of HAM [11].

The practice of pharmacy students was evaluated by asking which resources they used when they need information about HAM. It is a part of measuring the practice but would not comprise practice as a whole. A list of common resources used by pharmacists in Türkiye as well as an open section to type in alternative resources was provided in the questionnaire.

For the descriptive and inferential statistics, IBM SPSS Statistics version 26 and Microsoft Excel for Windows version 2016 were used. Categorical variables were described with numbers and percentages. Parametric continuous variables were described with the mean ± standard deviation (SD). Normality was checked among continuous variables. Based on the variables, ANOVA and Post Hoc tests were used to see meaningful differences between student groups. For post hoc analysis, Benforoni test was chosen because it is applicable for equal or unequal size of students groups.

RESULT AND DISCUSSION

A total of 124 pharmacy students participated in the study (response rate: 21.1%). The majority of the students were female (80.6%). The mean ± SD of the students was 23.3 ± 1.37 years. The percentages of the 3rd, 4th and 5th-year students were 51.6%, 16.1% and 32.3%, respectively. The mean ± SD score of the students was 6.8 ± 3.28 out of 20. (Table 1).

Table 1. Demographics of the pharmacy students

Demographics, n=124	**Value**
Female, n (%)	100 (80.6)
Age, mean ± SD	23.30 ± 1.37
Education year	
Third year	64 (51.6)
Fourth year	20 (16.1)
Fifth year	40 (32.3)

There were statistically significant differences in knowledge scores between 3rd, 4th and 5th-year students (p=0.003). The 5th-year students (mean ± SD: 8.0 ± 2.90) were more likely to get higher scores compared to 3rd- year students (mean ± SD: 5.89 ± 3.15) (p=0.003). The scores of 4th-year students

(mean ± SD: 7.6 ± 3.61) were not statistically different from 3th (p=0.103) and 5th-year students (p=0.861).

At least half of the students answered correctly to the Item 4 of the questionnaire, which was about port-A route (50.0%), Item 5, which was about insulin syringe (50.8), Item 7, which was about Ca gluconate and $CaCl_2$ (50.8%), Item 11, which was about fentanyl skin patch (58.9%), Item 12, which was about distinctive labelling of medication (91.9%), and Item 13, which was about heparin and insulin storage (56.5%) (Table 2).

Table 2. Answers to the high alert medications questions

Questions	True, n (%)	False, n (%)	Do not know, n (%)	Correct answer, n (%)
1. 'cc' or 'ml' is the dosage expression for insulin injection.	72 (58.1)	38 (30.6)	14 (11.3)	38 (30.6)
2. When an emergency such as ventricular fibrillation happens, push fast 7.5% KCl 10 ml into IV.	23 (18.5)	13 (10.5)	88 (71.0)	13 (10.5)
3. Fast IV infusion of 3% NaCl 500 ml for a patient who has a low sodium level.	38 (30.6)	25 (20.2)	61 (49.2)	25 (20.2)
4. The Port-A route can be used for blood withdrawal and drug injection generally.	41 (33.1)	62 (50.0)	21 (16.9)	62 (50.0)
5. The insulin syringe can be replaced by 1 ml syringe.	21 (16.9)	63 (50.8)	40 (32.3)	63 (50.8)
6. Fast IV push 1:1000 epinephrine 1 ampule for a patient who has a mild allergic reaction.	35 (28.2)	24 (19.4)	65 (52.4)	24 (19.4)
7. 10% Ca gluconate and 10% $CaCl_2$ are the same drug and interchangeable.	11 (8.9)	63 (50.8)	50 (40.3)	63 (50.8)
8. 7.5% KCl is better added to Ringer's solution for rapid infusion.	23 (18.5)	9 (7.3)	92 (74.2)	9 (7.3)
9. When an emergency happens, fast IV push 10% $CaCl_2$ 10 ml in 1-2 minutes.	29 (23.4)	7 (5.6)	88 (71.0)	7 (5.6)
10. For chemotherapy dose calculation, adults are based on body weight, while children are based on body surface area.	78 (62.9)	13 (10.5)	33 (26.6)	13 (10.5)
11. Taken fentanyl skin patch as regulated narcotic.	73 (58.9)	7 (5.6)	44 (35.5)	73 (58.9)
12. Use distinctive labelling on look-alike drugs.	114 (91.9)	5 (4.0)	5 (4.0)	114 (91.9)
13. For convenience, heparin and insulin should be stored together in the refrigerator.	23 (18.5)	70 (56.5)	31 (25.0)	70 (56.5)
14. Use 'Amp' or 'Vial' for dose expression instead of 'mg' or 'gm'.	58 (46.8)	44 (35.5)	22 (17.7)	44 (35.5)
15. If award stores atracurium for tracheal intubation, the drug should be stored with other drugs and easily accessed by nurses.	22 (17.7)	33 (26.6)	69 (55.6)	33 (26.6)
16. 7.5% KCl is frequently used, so it should be easily and freely accessed by nurses	41 (33.1)	21 (16.9)	62 (50.0)	21 (16.9)
17. If a patient can tolerate it, potassium can be administered orally instead of IV route.	39 (31.5)	23 (18.5)	62 (50.0)	39 (31.5)
18. Each drug better has multiple concentrations for a nurse to choose.	32 (25.8)	59 (47.6)	33 (26.6)	59 (47.6)
19. For paediatric dose, use teaspoon for dose expression.	63 (50.8)	42 (33.9)	19 (15.3)	42 (33.9)
20. Use 'U' instead of 'unit' for dose expression.	69 (55.6)	36 (29.0)	19 (15.3)	36 (29.0)

KCl: potassium chloride; Ca: calcium; NaCl: sodium chloride; $CaCl_2$: calcium chloride; IV: intravenous

Less than a quarter of the students were able to answer correctly to the questions mainly about the administration of the HAM. However, more than a quarter of them were able to answer the questions associated with the storage and prescription of HAM (Table 2). For example, a few students answer correctly to the Item 2 of the questionnaire, which was about emergency use of KCl (10.5%), Item 3, which was about administering intravenous (IV) infusion to the patient with low sodium level (20.2%), Item 6, which was about administration of epinephrine in allergic reactions (19.4%), Item 8, which was about mixing KCl and Ringer's solution (7.3%), Item 9, which was about administering fast $CaCl_2$ in an emergency (5.6%), Item 10, which was about calculation of the dose of chemotherapy (10.5%), and Item 16, which was about accessibility of KCl by nurses (16.9%) (Table 2).

The top 5 resources that students used for HAM were RxMediaPharma® (local online drug information database) (75.0%), pharmacists (59.7%), Pubmed®/Medline® (49.2%), Hacettepe University Drug and Poison Information Centre (HIZBIM) (12.9%), Medscape® (11.3%) and Drugs.com (11.3%), respectively (Figure 1).

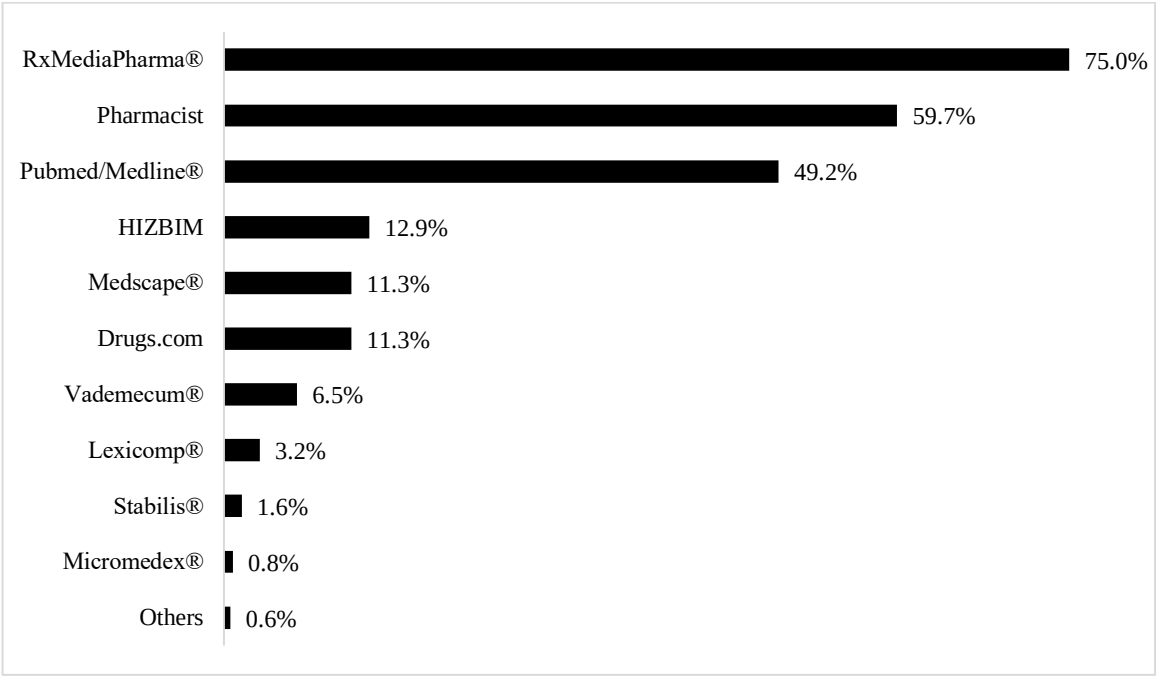

Figure 1. Students' answers for the information resources used for high alert medications
HIZBIM: Hacettepe University Drug and Poison Information Center; Others: tebrp® (Turkish Pharmacists' Association Pharmaceutical Care Assistant, ndrugs.com, FDA, CDC and NCBI

According to the results of this study, pharmacy students' knowledge about HAM is poor. Since pharmacists are the health care professionals who provide counselling and review about mediation use, it is crucial to teach them about HAM to avoid medication-related harms to the patients. However, to the best of our knowledge there is no study that evaluates the knowledge of pharmacy students about HAM.

A high number of medication errors reported in studies needs pharmacists' interventions [12]. Multiple interventions to reduce medication errors showed that pharmacists play a key role to eliminate the errors [12]. One way of the intervention is to make pharmacists educate other health care professionals. The other way of intervention, which is a prior step, is to educate pharmacists to improve their knowledge so that medication errors can be reduced through their involvement. This is because the lack of pharmaceutical knowledge has an important effect on the occurrence of medication errors and their prevention [13]. Education seemed to be one of the solutions to reduce medication errors [14].

In this study, the knowledge of pharmacy students was investigated as a starting point to see if future educational interventions/modules/seminars were needed to be placed in the pharmacy

curriculum. This was in line with the Accreditation Council for Pharmacy Education (ACPE) guideline, which requires that pharmacy students can apply "*quality improvement strategies, medication safety and error reduction programs and research processes to minimize drug misadventures and optimize patient outcomes*" when they graduate [15]. In response to this requirement, investigators from the United States developed an Educating Pharmacy Students and Pharmacists to Improve Quality Program. This program aimed to improve the knowledge and skills of pharmacy students to reduce medication errors and to apply quality improvement techniques for patient safety [16]. This program was inserted into the pharmacy curriculum of the 19 colleges and schools of pharmacy in the United States [16]. Another approach is interprofessional education of medication safety on the medication error [14]. The education program targeted the physicians, nurses and clinical pharmacists at intensive care units [14]. Interprofessional education enables two or more professionals to learn together and each other as well as improves teamwork and quality of care [14]. After the program implementation medication errors were reduced [14]. Therefore, interprofessional education can be effective for medication safety.

The questions on prescribing, dosage, storage, delivery route, and regulation about HAM are more likely to be answered correctly by the pharmacy students compared to those on administration. One possible reason is that pharmacists in Türkiye do not administer medications. It is mainly the nurses' responsibility. Pharmacy students might have felt that they did not need to know the administration of medications, or they have never learned before. However, they must still know the information about medication administration to provide appropriate answers in case they are asked by nurses or other health care professionals in clinical settings.

One of the roles of pharmacist regarding HAM is monitoring safety of high alert and look alike and sound alike medicines, developing risk-specific protocols, and conducting double checks for medications [17]. A study was conducted to monitor of HAM by clinical pharmacists in a hospital setting [18]. Clinical pharmacist developed a rapid HAM feedback system to evaluate HAM use so that patient harm would be eliminated [18]. Pharmacists involvement in HAM use can eliminate medication associated fatal events.

Gaps is education and knowledge about HAM can result to medication harms and errors [19]. Because HAM has the highest risk to cause patient harm [20]. Pharmacists are responsible for safe medication use. It is their one of the crucial roles to eliminate medication harms. This role is defined by American Society of Health System Pharmacists (ASHP) as a medication safety expert and leader [21]. Pharmacists must have enough knowledge of good practices relating to the process of prescribing, preparation, dispensing, administration, and monitoring of medications to ensure patient safety in each process [20]. The training of pharmacists must include analytic abilities and organized approach to develop and monitor patient safety [20].

In our faculty, pharmacology, analytical chemistry lectures are included in the curriculum starting from 2nd year while other lectures such as pharmaceutical technology, pharmaceutical chemistry, pharmacotherapy and toxicology lectures are included starting from 3rd year. Thus, students are expected to have certain level of knowledge in chemical and formulation perspective about HAM starting from 3rd grade. Since the study was conducted in May, it means third year students were about to complete their lectures in the year. However, such topics covered by the HAM questionnaire are not covered in clinical pharmacy lectures. Special attention must be given in clinical pharmacy lectures so that students will understand more about clinical perspective of HAM.

Most of the students who participated in the questionnaire were female. In Türkiye, the number of admissions of female pharmacy students is higher than those of male students. Thus, the participant can represent the general pharmacy student population. Fifth year students were more likely to get higher scores. It was also expected that higher year students had more internships or lectures that they could learn more about HAM. However, there were several limitations of this study. Due to the low response rate and conducting in a single center, it limited the generalizability of the findings. The non-responders and those who stopped completing the survey halfway were not recorded. Further studies are needed to investigate more on the knowledge of HAM among pharmacy students and strategies on how to improve the use of HAM to eliminate medication errors. This study can highlight the issue of poor knowledge of HAM and the necessity of place for HAM in the clinical pharmacy curriculum as well as guide other institutions to take any actions for strengthening HAM use.

In conclusion, the findings of this study highlighted the poor knowledge about HAM among pharmacy students. It is important to have high alert medication education in the clinical pharmacy curriculum. Especially in faculties where the knowledge of HAM is poor, it is crucial to educate students to reduce future medication errors and hence to promote patient safety in the future.

ACKNOWLEDGEMENTS

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

AUTHOR CONTRIBUTIONS

Concept: A.S., B.O.; Design: A.S., B.O; Control: A.S., B.O; Sources: A.S., B.O; Materials: A.S.; Data Collection and/or Processing: A.S.; Analysis and/or Interpretation: A.S.; Literature Review: A.S., B.O; Manuscript Writing: A.S., B.O; Critical Review: A.S., B.O.; 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 Ethics Committee for Human Research of Ankara University is approved the study (Date: April 26, 2021; No: 07-87).

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 800-807, 2023
Doi: 10.33483/jfpau.1294563

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SIMULTANEOUS SPECTROPHOTOMETRIC DETERMINATION OF FLUOXETINE AND OLANZAPINE GREENNES ASSESSMENT

FLUOKSETİN VE OLANZAPİN'İN EŞ ZAMANLI SPEKTROFOTOMETRİK TAYİNİ VE YEŞİL KİMYA UYGULAMALARI

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ABSTRACT

Objective: *In our study, the simultaneous determination of fluoxetine (FLX) and olanzapine (OLZ) was performed by absorbance subtraction and absorbance correction spectrophotometric methods.*
Material and Method: *The active substances were determined by choosing the isosbestic point of 232 nm in the absorbance subtraction method and 255 nm and 245 nm wavelengths in the absorbance correction method. The accuracy of the methods was determined by applying the percentage recovery studies to the laboratory mixtures. The percent recovery values were found in the range of 98.1-100.2 for OLZ and 96.8-105.3 for FLX. The concentration range studied was 3.12-15.62 and 3.45-17.28 µg/ml for OLZ and FLX, respectively.*
Result and Discussion: *In the study, two active substances used in antidepressant treatment were determined simultaneously. Today, these active substances used in the treatment have started to be used in combination in order to achieve a better effect of the treatment. Therefore, simultaneous analysis of two active substances becomes important. Two different spectrophotometric methods were used for analysis. The methods have been successfully applied and validated for the simultaneous determination of antidepressant active substances. Since the applied methods do not require pre-separation and can be applied directly, the amount of waste generated is reduced. Environmentally sensitive methods have been applied.*
Keywords: *Determination, fluoxetine, olanzapine, spectrophotometry*

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Submitted / Gönderilme : 09.05.2023
Accepted / Kabul : 01.06.2023
Published / Yayınlanma : 20.09.2023

ÖZ

Amaç: *Çalışmada fluoksetin (FLX) ve olanzapinin (OLZ) etken maddelerinin aynı anda tayini absorbans çıkarma ve absorbans düzeltme spektrofotometrik yöntemleri uygulanarak yapılmıştır.*
Gereç ve Yöntem: *Etken maddeler absorbans çıkarma yönteminde isosbestik nokta olan 232 nm ve absorbans düzeltme yönteminde 255 nm, 245 nm dalga boyları seçilerek tayin edilmiştir. Yüzde geri kazanım çalışmaları laboratuvar karışımlarına uygulanarak yöntemlerin doğruluğu yapılmıştır. Yüzde geri kazanım değerleri OLZ için 98.1-100.2, FLX için 96.8-105.3 aralığında bulunmuştur. Çalışılan konsantrasyon aralığı, OLZ ve FLX için sırasıyla 3.12-15.62 ve 3.45-17.28 µg/ml dir.*
Sonuç ve Tartışma: *Çalışmada antidepresan tedavisinde kullanılan iki etken madde aynı anda tayin edilmiştir. Günümüzde tedavide kulanılan bu etken maddeler tedavinin etkisinin daha iyi sağlanabilmesi için kombinasyon şeklinde kullanıma sunulmaya başlanmıştır. Bu nedenle iki etken maddenin aynı anda analizi önem kazanmaktadır. Analiz için iki farklı spektrofotometrik yöntem uygulanmıştır. Yöntemler antidepresan etken maddelerin aynı anda tayini için başarıyla uygulanmış ve valide edilmiştir. Uygulanan yöntemler ön ayırma gerektirmediği ve direkt olarak uygulanabilmesi sayesinde oluşan atık miktarı azalmaktadır. Çevreye hassas olarak yöntemler uygulanmıştır.*

Anahtar Kelimeler: *Fluoksetin, kantitatif belirleme, olanzapin, spektrofotometri*

INTRODUCTION

Low mood is an indicator of depression, an illness that involves abnormalities in numerous brain networks. Research has shown that one of the most significant disorders affecting human health and longevity is depression. Antidepressants are frequently used to treat various forms of depression, bipolar affective disorder, anxiety [1,2].

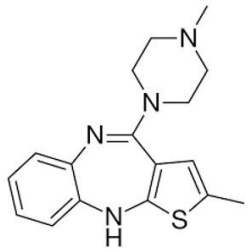

Figure 1. Chemical structure of OLZ

The combined use of the active ingredients of olanzapine (OLZ), an atypical antipsychotic, and fluoxetine (FLX), a selective serotonin reuptake inhibitor, is more effective in alleviating bipolar I depression than the use of OLZ alone [3]. OLZ chemical name is 2-methyl-4-(4-methylpiperazin-1-yl)-10*H*-thieno[2,3-b][1,5]benzodiazepine (Figure 1). FLX, *N*-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine (Figure 2) is used as an antidepressant.

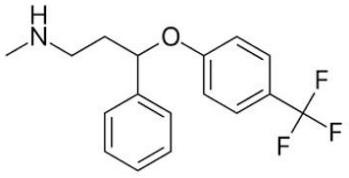

Figure 2. Chemical structure of FLX

In green chemistry applications, it is important to reduce the amount of waste generated, to use less harmful solvents, to reduce the amount of solvent used, to make direct determination without pre-preparation steps and to reduce energy use [4]. Methods are being developed to reduce the damage to

the environment due to the analysis. The method we have developed has also been applied in an environmentally friendly manner. To apply the experimental used device was consumed lower energy. Complex devices such as HPLC, LC-MS consume more energy. To apply the experimental used device was consumed lower energy. Also, no pre-preparation, extraction or derivatization process was applied in the proposed method. Experiments were carried out by dissolving the active ingredients directly in a single solvent. The effects of waste on the environment have been minimized due to their direct and indirect effects on our health.

The use of active substances in combination is increasing. therefore, it is important to be able to analyze drugs simultaneously. There are several studies in the literature conducted with different methods. In the literature, two active substances were determined simultaneously by the HPLC method [5,6]. Liquid-liquid extraction was analyzed by GC-MS, HPLC-MS/MS and LC-MS/MS methods [7-10]. Analysis was performed in LC-MS/MS plasma with a protein precipitation process [11], and in another study, analysis was performed from plasma by applying a microextraction process [12] in human plasma with solid phase extraction and LC-MS/MS methods [13,14]. These analyzes are made using expensive devices, the energy consumption is high and the sample preparation processes are laborious.

There are also several derivative spectrophotometric methods when the literature is searched. First derivative or ratio derivative methods are used in the methods [15,16]. The active ingredient of OLZ was determined by the spectrophotometric method during sample preparation [17,18]. FLX was quantified by spectrophotometric or spectrofluorimetric method [19,20].

The use of combined drugs in the treatment of depression is increasing. For this reason, it is important to analyze drugs simultaneously. Expensive devices and many chemicals were used in previous methods. Quantitative determination was made simply and easily with the new spectrophotometric methods applied.

The applied analysis method is simple, fast, can be applied in routine analysis laboratories without requiring preliminary preparation and expensive programs. In this study, the simultaneous determination of the active ingredients of OLZ and FLX was analyzed using two spectrophotometric methods. The first method is absorbance subtraction, and the second method is the absorbance correction method. The methods were applied directly for the determination of active substances. Validation studies have been carried out. The method was applied without the need for complex devices and was applied directly. No additional chemical agents were added. Because of these features, the developed method is sensitive to the environment. Our aim in this study is to analyze two active substances, which are important to analyze simultaneously, simply, using simple devices, in accordance with green chemistry.

MATERIAL AND METHOD

Instrument and Software

The spectra of the active substances were taken in the spectrophotometer device, in the UV region, between 200-400 nm. A quartz cuvette was used for measurements. The brand and model of the dual-beam spectrophotometer device used is Shimadzu UV 1800. UV probe 2.52 was used as software for the spectra. Excel program was used to create calibration curves and apply data.

Used Chemicals

Used all materials were of analytical grade. Reference standards were kindly supplied by Abdi Ibrahim Pharmaceutical Industry, Turkey. Liquid chromatography grade methanol was purchased from Merck (Darmstadt, Germany).

Preparation of Standard Solutions

By transferring the 10.0 mg of active substances into 50.0 ml volumetric flasks containing methanol, standard stock solutions of each of the two analytes, equal to (0.2 mg/ml), of OLP and FLX were prepared separately. Active substances solutions containing 3.12-15.62 µg/ml of OLP, 3.45-17.29 µg/ml of FLX were solved separately in methanol. Laboratory mixtures were prepared three replicates in certain proportions and measurements were made and percent recovery values were calculated.

RESULT AND DISCUSSION

In the study, two active substances were analyzed by absorbance subtraction and absorbance correction methods. The spectra of OLZ and FLX between 200-300 nm are given in Figure 3. The methods are applied direct prepared laboratory mixture.

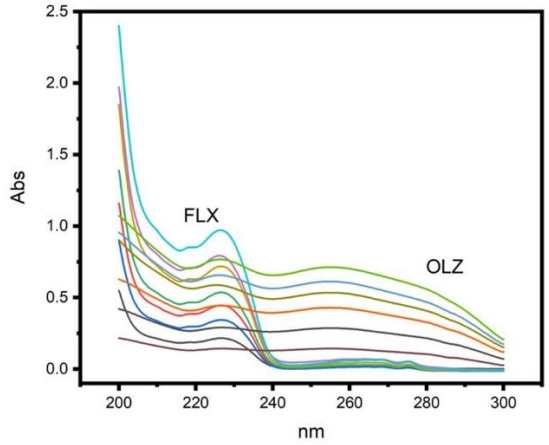

Figure 3. Zero order spectrum of active ingredient

Absorbance Subtraction Method

The first of the applied methods is the absorbance subtraction method. This method is based on the isoabsorption point spectrophotometric method. At the point of isoabsorption, the drug mixture acts as a single ingredient as they exhibit equal absorptive value. Thus, by measuring the absorbance value at the selected isoabsorption point (λiso), the total concentration of both drugs (λiso) can be calculated using the absorbance [21].

The isosbestic point of 232 nm was chosen to apply the method. The isosbestic points of the active ingredients are given in Figure 4. 280 nm was chosen as the second wavelength. The absorbance factor value for OLZ was calculated by dividing the absorbance at the isosbestic point by the absorbance at 280 nm and its value was found to be 1.29.

To determine OLZ from the mixture, the absorbance of the mixture at 280 nm was multiplied by the absorbance factor value. The concentration was calculated with the help of the calibration curve created according to the concentration versus the absorbance at the isosbestic point. The parameters of the method are given in Table 1.

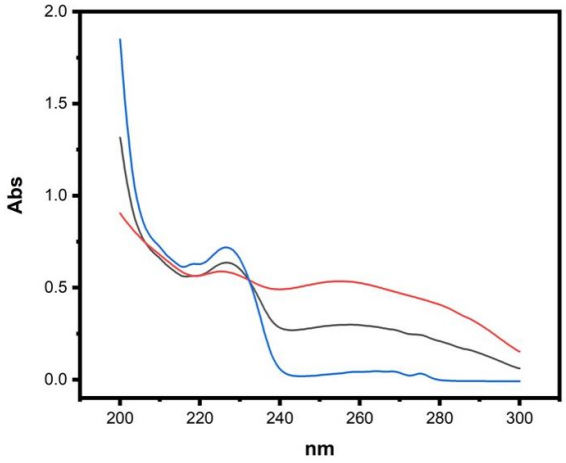

Figure 4. Isosbestic point of active ingredient

Table 1. The parameter of the absorbance subtraction method

Parameters	OLZ	FLX
Concentration range (µg/ml)	3.12-15.62	3.45-17.28
Wavelength, nm	232	232
Intercept value	-0.0053	-0.0053
Slope value	0.0461	0.0461
Correlation coefficient, R^2	0.9965	0.9965

Absorbance Correction Method

Two wavelengths, 245 and 255 nm, were chosen to apply the absorbance correction method. To calculate the absorbance correction factor value, the absorbance value of OLZ at 255 nm was mathematically divided by the absorbance value at 245 nm. The wavelength of the mixture at 245 nm was multiplied by the absorbance correction factor value. In order to calculate the FLX concentration, this calculated value from the absorbance value of the mixture at 255 nm was subtracted. The concentration of OLZ in the mixture was calculated. Absorbance at 255 nm versus concentration was plotted to generate the calibration curve for the method. The calibration curve parameters are given in Table 2.

Absorbance correction factor $F_{OLZ} = Abs_{255\,nm} / Abs_{245\,nm}$

Table 2. The calibration curve parameter of the absorbance correction method

Parameters	OLZ	FLX
Concentration range (µg/ml)	3.12-15.62	3.45-17.28
Wavelength, nm	255	255
Intercept value	0.0027	-0.0154
Slope value	0.0464	0.0036
Correlation coefficient, R^2	0.9965	0.9965

Recovery Results for Methods

Recovery studies were calculated for the laboratory mixture. These mixtures were prepared in different concentration series. The recovery results were shown in Table 3.

Table 3. The recovery results of absorbance correction and subtraction method

Sample	Added concentration		Recovery %	
	OLZ	FLX	OLZ	FLX
Absorbance correction method				
Sample I	6.25	6.91	100.2	105.3
Sample II	9.37	6.91	100.3	96.7
Sample III	6.25	10.37	103.2	97.4
Absorbance subtraction method				
Sample I	6.25	6.91	97.2	96.9
Sample II	9.37	6.91	98.4	96.8
Sample III	6.25	10.37	98.1	99.2

Precision Results for Methods

Precision studies were carried out to demonstrate the reproducibility of the methods. The calculated results are given in Table 4.

Table 4. The precision results of absorbance correction and subtraction method

Sample	Added concentration		Found concentration %	
	OLZ	FLX	OLZ	FLX
Absorbance correction method				
1. day	10.93	10.37	102.2	104.3
2. day	10.93	10.37	100.9	98.7
3. day	10.93	10.37	103.4	99.4
Absorbance subtraction method				
1. day	10.93	10.37	97.9	97.9
2. day	10.93	10.37	99.4	99.8
3. day	10.93	10.37	98.8	101.2

In the method applied, no separation process was applied and no additional chemical was used, and it was determined directly (Table 5).

Table 5. Comparison table with other studies

Analyte	Reagent	Linear range (µg/ml)	Correlation coefficent	Recovery %	Reference
FLX and OLZ	-	3.45-17.28 and 3.12-15.62	0.9965	97.4-100.2	Proposed method (Absorbance correction)
FLX and OLZ	-	3.45-17.28 and 3.12-15.62	0.9965	99.2-98.1	Proposed method (Absorbance subtraction)
FLX and OLZ	-	100.00-600.00 and 5.00-17.50	0.9998 and 0.9999	100.78 and 100.28	15
FLX and OLZ	HCl	8.00-80.00 and 2.00-20.00	0.9991 and 0.9997	98.9 and 95.2	16
OLZ	HCl and phosphate buffer saline	3.00-18.00	0.9999 and 0.9998	99.8-101.5	17
FLX	HCl and 3,4-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt	9.00-54.00	0.9995	100.5	19

Assessment of Greenness for Methods

When the method is evaluated for the environment, it is superior to other applied methods in that it does not use a complex device, does not have complex pre-preparation processes and does not have an extraction step.

A green analytical procedure index (GAPI) evaluation was made for the method. According to the GAPI, the methods are examined according to 15 different parameters such as sample preparation, solvents used, and energy consumption [22]. The applied method was examined according to the GAPI and the GAPI results for the method we applied are shown in Figure 5.

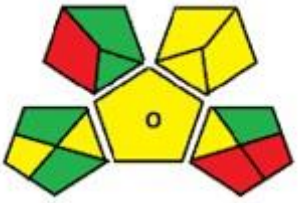

Figure 5. GAPI result of applied methods

In conclusion, two spectrophotometric method was applied active ingredient of FLX and OLZ. The method applied as simple, practically and without pre-preparation process the comparison of the other method. The active ingredients were solved simple and were measured directly. Thanks to the applied methods two substances were determined simultaneously without expensive devices. Therefore, the energy consumption is reduced. The chemical reagents were not used because of pretreatment procedure. The applied methods were greenness for environmental for this reason. These methods can be applied routine analysis laboratory.

AUTHOR CONTRIBUTIONS

Concept: G.T., N.E.; Design: G.T., N.E.; Control: G.T., E.O., N.E.; Sources: G.T., E.O., N.E.; Materials: G.T., E.O., N.E.; Data Collection and/or Processing: G.T., E.O., N.E.; Analysis and/or Interpretation: G.T., E.O., N.E.; Literature Review: G.T., E.O., N.E.; Manuscript Writing: G.T., N.E.; Critical Review: G.T., N.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 declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 808-815, 2023
Doi: 10.33483/jfpau.1298235

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EFFECTS OF COLOR AND BRIGHTNESS OF BREWED BLACK TEA ON ITS FLUORIDE CONCENTRATION IN TÜRKİYE

TÜRKİYE'DE DEMLENMİŞ SİYAH ÇAYIN RENK VE PARLAKLIĞININ FLORÜR KONSANTRASYONUNA ETKİSİ

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ABSTRACT

Objective: *The main purpose of this study is to determine the effect of the tea brewing time on the total color, brightness, and fluoride ion concentration of tea infusions in Türkiye.*
Material and Method: *250 ml of boiling distilled water was added to 6 g of granulated tea sample and brewed against increasing time. The total color and brightness of the tea infusions were determined by a spectrophotometer at 460 nm, while a potentiometric method was used to determine the fluoride ion concentration.*
Result and Discussion: *We conclude that the concentration of fluoride ions and other bioactive compounds in the tea infusion is strongly dependent on the tea preparation and brewing preferences of consumers. According to our results, fluoride concentrations in tea infusions reach the plateau after 20 minutes in a Turkish way of tea brewing. Therefore, tea-brewing time is an important variable in calculating the daily fluoride intake for extreme tea-consuming populations like Türkiye.*
Keywords: *Black tea, brewed tea, extractable fluoride, percent brightness, total color*

ÖZ

Amaç: *Bu çalışmanın temel amacı, çay demleme süresinin Türkiye'deki çay infüzyonlarının toplam renk, parlaklık ve florür iyon konsantrasyonu üzerindeki etkisini belirlemektir.*
Gereç ve Yöntem: *6 g granül çay örneğine 250 ml kaynayan distile su ilave edilerek artan zamana karşı demlendi. Çay infüzyonlarının toplam rengi ve parlaklığı 460 nm'de bir spektrofotometre ile belirlenirken, florür iyonu konsantrasyonunu belirlemek için potansiyometrik bir yöntem kullanıldı.*
Sonuç ve Tartışma: *Çay infüzyonundaki florür iyonlarının ve diğer biyoaktif bileşiklerin konsantrasyonunun, tüketicilerin çay hazırlama ve demleme tercihlerine güçlü bir şekilde bağlı olduğu sonucuna vardık. Elde ettiğimiz sonuçlara göre, çay infüzyonlarındaki florür*

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Submitted / Gönderilme : 17.05.2023
Accepted / Kabul : 04.07.2023
Published / Yayınlanma : 20.09.2023

konsantrasyonları, Türk usulü çay demlemede 20 dakika sonra platoya ulaşmaktadır. Bu nedenle çay demleme süresi, Türkiye gibi aşırı çay tüketen toplumlar için günlük flor alımının hesaplanmasında önemli bir değişkendir.
Anahtar Kelimeler: *Demleme çay, ekstrakte edilebilir florür, siyah çay, toplam renk, yüzde parlaklık*

INTRODUCTION

The tea plant is an evergreen shrub or small tree of the *Camelia* genus, native to China, with dark green shiny leaves and white flowers. Processing of tea leaves allows the production of various types of tea: white, yellow, oolong, green, black, and pu-erh, depending on the extent of the oxidation/fermentation process [1]. Black tea is formed by oxidation process that is catalyzed by the enzyme polyphenol oxidase [2]. Tea is one of the most widely consumed beverages in the world and is second only to water [3]. Also, it is an antioxidant agent in daily consumption. However, considering the high consumption rate, the positive or negative health effects of tea can have serious consequences for public [4]. The positive effects of tea are quite remarkable. Pure catechins and phenolic acids contained in tea are a stronger antioxidant than vitamins C, E and β-carotene [5]. According to the studies published so far, it has been reported that tea has positive impacts on health such as protective effects on the cardiovascular system [6], anti-inflammatory [7], anticancer [8] and anti-diabetic [9]. Despite these beneficial effects of tea consumption, the fluoride content of tea might be a health concern for the highly tea-consumed populations due to the possibility of exceeding the safe daily fluoride intake level.

It has long been known that tea contains a certain amount of fluoride [10,11]. The amount of fluoride in tea may depend according to the geography and climatic conditions in which it is grown. The fluoride accumulated in the leaves of the tea plant is higher in the old leaves than in the young shoots [12]. Low-land tea contained more fluoride than high-land tea. Tea plucked in May has the highest fluoride content and the processing method does not affect the fluoride content of tea [13].

The World Health Organization recommends a maximum of 1.5 mg/l fluoride in drinking water. It has additionally been reported that if the amount of fluoride from other sources is more than 6 mg/day, this upper limit value of fluoride in drinking water might be set lower at the local scale [14]. The Food and Nutrition Board (FNB) has established Tolerable Upper Intake Levels (ULs) for fluoride from all sources for healthy individuals based on levels associated with dental and skeletal fluorosis. Accordingly, 10 mg is the daily UL of fluoride for both males and females aged over 9 [15].

There is no universally accepted method for the preparation and presentation of tea infusion. In some countries, tea is brewed by soaking a disposable tea bag (1.8-2.4 g) in 200-250 ml of hot water for 3-5 minutes. In Japan green tea, which is the most preferred, is presented with a ceremony. It is common in England to add a certain proportion of milk to black tea. Peppermint-flavored tea is preferred in North African countries, especially in Morocco. Türkiye is the country that consumes the most black tea in the world. Tea in Türkiye is served in traditional tea glasses called thin-waist tea glasses after brewing it in a traditional teapot and infuser. The most common way of brewing tea in Türkiye is displayed in Figure 1 [16,17].

Tea is a part of daily life and can be considered a cultural symbol and socialization tool in Türkiye. First of all, the color of the tea is essential for extreme consumers. The most preferred color is bright and dark red, which is named "tavşankanı" in Türkiye [18]. This color preference strongly influences the fluoride concentration of the tea, which contributes to the daily fluoride intake.

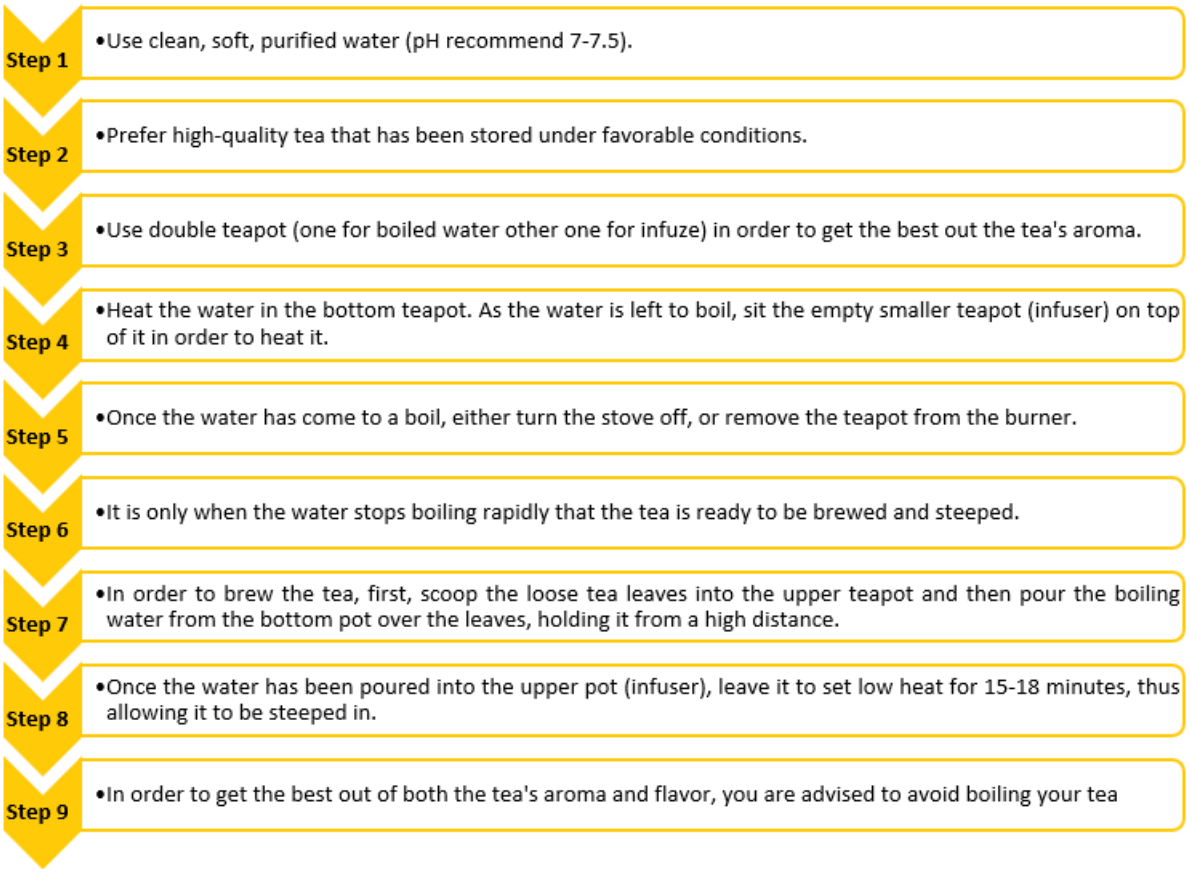

Figure 1. Outline of brewing black tea in Türkiye [15,16]

MATERIAL AND METHOD

Instruments and Chemicals

All chemicals used were analytical reagent grade. Fluoride ion selective electrode (Orion Fluoride Electrodes 9609BNWP) was immersed in the solutions and readings on the ion analyzer (Orion Star A324 pH/ISE Portable Multiparameter Meter) were recorded. Total ionic strength adjustment buffer (TISAB II, Orion 940909) regulates the ionic strength of samples and standard solutions and adjusts the pH, and also avoids interferences. The optical densities at 460 nm were measured with HITACHI U-1800 spectrophotometer.

Preparation of Tea Liquors

Tea liquors were prepared based on Roberts and Simit [18]. Accordingly, 250 ml of boiling water was added to 6 g of dry tea and brewed. After the specified brewing time, it was filtered through a cotton sieve and cooled to room temperature. It was carried out triple replicas every step. Tea liquor preparation steps are summarized in Figure 1.

The Determination of Total Color and Brightness in Tea Liquor

The determination of Total Color and Brightness in Tea Liquor was made according to Roberts and Simit [19]. Steps of method are summarized in Figure 2.

Roberts and Simit [19] have evaluated the sum of the optical densities of the A and B solutions as a measure of the color of the infusion. Thus the total color is calculated from the results of the measurements at 460 nm in the spectrophotometer with Equation 1.

$$\textbf{Total Color} = 6.25x(E_A + 2E_B) \qquad \text{(Equation 1)}$$

Roberts and Simit (1963) stated that the hue of the color, rather than the depth of color, is important in evaluating tea liquors and tea tasters refer to it as 'brightness'. The percent brightness values were calculated with Equation 2 from the results of the measurements at 460 nm in the spectrophotometer.

$$\textbf{Brightness\%} = 100xE_C/(E_A + 2E_B) \qquad \text{(Equation 2)}$$

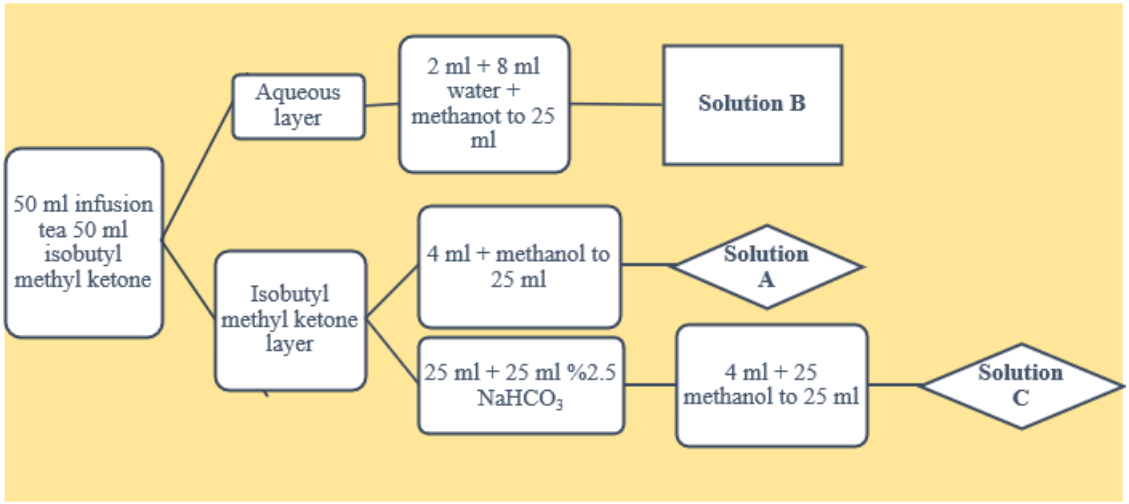

Figure 2. Sample preparation steps for Total Color and Brightness in Tea Liquor

Determination of Fluoride Ion in Tea Liquor:

Method 9214 [20] published by the United States Environmental Protection Agency (EPA) was used. The method is based on the potentiometric determination of total fluoride using a fluoride ion-selective electrode (ISE). The samples and fluoride standard solutions were diluted 1:1 with the TISAB II.

Calibration Solution:

For 1000 mg/l Fluoride (F^-) stock solution: 0.2210 g sodium fluoride (NaF, dried at 110°C for two hours and allowed to come to room temperature in a desiccator) is dissolved in 100 ml polyethylene measuring flask with distilled water.

For 100 mg/l Fluoride (F^-) standard solution: Dilute 10.0 ml of 1,000 mg/l fluoride calibration stock solution to 100 ml with water in a polyethylene volumetric flask.

Calibration curve was determined against the measured mV values, respectively, using the fluoride ion-selective electrode (ISE). Fluoride ion content of infusion tea samples was determined from the calibration curve (Table 1).

Table 1. Fluoride Calibration Standards

ml of 100 mg/l F^- Solution	Concentration when diluted to 50.0 ml (mg/l F^-)
0.050	0.100
0.150	0.300
0.500	1.00
1.500	3.0
5.000	10.0

RESULT AND DISCUSSION

Linearity of the Standard Curve

The linearity was obtained in the range of 0.1-10 mg/l fluoride concentration as Figure 3. The measured potential from fluoride ion selective electrode corresponding to the level of fluoride ion in solution is described by the Nernst equation.

$$\mathbf{E = S \log C + {}^{\circ}E} \qquad \text{(Equation 3)}$$

Where C is the mg/l fluoride yielding a millivolt potential of E, S is the slope, and ˚E is reference potential. A slope range 58.6 mV. The calibration curve was generated for each measurement with R^2=0.999.

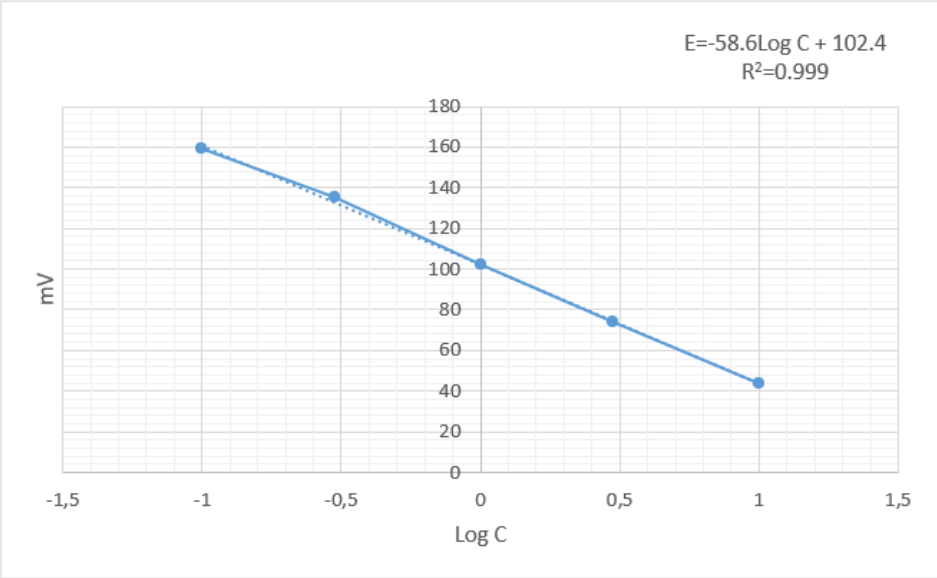

Figure 3. Standard calibration curve of fluoride

Time-dependent Change in Total Color and Brightness % in Tea Liquor

It was concluded that the Total Color parameter in the infused tea would not change significantly after the 10th minute of brewing time (Figure 4).

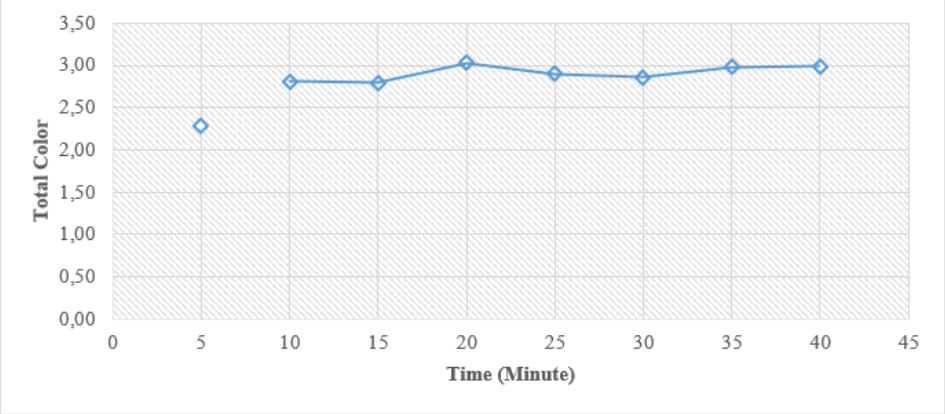

Figure 4. Effect of tea infusion on Total Color depending on brewing time

However, the Brightness % values of the tea infusions tended to decrease continuously. It is clear that this situation would affect consumption preference negatively. Therefore, it is recommended to consume the tea brewed in accordance with Turkish traditions within 30-35 minutes at most.

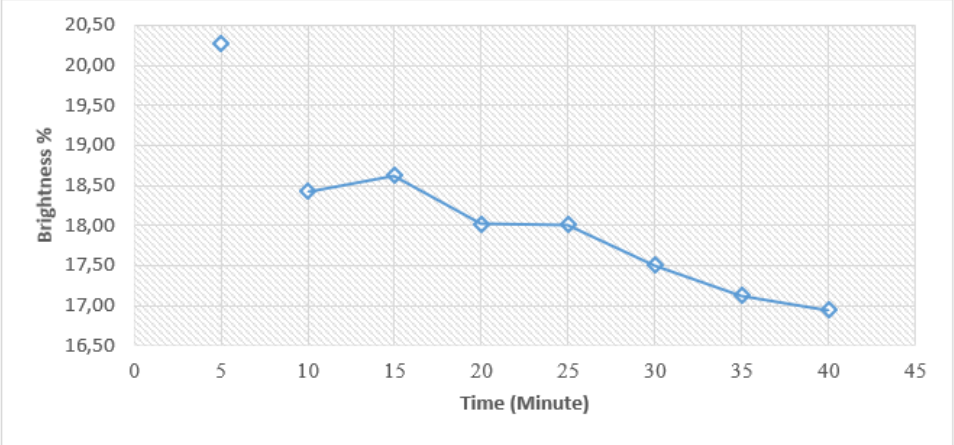

Figure 5. Effect of tea infusion on Brightness % depending on brewing time

There is a rapid increase in fluoride ion extraction immediately after the granule tea encounters boiling water (Figure 6). The rate of fluoride release increases relatively until the 5th minute, and the increases after the 15th minute are not significant. At the 15th minute, the fluoride ion concentration reaches its maximum and remains constant, while the brightness decrease is continuous.

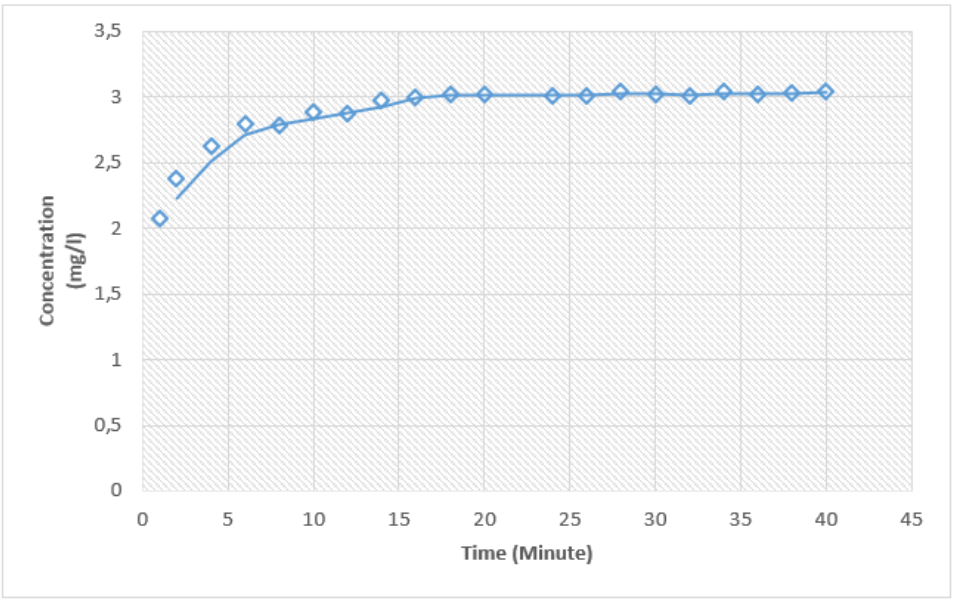

Figure 6. Effect on fluoride ion extraction depending on brewing time on tea infusion

The color of food is one of the most important features that directly affect the food preference and desires of consumers [21]. From the tea color point of view, it is not possible to evaluate consumer preferences using a standard analytical method. In Türkiye, it generally is asked the customer; how color do you take your tea, dark or light? The answer to this question is just a preference that varies from

person to person. However, the preferred color determines the fluoride concentration in tea and finally contributes to daily fluoride intake.

Tea naturally contains fluoride ions. According to the available reports, the fluorine content of granulated tea samples varies between 3.2 and 400 ppm in Türkiye [22]. This is quite a wide range and is the precursor to the fluoride ion that would migrate into the tea infusion. It is, of course, the color choice that would determine the exact chemical content of tea.

It is well known that fluoride has many beneficial effects on human health at a certain daily intake level. A high level of daily fluoride intake, however, may cause some health problems such as dental/enamel fluorosis and skeletal fluorosis. The safe daily fluoride intake level is especially significant for the populations consuming high levels of tea due to the high level of fluoride content of the black tea. The preparation and presentation methods of tea can also affect the concentration of fluoride in the tea infusion that would ultimately be consumed.

According to our results, it was concluded that the fluoride concentration reaches a plateau within 20 minutes of brewing (by Turkish traditions). Generally, the in-house brewing period of Turkish tea is much longer than 20 minutes. So, it means the tea infusion reaches its maximum fluoride concentration in the infuser. In our study, when brewing black tea (6 g/250 ml) by Turkish traditions, the fluoride concentration in the infuser reached a steady state after 20 minutes at 3 mg/l. After this stage, the color of the tea we prefer becomes important. Because the tea infusion in the teapot is diluted until the desired color is obtained in the tea glass. In general, the desired bright and dark red color (tavşankanı) is obtained in the tea glass by adding one portion of brewed tea from the teapot and one portion of boiling water. Thus, the fluoride concentration is diluted by half (this color preference is crucial from the daily fluoride intake point of view).

Using the above information, it is possible to calculate the daily fluoride intake that stems from tea consumption. Although the volumes of tea glasses in Türkiye vary between 100 and 175 ml, 125 ml is the most preferred glass for drinking tea. The final fluoride concentration will be 0.19 mg [3x(125/2)/1000] when we put one portion of brewed tea and one portion of boiling water in this tea glass. Heavy tea consumers drink 15-20 glasses of tea daily. The fluoride intake would be 3.8 mg/day (0.19x20) for these extreme consumers, which represents a worst-case exposure scenario. As mentioned before, 10 mg is the daily UL of fluoride for both males and females aged over 9 [15]. Accordingly, considering the population that consumes excessive tea in Türkiye, it is foreseen that the amount of fluoride that can be safely taken from sources other than tea is 6.2 mg.

This study should be considered as an attempt to assess the daily fluoride intake that stems from daily tea consumption. It is well known that different tea samples contain different amounts of fluoride. Therefore, in our next study, we have aimed to expand this study by using different tea samples to make a more precise exposure assessment for fluoride in Türkiye.

ACKNOWLEDGEMENTS

The authors are grateful to Ankara University (Project no: 21L0237007) for financial support.

AUTHOR CONTRIBUTIONS

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

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., 47(3): 816-826, 2023
Doi: 10.33483/jfpau.1296874

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANATOMICAL, PALYNOLOGICAL AND MICROMORPHOLOGICAL INVESTIGATIONS ON ENDEMIC *THYMUS BRACHYCHILUS* JALAS (LAMIACEAE)

ENDEMİK THYMUS BRACHYCHILUS JALAS (LAMIACEAE) ÜZERİNDE ANATOMİK, PALİNOLOJİK VE MİKROMORFOLOJİK ARAŞTIRMALAR

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ABSTRACT

Objective: *In this study, it was aimed to investigate the anatomical and palynological features of endemic Thymus brachychilus Jalas.*
Material and Method: *The plant material was fixed in 70% alcohol for anatomical investigation. Cross sections of the stem, leaves, and leaf surface sections were stained with sartur solution, and the anatomical aspects were examined. The trichome architectures, mesophyll cells, stomatal index, and glandular and non-glandular trichomes features of leaves and stem have been investigated and compared with the other Thymus species anatomical findings. In addition, measurements of anatomical structures were made. Pollen, flower parts, epidermis and trichome structure of the leaves, were examined in SEM.*
Result and Discussion: *This is the first anatomical, palynological and micromorphological study on Thymus brachychilus. The epidermis of leaf consists of a single row of elongated shaped cells and is covered with a thin cuticle. There are nonglandular and glandular trichomes in the upper and lower epidermis. Two different types of nonglandular trichomes were found. Stomata are diacytic, and the epidermis is amphistomatic. The mesophyll layer is dorsiventral. The stem cross-section is square. The outermost single-row, elongated, square or rectangular epidermis is covered with a thin cuticle. The pollen symmetry is isopolar, shape is suboblate. It has hexacolpate and bireticulate properties.*

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Submitted / Gönderilme : 15.05.2023
Accepted / Kabul : 12.07.2023
Published / Yayınlanma : 20.09.2023

Keywords: *Aladağlar, micromorphology, palynology, plant anatomy, Thymus brachychilus*

ÖZ

Amaç: *Bu çalışmada, endemik Thymus brachychilus Jalas'ın anatomik ve palinolojik özelliklerinin araştırılması amaçlanmıştır.*
Gereç ve Yöntem: *Anatomik inceleme için bitki materyali %70 alkol içinde fikse edildi. Gövde ve yaprak enine kesitleri, yaprak yüzey kesitleri sartur solüsyonu ile boyandı ve anatomik açıdan incelendi. Yaprak ve gövdenin trikom yapıları, mezofil hücreleri, stoma indeksi, salgı ve örtü tüyleri incelendi, aynı zamanda diğer Thymus türlerinin anatomik bulguları ile karşılaştırıldı. Ayrıca anatomik yapıların ölçümleri yapıldı. SEM'de polen, çiçek kısımları, yaprakların epidermis ve trikom yapısı, kısımları incelenmiştir.*
Sonuç ve Tartışma: *Thymus brachychilus üzerinde yapılan ilk anatomik, palinolojik ve mikromorfolojik çalışmadır. Yaprağın epidermisi tek sıra uzun şekilli hücrelerden oluşur ve ince bir kütikül ile kaplanmıştır. Üst ve alt epidermiste glandüler olmayan ve glandüler trikomlar vardır. İki farklı tipte nonglandüler trikom bulundu. Stomalar diastiktir ve epidermis amfistomatiktir. Mezofil tabakası dorsiventraldir. Gövde enine kesiti karedir. En dıştaki tek sıralı, uzun, kare veya dikdörtgen epidermis ince bir kütikül ile kaplıdır. Polen simetrisi izopolardır ve şekli suboblate. Hexacolpate ve bireticulate özelliklere sahiptir.*
Anahtar Kelimeler: *Aladağlar, mikromorfoloji, palinoloji, bitki anatomisi, Thymus brachychilus*

INTRODUCTION

Thymus L. has 341 species in the world and 46 species in Türkiye, 19 of which are endemic [1-3]. *Thymus* species are generally woody at the base, small shrubs or cushion plants. Leaves are entire, sessile or petiolate, with flat or revolute edges, and frequently ciliate at base of lamina. Sessile, colorless to brilliant red glands are present on the bracts, calyces, and leaves. Verticillasters with two or more flowerings, subtending floral leaves, or clustered into a terminal head with distinct bracts. Calyx clearly bilabiate; tube cylindrical to campanulate, straight, with 10-13 veins; upper lip broad patent or recurved. Corolla is purple, pink, cream, or white; tube is straight; the top and lower lips are emarginate and straight. Nutlets are glabrous. The stems and leaves of *Thymus brachychilus* are similar to *T. leucotrichus* but have distinct coarse trichomes, more narrow leaves, weak differentiation between the inflorescence and the vegetative shoots, oil glands are primarily orange or red. Upper lip of the calyx is shorter than lower teeth; bracts are practically leaf-like, 0.8-1.5 mm broad, narrowly rhombic, and progressively narrow into a short petiole. Flowering time is between 6 and 8 months. Grows in 1800-3660 m., rocks and screes. *Thymus brachychilus* grows naturally in the provinces of Adana, Erzincan, İçel, Niğde, Tunceli, Van in the Southern and Central Anatolian regions of Türkiye [3].

For many years, people in Anatolia have used several *Thymus* species, with a variety of outcomes. *Thymus brachychilus* is known as "Mor kekik" in the Niğde [4]. According to ethnobotanical studies carried out in various regions of Türkiye, it is used for abdominal pain, anxiety, anorexia, asthma, backache, breathing problems, bronchitis, cancer, colds, diabetes, edema, enteralgia, flu, gastritis, halitosis, hemorrhoids, high cholesterol, hypertension, kidney diseases, renal inflammations, prostrate, stomachache, and tonsillitis [5-15]. *Thymus* species grown in Türkiye are especially important with their rich essential oil content. The essential oil contents of *Thymus* species grown in Türkiye contain borneol, carvacrol, carvacrol methyl etherl, 1,8-cineole, cymene, farnesol, limonene, linalool, γ-terpinen, thymol, α-pinene [16-20]. It is important to determine the anatomical, palynological and micromorphological characteristics of *Thymus* species which are so important in terms of medicine and widely used in traditional medicine of Anatolia. These findings are also valuable in helping to accurately identify *Thymus* species. This study includes anatomical and palynological studies on the endemic *Thymus brachychilus* (TB). It is the first research on the anatomy and palynology of this species.

MATERIAL AND METHOD

Plant Material

Samples of the *Thymus brachychilus* were collected in the Maden Valleys of the Aladağlar

mountain range (Niğde-Türkiye) between 2500 and 3500 meters in July 2022 (Figure 1). After identification of the plant species by authors, voucher specimens were prepared. These voucher specimens were kept in the Herbarium of the Pharmacy Faculty, Altınbaş University (HERA 1074).

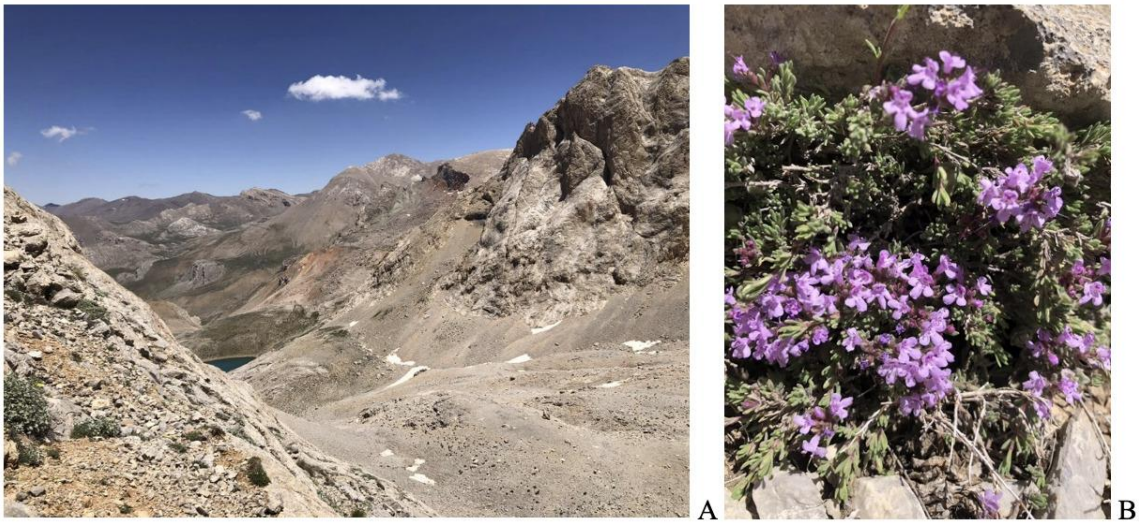

Figure 1. A- Habitus of *Thymus brachychilus* in Aladağlar (Niğde, Türkiye), B- General view of *Thymus brachychilus*

Anatomical Studies

The material was fixed in 70% alcohol for anatomical investigation. Parts of the stem, leaves, and leaf surface were stained with sartur solution. The well-stained slices were photographed using an Soif Optical Instruments Trinocular Stereo microscope and Zeiss light microscope. Investigations have been done in the anatomical characteristics of stems, leaves and leaf surfaces. Anatomical studies were conducted on cuticle, epidermis cells, mesophyll cells, stomatal index, stomata structures, non glandular and glandular trichomes of the leaf; cuticle, epidermis cells, cortex layer, collenchyma, parenchyma endodermis cells, trachea of the stem. Additionally, calculations have been made for the micro-anatomic measurements of cells and tissues.

Palynological and Micromorphological Studies

Pollens were removed with the help of arrowheads. Pollens and epidermis, trichome structure of the leaves, flos, calix parts adhered to the sample holder (stab) with double-sided tape. The prepared samples were coated with gold and examined in detail under a table-top scanning electron microscope (FEI Versa 3D dual beam) and photographed. In pollen morphology, Walker and Doyle [21] terminology was used.

RESULT AND DISCUSSION

This is the first anatomical, palynological and micromorphological study on *Thymus brachychilus*.

Anatomical Studies

Leaves Anatomy:

The outermost layer, the epidermis, consists of a single row of elongated shaped cells and is covered with a thin cuticle. The upper cuticle thickness is 2.81 µm, lower side cuticle thickness is 3 µm. The upper epidermis cell length is 11.02 µm and the width is 17.31 µm. The lower epidermis cell length is 12.74 µm and the width is 11.21 µm. The epidermis cells on the upper side are wider. There are

nonglandular and glandular trichomes in the upper and lower epidermis. Two different types of nonglandular trichomes were found. Non-glandular trichomes are simple, erect mostly 3-5 cells and the other type nonglandular trichomes are simple with one cell (Figure 2). Along the leaf surface, two different types of glandular trichomes were found. One of them has a base, a stalk, and a unicellular head, and is called a capitate glandular trichome (Figure 3). Peltate glandular trichomes are the other type; they feature a base, a short sessile stalk, and a multicellular head. Stomata are diacytic, and they were identified as being at the same level as the epidermis (amphistomatic). The upper stomata length is 26 µm and the width is 17 µm. The lower stomata length is 18 µm and the width is 14 µm. It was determined that the stomata on the upper side were larger. The upper side stomata index is 17.78 while the lower side stomata index is 18.10 (Table 1). The stomatas are more dense on the lower side (Figure 4). The mesophyll layer is dorsiventral. It consists of 1-2 rows of palisade and 3-4 rows of sponge parenchyma. Vascular bundles are collateral. On the midrib region, the xylem is directed towards the upper epidermis and the phloem towards the lower epidermis. Just below the xylem, multilayered (10-15) sclerenchymatic bundles are seen. Phloem has 2-3 rows. Oval collenchyma is located under the upper and lower epidermis on the middle vein region. Round parenchyma seen below the collenchyma (Figure 5).

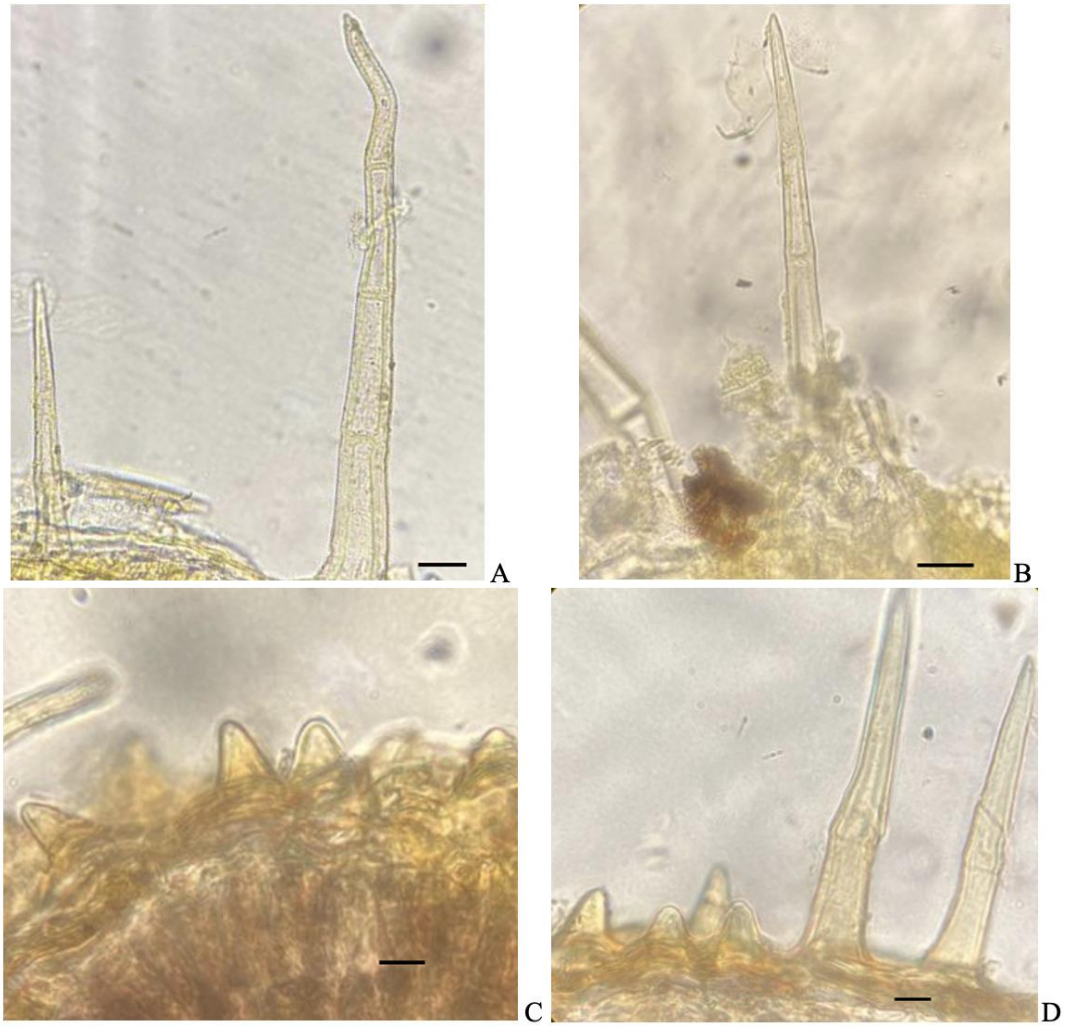

Figure 2. Nonglandular trichomes observed on studied TB. A-4 celled nonglandular trichomes on abaxial surface (Scale bars: 25 µm); B-4 celled nonglandular trichomes on adaxial surface; C-Single celled nonglandular trichomes (Scale bars: 100 µm); D-Single celled and 2-celled nonglandular trichomes (Scale bars: 100 µm)

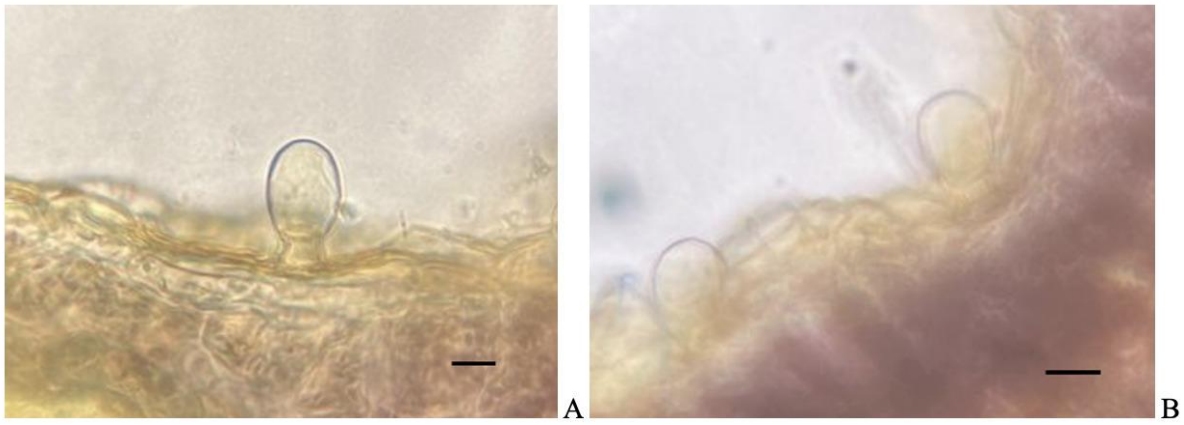

Figure 3. Glandular trichomes types observed on studied TB. A-abaxial surface; B-adaxial surface (Scale bars: 10 µm)

Table 1. Anatomical measurements of leaf

Anatomical Structures	**TB**
Upper epidermis	
Stomata type	diacytic
Stomata index	17.78
Stomata length (µm) (Avr. ± Sd)	26±2.23
Stomata width (µm) (Avr. ± Sd)	17±2.09
Cuticle thickness (µm) (Avr. ± Sd)	2.81±0.74
Epidermis cell length (µm) (Avr. ± Sd)	11.02±1.65
Epidermis cell width (µm) (Avr. ± Sd)	17.31±0.88
Lower epidermis	
Stomata type	diacytic
Stomata index	18.10
Stomata length (µm) (Avr. ± Sd)	18±3.26
Stomata width (µm) (Avr. ± Sd)	14±2.85
Cuticle thickness (µm) (Avr. ± Sd)	3±1.22
Epidermis cell length (µm) (Avr. ± Sd)	12.74±1.17
Epidermis cell width (µm) (Avr. ± Sd)	11.21±1.85

Avr: average, Sd: standard deviation

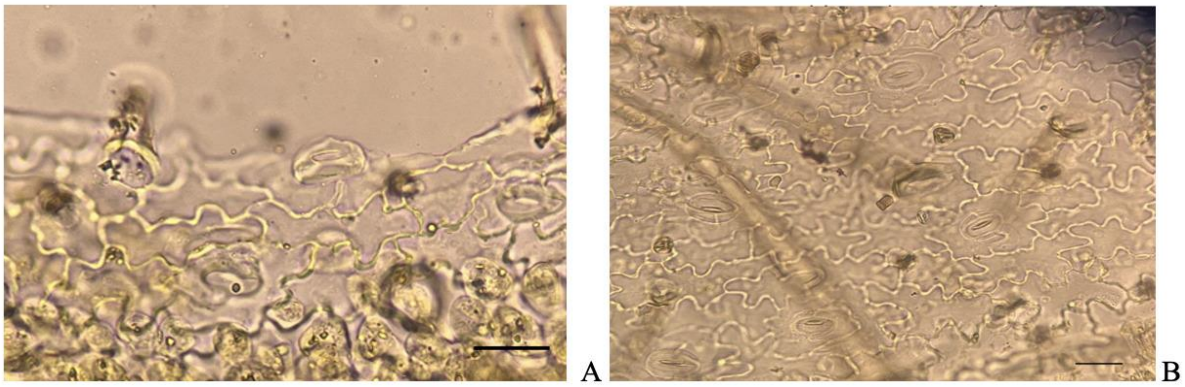

Figure 4. Lamina epidermal surface of TB. A- abaxial surface; B-adaxial surface (Scale bars: 25 µm)

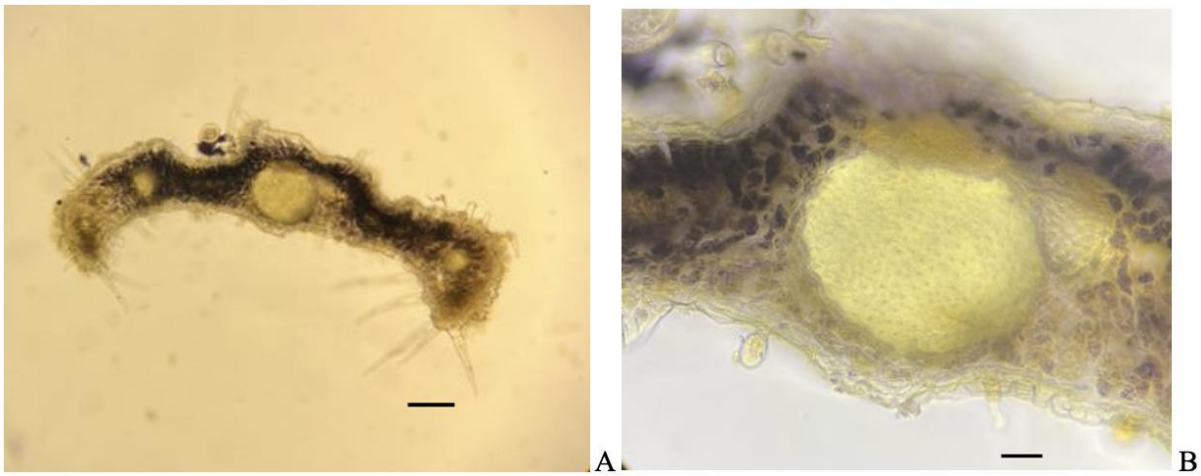

Figure 5. A- Cross section of leaves (Scale bars: 100 µm); B- Cross section of leaves (Scale bars: 25 µm)

Stem Anatomy:

The stem cross-section is square and hollow. The outermost single-row, elongated, square or rectangular epidermis is covered with a thin cuticle. The epidermis cell length is 16.90 µm and width is 16.87 µm. Glandular and nonglandular trichomes are observed on the epidermis. Nonglandular trichomes 1-6 cells, unbranched. The glandular trichomes are seen in the capitate type. Just below the epidermis is the cortex layer. This layer consists of collenchyma (3-5 rows), parenchyma (3-4 rows) and endodermis (1-2 rows). The collenchyma layer is 37.48 µm in the corner while it is 15.08 µm between corners. The parenchyma layer is 24.99 µm. The phloem is 3-6 rows and phloem layer is 20.81 µm. The xylem encircled the pith in an annular manner and the xylem layer is 31.22 µm (Table 2). The pith is composed of polygonal shaped, thin-walled parenchymatic cells (Figure 6).

Table 2. Anatomical measurements of stem

Anatomical Structures	**TB**
Epidermis cell length (µm) (Avr. ± Sd)	11.29±0.65
Epidermis cell width (µm) (Avr. ± Sd)	10.5±1.22
Pith ray cell	15.51±2.30
Trachea (µm) (Avr. ± Sd)	9.68±0.95
Endodermis cell length (µm) (Avr. ± Sd)	16.90±2
Endodermis cell width (µm) (Avr. ± Sd)	16.87±3.18
Collenchyma cell	12.52±0.59
Collenchyma layer (Corners)	37.48±3.19
Collenchyma layer (Between corners)	15.08±0.49
Parenchyma layer	24.99±6
Phloem layer	20.81±1.49
Xylem layer	31.22±2.26

Avr: average, Sd: standard deviation

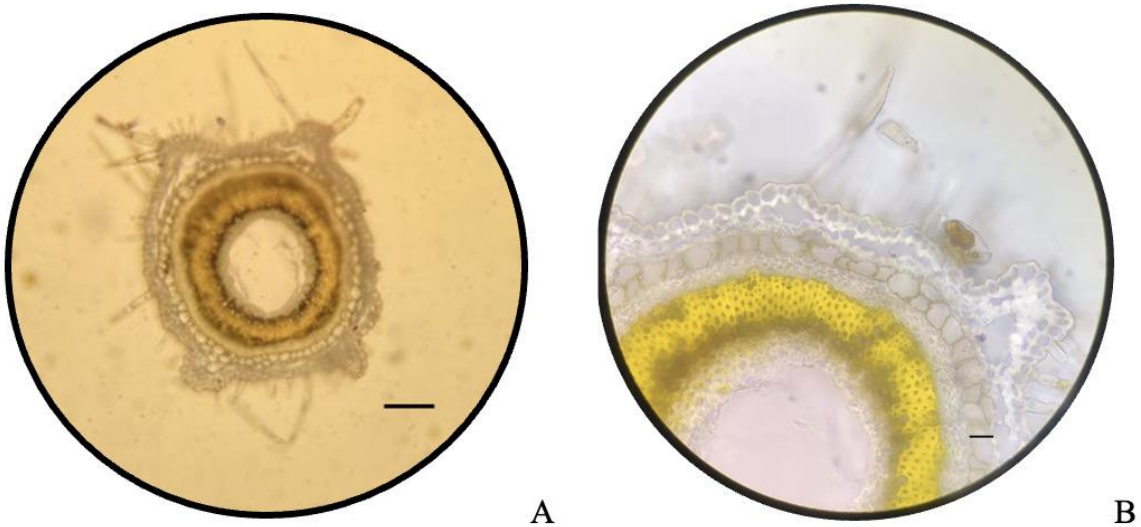

Figure 6. A- Cross section of stem (Scale bars: 100 µm); B- Cross section of stem (Scale bars: 25 µm)

Palynological and Micromorphological Studies:

In morphological pollen analysis, monad pollen is small-medium sized and its symmetry is isopolar (Figure 7). Pollen polar axis on average is 22.39 µm, equatorial axis on average is 26.45 µm. According to the P/E ratio, pollen shape is suboblate. Apertures are colpate, with an average colpus width of 1.7 µm and a length of 19.95 µm. 6 colpus makes pollen as a hexacolpate. Sculpture is a bireticulate (Table 3).

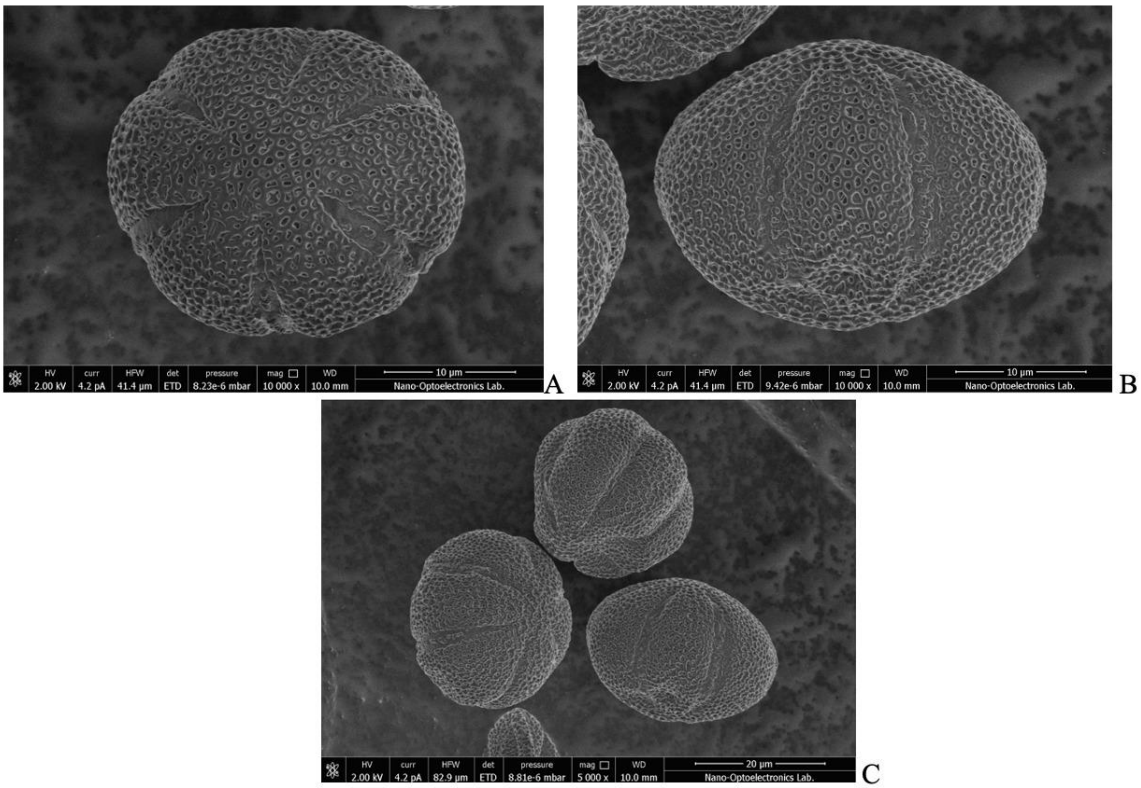

Figure 7. SEM images of TB, A- Polar view; B- Equatorial view; C- General view

Table 3. Palynological features and measurements

Ornamentation	**Bi-reticulate**
Aperture type	Colpus
Pollen shape	Suboblate
Aperture number	6
Colpus width	1.7±0.54 μm
Colpus length	19.95±0.64 μm
Polar axis (P)	22.39±0.24 μm
Equatorial axis (E)	26.45±1.26 μm
Pollen symmetry	isopolar
P/E	0.85

In leaf micromorphology, leaf arm tips are revolute. It was observed that the nonglandular trichomes on the lower and upper surfaces were similar to each other. The glandular trichomes were densely peltate and sparsely capitate type on both sides. Calyx has the nonglandular trichomes and peltate glandular trichomes (Figure 8-10).

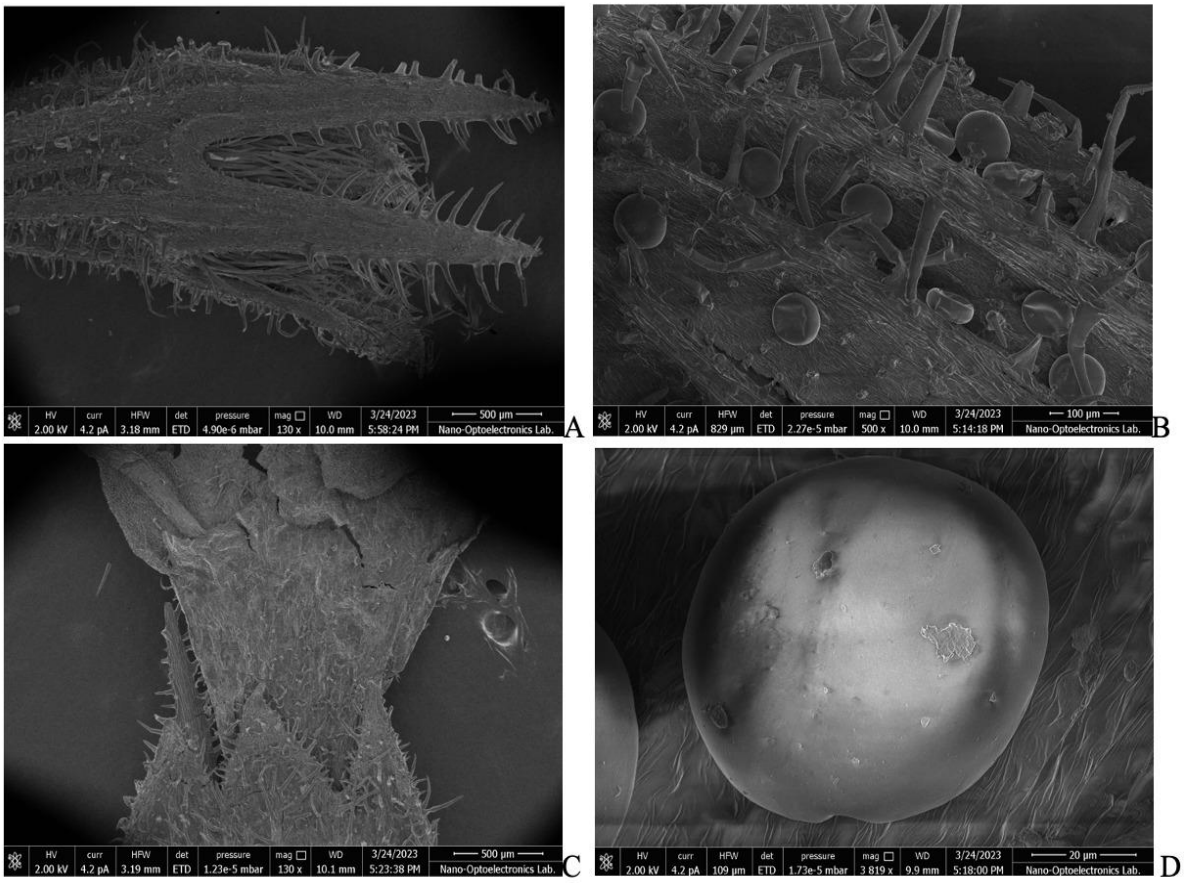

Figure 8. A, B- Calyx of TB; C- Calyx and Corolla of TB; D- Glandular trichomes of TB

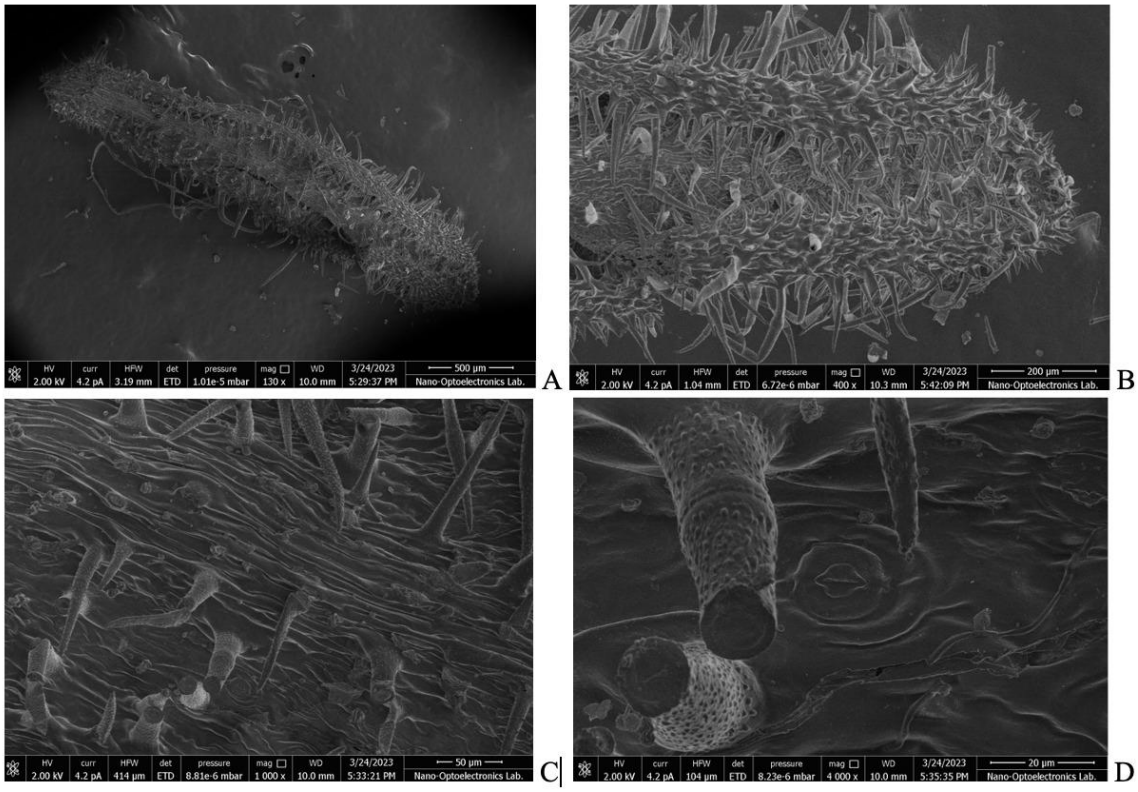

Figure 9. A, B, C, D- Lamina abaxial surface of TB

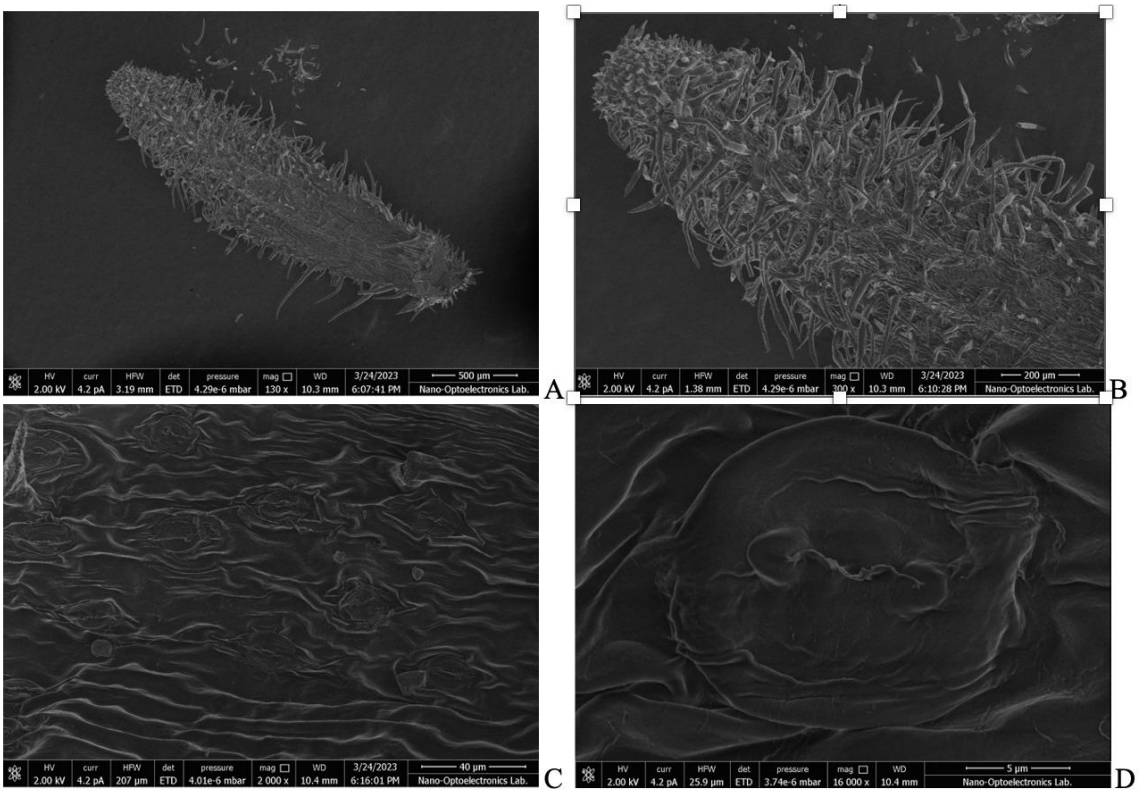

Figure 10. A, B, C, D- Lamina adaxial surface of TB

The results of the study were compared with previous anatomical, micromorphological and palynological results of *Thymus* species. According to an anatomical study on *Thymus cherlerioides* and T. pulvinatus, stomata index of *T. brachychilus* leaves is very similar to *T. cherlerioides* and less than *T. pulvinatus*. The stomata size of *T. brachychilus* is bigger than *T. cherlerioides* and *T. pulvinatus*. Glandular and nonglandular trichome types are very similar with this two species. The stem cross-section is of *T. brachychilus* is similar to *T. pulvinatus* [22]. In a micromorphological study of *Thymus* genus the most common epidermal cell shape was a slightly elongated form. Only one of the 32 species had isodiametric-shaped epidermal cells, while 18 of them had slightly elongated epidermal cells. The *T. brachychilus* epidermal cells of leaf and stem are elongated. Except for two species, all of the leaves of *Thymus* contained stomata on both the adaxial and abaxial surfaces. Only in *T. spahulifolius* were stomata visible, however this might be because of the extremely extensive indumentum structure on the leaf surface. *T. pseudopulegioides* abaxial leaf surface was devoid of stomata. *T. brachychilus* have stomata on both surface. The shape of nonglandular trichome is erect (*T. leucostomus* var. *gypsaceus, T. cherlerioides* var. *isauricus*), curved (*T. longicaulis* subsp. *longicaulis* var. *longicaulis, T. kotschyanus* var. *glabrescens, T. argaeus*) or appressed (*T. migricus, T. fedtschenkoi* var. *handelii*) and Glandular trichomes are either capitate or peltate [23]. *T. brachychilus* usually has erect nonglandular trichomes, capitate and peltate glandular trichomes. Pollen morphological features of five *Thymus* taxa examined by Kızılpınar et al (2009). According to this study *T. longicaulis* subsp. *longicaulis* var. *subisophyllus* has oblate-spheriodal, *T. sipyleus* subsp. *sipyleus* var. *sipyleus* has spheriodal, *T. sipyleus* subsp. *rosulans* and *T.cappadocicus* var. *globifer* suboblate, *T. leuchotricus* var. *leuchotricus* has prolate-spheriodal pollen shape. In our study, *T. brachychilus* has suboblate pollen shape [24].

ACKNOWLEDGEMENTS

The authors thank DÜAGEM (Altınbaş University Natural Products Research and Development Center) and BAP-Altınbaş University Project number PB2020-ECZ-1.

AUTHOR CONTRIBUTIONS

Concept: E.Ö.N., S.S.; Design: E.Ö.N., S.S.; Control: E.Ö.N, S.S.; Sources: E.Ö.N., S.S.; Materials: E.Ö.N., S.S.; Data Collection and/or Processing: E.Ö.N., S.S.; Analysis and/or Interpretation: E.Ö.N., S.S.; Literature Review: E.Ö.N., S.S.; Manuscript Writing: E.Ö.N., S.S.; Critical Review: E.Ö.N., S.S.; 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., 47(3): 827-835, 2023
Doi: 10.33483/jfpau.1264321

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIOXIDANT, ENZYME INHIBITORY, AND CYTOTOXIC ACTIVITY SCREENING OF *MYRTUS COMMUNIS* L.

MYRTUS COMMUNIS L.'NİN ANTİOKSİDAN, ENZİM İNHİBİTÖR VE SİTOTOKSİK AKTİVİTE TARAMALARI

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ABSTRACT

Objective: *The present study aimed to evaluate the biological activities of Myrtus communis L., which has traditional medicinal use for different purposes, from a broad perspective.*
Material and Method: *The antioxidant (DPPH, ABTS, total phenol, and flavonoid amounts) anti-inflammatory (LOX enzyme inhibition), antidiabetic (α-glucosidase enzyme inhibition), and cytotoxic properties (MTT assay) of a 70% methanol extract made from leaves and dichloromethane, butanol, ethyl acetate, and the residual water fractions were investigated.*
Result and Discussion: *The DPPH radical scavenging effect of the ethyl acetate fraction, with the highest total phenol and flavonoid content, was found to be 1.4 µg/ml, and the ABTS radical scavenging effect was 2.58 mmol/Trolox. The most potent inhibitor of LOX (IC_{50}: 31.17 µg/ml) and α-glucosidase (96% inhibition at 0.5 mg/ml) enzymes was determined as ethyl acetate extract. Dichloromethane fraction was shown to have the most cytotoxic activity in both Hela and Colo cell lines. This research has given us a better understanding of the traditional use of the M. communis plant, which stands out for its therapeutic properties.*
Keywords: *Antioxidant, cytotoxic activity, enzyme inhibition, Myrtus communis*

ÖZ

Amaç: *Bu çalışmada farklı amaçlarla geleneksel tıbbi kullanıma sahip olan Myrtus communis* L.*'nin biyolojik aktivitelerinin geniş bir perspektiften değerlendirilmesi amaçlanmıştır.*

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Submitted / Gönderilme : 13.03.2023
Accepted / Kabul : 19.07.2023
Published / Yayınlanma : 20.09.2023

Gereç ve Yöntem: *Yapraklardan yapılan %70 metanol ekstresi'nin ve diklorometan, butanol, etil asetat ve kalan su fraksiyonlarının antioksidan (DPPH, ABTS, toplam fenol ve flavonoit miktarları), antienflamatuvar (LOX enzim inhibisyonu), antidiyabetik (α-glukozidaz enzim inhibisyonu) ve sitotoksik özellikleri (MTT testi) incelenmiştir.*

Sonuç ve Tartışma: *En yüksek toplam fenol ve flavonoit içeriğine sahip etil asetat fraksiyonunun DPPH radikal süpürücü etkisi 1.4 µg/ml ve ABTS radikal süpürücü etkisi 2.58 mmol/lTrolox olarak bulunmuştur. LOX (IC_{50}: 31.17 µg/ml) ve α-glukozidaz (0,5 mg/ml'de %96 inhibisyon) enzimlerinin en güçlü inhibitörü etil asetat ekstresi olarak belirlenmiştir. Diklorometan fraksiyonunun hem Hela hem de Colo hücre hatlarında en fazla sitotoksik aktiviteye sahip olduğu gösterilmiştir. Bu araştırma, tedavi edici özellikleriyle öne çıkan M. communis bitkisinin geleneksel kullanımını daha iyi anlamamızı sağlamıştır.*

Anahtar Kelimeler: *Antioksidan, sitotoksik aktivite, enzim inhibisyonu, Myrtus communis*

INTRODUCTION

There is a balance in the organism between free radicals and their scavenger antioxidants. Disruption of this balance indicates oxidative stress [1]. When the mechanisms to eliminate the negative effects of free radicals are not sufficient, free radicals can cause cytotoxic and genotoxic effects by damaging basic biomolecules such as lipids, proteins, and DNA [2,3]. In addition, various diseases such as the destruction of immune cells, cardiovascular diseases, diabetes, gastrointestinal problems, and cancer types can occur [3]. Maintaining the balance between antioxidants and oxidants is important in this regard [4]. Antioxidant systems and chemicals protect living organisms from oxidative harm [1]. Secondary metabolites found in plant essential oils or extracts have recently received a lot of attention as natural antioxidants against oxidative damage [5].

The Myrtaceae is a huge plant family with 120 genera and 3850 species. *Myrtus* is a minor genus in this family that grows in tropical, subtropical, and temperate climates. *Myrtus* is more common in the Mediterranean and Middle East [6]. One of the most significant medicinal and aromatic species in this plant family is *Myrtus communis* L. [7]. It is a widespread species in the Mediterranean area (Southern Europe, North Africa, and Western Asia) [8]. It can be found naturally in coastal provinces such as Adana, Antalya, Mersin, Çanakkale, Istanbul, Zonguldak, Trabzon, Izmir, Samsun, Muğla, and Hatay in Turkey [5].

An aromatic, perennial, evergreen shrub, *M. communis*, [5] is known as 'Mersin' and 'Murt' by the local people in Turkey [9]. Traditionally, its leaves and fruits have been used as hypoglycemic, antimicrobial, anti-hemorrhagic, appetizing, and wound healing, as well as for therapeutic purposes in cough, constipation, nausea, and oral diseases. In Mersin, it has been reported that the fruits of the plant are used as an eye tonic, and the leaves are used internally by chewing or decoction against diabetes and chokes. In addition, it has been recorded in ethnobotanical studies that aromatic water is consumed against ailments such as diabetes and high cholesterol, and the shoots are kept in raki against asthma and bronchitis [10]. The essential oil obtained from its leaves is used in the treatment of lung diseases [8,11]. Tannins, flavonoids including quercetin, catechin, and myricetin derivatives, and coumarins (myrtucommulone (MC) A and B, semimyrtucommulone (S-MC)), are found in the leaves [12]. The fruits are also rich in flavonoids and anthocyanins [5]. Pharmacologically, it is known as a result of studies that the *M. communis* plant has anti-inflammatory, anti-cancer, anti-microbial, anti-diarrheal, anti-ulcer, antioxidant, anti-fungal, anti-mutagenic, and antiviral properties [12].

Given the numerous health advantages of *M. communis* and its use in foods, it was thought that the pharmacological effects of this plant should be investigated further. Hence, the present study aimed at investigating the antioxidant, antidiabetic, and cytotoxic properties of the leaf extracts of *M. communis*. Total phenolic and flavonoid amounts were determined by spectrophotometric methods. The antioxidant activity was measured using radical scavenging assays, whereas the anti-diabetic impact was measured using the α-glucosidase enzyme inhibition test. The cytotoxic impact of the extracts on the Colo and Hela cell lines as well as their ability to inhibit the LOX enzyme were assessed.

MATERIAL AND METHOD

Plant Material and Extraction Procedure

Myrtus communis was collected from the Silifke district in September 2018. The herbarium specimen of the plant (GK-1010) is stored in the Erciyes University Faculty of Pharmacy, in Kayseri, Turkey.

Dried leaves were pulverized and macerated in 70% methanol four times. Each maceration was carried out in a shaking water bath for 24 h at room temperature. The obtained extracts were combined and concentrated with a rotary evaporator under a vacuum. The powdered extracts were first dispersed with water to be fractionated and then subjected to a liquid-liquid fractionation with dichloromethane, ethyl acetate, and n-butanol, respectively. All prepared sub-fractions and the leftover water sub-fraction were lyophilized after being withdrawn from their solvents.

Antioxidant Activity

DPPH• Radical Scavenging Activity

In order to determine the DPPH radical scavenging effects of the extracts, the method reported by Hatano et al. (1989) was modified and applied [13]. 100 µl of the extracts' solutions prepared at varying concentrations were distributed on 96-well plates, and then 100 µl of DPPH (0.1 mM, in ethanol) solution was added. The radical scavenging effect was calculated by measuring the absorbance at 517 nm after it was kept in the dark at 37°C for 30 minutes. Experiments were carried out in 3 repetitions. IC_{50} values were calculated using nonlinear regression curves (Sigma Plot 2001 version 7.0, SPSS Inc., Chicago IL).

$$\% \text{ Inhibition} = [(\text{Absorbance control} - \text{Absorbance sample}) / (\text{Absorbance control})]x100 \quad \text{Eq 1.}$$

$ABTS^{\bullet+}$ Radical Scavenging Activity

$ABTS^{\bullet+}$ radical was formed with an aqueous solution of ABTS (7 mM) and potassium persulfate ($K_2S_2O_8$) (2.45 mM, final concentration) by keeping it in the dark for 12-16 hours and its absorbance at 734 nm was adjusted to be 0.700 (±0.020). Extracts were prepared at two different concentrations (0.25 and 0.5 mg/ml). The prepared radical solution and the extract were mixed to 990 µl as 10 µl. For a total of 30 minutes, reaction kinetics were monitored at 734 nm once each minute. It was determined that percentages of inhibition evaluated versus concentration were equal to Trolox (TEAC).

Enzyme Inhibition Tests

Lipoxygenase Inhibitory Activity

In order to determine the anti-inflammatory effects of the extracts, their effects on the lipoxygenase (LOX) enzyme were determined by performing the modified FOX (ferric oxidation of xylenol orange)-test [14]. In a 96-well microplate, 50 µl of LOX (in 50 mM pH 7.4 Tris HCl, final concentration 100 ng protein/mL) and 20 µl of sample solution (extract or standard inhibitory substance) were incubated at 25°C for 5 minutes. The reaction was started by adding 50 µl of linoleic acid solution (50 mM pH 7.4 in Tris HCl, final concentration 140 µM) and the reaction mixture was incubated for 20 minutes at 25°C in the dark. With newly made FOX reagent (sulfuric acid (30 mM), xylenol orange (100 M), iron (II) sulfate (100 M), and methanol/water (9:1)) at 25°C for 30 minutes, the reaction was halted. Minutes later, using a microplate reader, absorbance readings were determined at 560 nm. Enzyme inhibitions were calculated using Eq 1.

α-Glucosidase Inhibitory Activity

The α-glucosidase enzyme inhibitory activity assay was performed according to the method reported by Liu et al. [15]. In this process, 50 µl of 2 U/ml α-glucosidase solution was mixed with 1000 µl of phosphate buffer and 200 µl of extract/acarbose. After incubation for 10 min at 37°C, 5 mM of 50 µl of *p*-nitrophenyl-α-D-glucopyranoside (pNPG) was added and the mixture was incubated again at

37°C for 20 min. Then, 2000 µl of 0.2 M sodium carbonate and 4700 µl of distilled water were added to stop the reaction, and absorbances were measured at 405 nm using a spectrophotometer. Inhibition % calculations were made using Eq 1.

Cell Culture

Colo 205 and Hela cell lines were supplied from the American Type Culture Collection (CCL-222; CCL-2). The cells were cultured in RPMI and DMEM, respectively, with 1% double antibiotics (penicillin and streptomycin) and 10% fetal bovine serum at 37°C and 5% CO_2.

Cytotoxic Activity

Colo and Hela cells were seeded in a 96-well plate at a density of 1×10^4 cells/mL 100 (100 µl per well) and divided into blank, control, and extracts (7.81; 15.6; 31.25; 62.5; 125; 250; 500, and 1000 µg/l) groups. After incubation for 24 hours, cells were treated with 100 µl of vehicle or samples for 24 hours. Next, MTT reagent (stock: 5 mg/ml in PBS) was added into each well and incubated at 37°C for 4 hours. Each well received 100 µl of DMSO, which was used to dissolve the formazan crystals generated by MTT. Using a microplate reader with a 540 nm wavelength, each well was read after 10 minutes.

Statistical Analysis

SPSS 18.0 (SPSS, Chicago, IL, USA) was used to evaluate the results, which were presented as mean±standard deviation (n=3). One-way analysis of variance (ANOVA) together with Tukey and Dunnett's tests were used to identify the significant differences ($P<0.05$) between groups.

RESULT AND DISCUSSION

The total phenol content of the methanol extract and fractions was found to be rich. The extract with the highest total phenol content was found to be the ethyl acetate fraction with a value of 542.44±13.62 mg GAE/g extract, and the dichloromethane fraction with the lowest content of 109.75±7.21 mg GAE/g extract. In total flavonoid content, methanol extract, which is the main extract, has the highest content with a value of 94.84±0.77 mg CA/g extract. The results are given in Table 1. The presence of compounds such as ferulic acid, caffeic acid, gallic acid, quercetin derivatives (quercetin 3-*O*-galactoside and quercetin 3-*O*-rhamnoside), catechin derivatives (epigallocatechin, epigallocatechin 3-*O*-gallate, epicatechin 3-*O*-gallate), and myricetin derivatives has been demonstrated in *M. communis* plant analyses [16]. The fact that it is rich in total phenolic and flavonoid content can be associated with having different secondary metabolite groups as stated in the literature.

Table 1. Total phenol/flavonoid content of *M. communis* extracts

Extracts	Total Phenol [mg_{GAE}/g_{extact}]	Total Flavonoid [$mg_{CA}/g_{extract}$]
M.c MeOH	273.54±10.59	94.84±0.77
M.c DCM	109.75±7.21	48.86±0.40
M.c EtOAc	542.44±13.62	76.74±1.45
M.c BuOH	363.57±2.13	69.64±1.50
M.c water	162.66±11.28	48.24±1.81

The data are presented as mean ± standard error (n=3), M.c MeOH: *M. communis* methanol extract, M.c DCM: *M communis* dichloromethane fraction, M.c EtOAc: *M. communis* ethyl acetate fractions, M.c BuOH: *M. communis* butanol fractions, M.c water: *M. communis* residual water fractions

All of the tested extracts significantly scavenged DPPH and ABTS radicals, as seen in Table 2. Methanol extract, butanol, and ethyl acetate fractions were found to have the same significance as rosmarinic acid in the scavenging effect of DPPH radical ($p>0.05$). The fraction with the lowest activity was found as the dichloromethane fraction with an IC_{50} value of 18±0.001 µg/ml. Except for the

methanol extract, all of the fractions were able to exhibit the ABTS radical scavenging activity with the same significance as rosmarinic acid at both concentrations studied ($p>0.05$). Although studies examining the antioxidant activities of the *M. communis* plant generally focused on the essential oil of the plant, it can be said that *M. communis* collected from Mersin has a very high activity when compared to studies examining the activity of the leaf extract. According to the results of the DPPH radical scavenging activity of the leaf methanol extracts of three species collected from different regions of Marmaris (Turkey) in a publication made in 2015, the IC_{50} values are above 1 mg/ml [17]. Tumen et al., on the other hand, reported that over 90% of DPPH radical scavenging activity at 2 mg/ml concentration of vegetative leaf methanol extract was collected from the Silifke district of Mersin [18]. It is thought that the strong antioxidant capacity of the plant is due to the flavonoids and secondary metabolites in the tannin structure [19].

Table 2. DPPH and ABTS radical scavenging activity of *M. communis* extracts

DPPH		ABTS TEAC mmol/lTrolox	
Extracts	**IC_{50} (µg/ml)**	**0.5 mg/ml**	**0.25 mg/ml**
M.c MeOH	1.9±0.0003[a]	2.52±0.003[b]	2.43±0.002[b]
M.c DCM	18±0.001[c]	2.57±0.01[a]	2.55±0.064[a,b]
M.c BuOH	1.9±0.0001[a]	2.584±0.002[a]	2.582±0.002[a]
M.c EtOAc	1.4±0.0003[a]	2.584±0.001[a]	2.584±0.003[a]
M.c water	4.63±0.0004[b]	2.57±0.01[a]	2.56±0.02[a]
RA	1.95±0.013[a]	2.585±0.02[a]	2.582±0.017[a]

Values presented as mean ± standard errors (n = 3), with statistical analyses performed using the Tukey comparison test. Same lowercase letter (a-b). M.c MeOH: *M. communis* methanol extract, M.c DCM: *M communis* dichloromethane fraction, M.c EtOAc: *M. communis* ethyl acetate fractions, M.c BuOH: *M. communis* butanol fractions, M.c water: *M. communis* residual water fractions

When the ability of the extracts to inhibit the lipoxygenase enzyme, which is connected to inflammation, is assessed, the ethyl acetate and butanol fractions, which are also rich in total phenolic content, come to the fore. As shown in Table 3, the dichloromethane fraction exhibited the least activity with an IC_{50} value of 113.33 µg/ml. Inflammation is associated with many illnesses, including cancer, stroke, cardiovascular and neurological disorders, and there is a need for novel anti-inflammatory chemicals and plant-derived molecules to be identified [20]. Previous studies with *M. communis* showed 89.2% inhibition of the chloroform fraction at a concentration of 200 µg/ml [21]. The butanol fraction similarly exhibited 83.5% inhibition. These findings are also consistent with our data.

Table 3. LOX enzyme inhibition IC_{50} values of *M. communis* extracts

Extracts	**LOX Enzyme Inhibition IC_{50} (µg/ml)**
M.c MeOH	56.42±0.005[a,b]
M.c DCM	113.33±0.015[c]
M.c BuOH	31.17±0.001[a]
M.c EtOAc	31.66±0.006[a]
M.c water	73.33±0.003[b]

Values presented as mean ± standard errors (n = 3), with statistical analyses performed using the Tukey comparison test. Same lowercase letter (a-c). M.c MeOH: *M. communis* methanol exract, M.c DCM: *M communis* dichloromethane fraction, M.c EtOAc: *M. communis* ethyl acetate fractions, M.c BuOH: *M. communis* butanol fractions, M.c water: *M. communis* residual water fractions

When treating diabetes, controlling oxidative stress and inhibiting glucosidase in the digestive

system are crucial anti-diabetic modes of action. Hyperglycemia, hyperglycemia-induced oxidative stress, inflammation, and the development and progression of type 2 diabetes mellitus are all strongly linked. Several studies have found that chronic low-grade inflammation increases the likelihood of developing type 2 diabetes, and that subclinical inflammation leads to insulin resistance and is connected to metabolic syndrome symptoms such as hyperglycemia. As carbohydrate hydrolyzing enzyme inhibitors, α-glucosidase and α-amylase provide an efficient technique for regulating or preventing hyperglycemia by reducing starch breakdown [22-24]. When the α-glucosidase enzyme inhibition of the extracts was evaluated, it was determined that the ethyl acetate fraction could inhibit the enzyme with the same significance as standard acarbose at both concentrations studied ($p>0.05$). Interestingly, the butanol fraction, which was active in LOX enzyme inhibition, showed low activity (35.52 % at 1 mg/ml concentration) in α-glucosidase enzyme inhibition (Figure 1). The results are given in Figure 1. In a recent study, the IC_{50} value of α-glucosidase enzyme inhibition of *M. communis* chloroform extract was found to be 22.33 µg/ml. Furthermore, PTP1B enzyme inhibition of the extract and isolated triterpenoid compounds demonstrated that *M. communis* contains active ingredients in Type 2 diabetes [25].

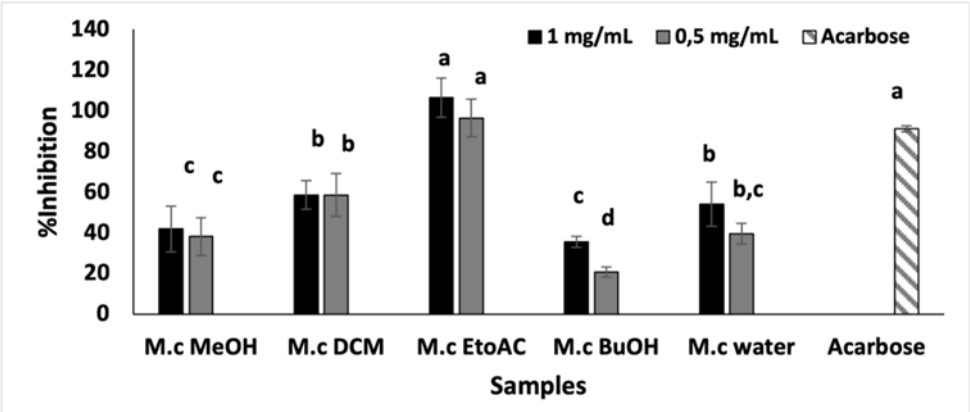

Figure 1. α-Glucosidase enzyme inhibition activities of *M. communis* extracts. M.c MeOH: *M. communis* methanol extract, M.c DCM: *M communis* dichloromethane fraction, M.c EtOAc: *M. communis* ethyl acetate fractions, M.c BuOH: *M. communis* butanol fractions, M.c water: *M. communis* residual water fractions

Despite their high phenolic content and antioxidant activity, the extracts did not exhibit strong cytotoxic activity against cancer cells. The most active fraction was determined as dichloromethane fraction in both the Hela cell line and Colo cell line, and IC_{50} values were calculated as 78.31 and 38.92 µg/ml, respectively. Butanol and the remaining water fractions did not show significant cytotoxic activity on viability in both cell lines. The cytotoxic effects of *M. communis* on Hela and Colo cell lines were investigated for the first time in this study. However, there are some studies in the literature investigating their cytotoxic effects against different cell lines. For example; in vitro cytotoxicity of methanol, ethyl acetate, *n*-butanol, and water extracts of *M. communis* and *Eucalyptus camaldulensis* Dehnh. were examined against two human breast cancer cell lines (MCF 7 and MDA-MB-231) using MTT and SRB assays. The results showed the significant cytotoxic potential of examined extracts, with IC_{50} values ranging from 7 to 138 µg/ml for *M. communis* [26]. In another research polyphenol-enriched fraction obtained from *M. communis* exerted a notable cytotoxicity towards HL60 with 19.87 µM and K562 with 29.64 µM IC_{50} values [27]. In a study performed with essential oils obtained by hydrodistillation from fresh aerial parts of two *Myrtus* cultivars, black and white (MB and MW), different in fruit color, both essential oils were screened for their cytotoxic activities against five cancer cell lines (PC3, MCF-7, A549, HepG2, HCT-116) and it was found that both EO of the *Myrtus* have cytotoxic activity against PC3 with IC_{50}; 4.7±0.15, 14.2±0.35 and MCF-7 with IC_{50}; 45.2±0.67, 50.5±0.6 respectively [28].

Table 4. Cytotoxic IC_{50} values of *M. communis* extracts on Hela and Colo cell lines

Extracts	Hela IC_{50} (µg/ml)	Colo IC_{50} (µg/ml)
M.c MeOH	270.83±22.23[b]	348.67±12.48[**]
M.c DCM	78.31±3.83[a]	38.92±1.73[*]
M.c EtOAc	243.16±19.71[b]	276.65±12.02[**]
M.c BuOH	356.56±2.83[c]	<500
M.c water	<500	451.52±6.70[***]

Values (µg/ml) are given as mean ±standard errors (n =3). Bars with the same lowercase letters (a–c), and symbols (*,***) are not significantly ($p > 0.05$) different. M.c MeOH: *M. communis* methanol extract, M.c DCM: *M communis* dichloromethane fraction, M.c EtOAc: *M. communis* ethyl acetate fractions, M.c BuOH: *M. communis* butanol fractions, M.c water: *M. communis* residual water fractions

After a thorough analysis of all the study's data, it was discovered that in assessments of antioxidant activity and enzyme inhibition, ethyl acetate and butanol fractions with high total phenol and flavonoid content excelled. In cytotoxicity experiments, the fact that the dichloromethane extract, which is the apolar fraction, had a lower IC_{50} value, revealed that it could be caused by the compounds in terpenic structure. This supports the plant's prospective use as a reliable alternative source of significant chemicals for use in industrial and medicinal applications.

AUTHOR CONTRIBUTIONS

Concept: E.K.Ş., S.İ., G.Ş.K.; Design: E.K.Ş., S.İ., G.Ş.K.; Control: S.A.M., G.Ş.K.; Sources: S.A.M., G.Ş.K.; Materials: E.K.Ş., S.İ., K.N.Ç., S.A.M., H.Y., G.Ş.K.; Data Collection and/or Processing: E.K.Ş., S.İ., K.N.Ç., S.A.M., H.Y., G.Ş.K.; Analysis and/or Interpretation: E.K.Ş., S.İ., K.N.Ç., S.A.M., H.Y., G.Ş.K.; Literature Review: G.Ş.K.; Manuscript Writing: K.N.Ç., S.A.M., G.Ş.K.; Critical Review: E.K.Ş., S.İ., S.A.M., G.Ş.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 ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 836-844, 2023
Doi: 10.33483/jfpau.1312637

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ISOIMPERATORIN-MEDIATED ANTICANCER ACTIVITY: ROLE OF MITOCHONDRIAL DYSFUNCTION IN HEPG2 CELLS

İZOİMPERATORİN ARACILIKLI ANTİKANSER AKTİVİTE: MİTOKONDRİYAL DİSFONKSİYONUN HEPG2 HÜCRELERİNDEKİ ROLÜ

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ABSTRACT

Objective: *The first goal of the present study is to investigate the role of mitochondria due to the Crabtree effect in HepG2 cells exposed to ISO in either glucose- or galactose-conditioned media. The second aim is to predict the interactions between electron transport chain (ETC) complexes and ISO, which might be the possible reason for mitochondrial dysfunction.*
Material and Method: *Cell viability and membrane damage for HepG2 cells exposed to ISO (12.5, 25, 50, 100, and 250 µM) were assessed by MTT and LDH leakage assays in either glucose- or*

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Submitted / Gönderilme : 11.06.2023
Accepted / Kabul : 20.07.2023
Published / Yayınlanma : 20.09.2023

galactose-conditioned media. The affinity of ISO to ETC complexes was also determined by a molecular docking study.
Result and Discussion: *MTT assay showed that 250 µM ISO leads to cytotoxic activity in glucose-conditioned media, while 25 µM and higher concentrations of ISO decrease cell viability in galactose-conditioned media. A membrane damage assay conducted in a glucose-conditioned media assay revealed that 250 µM ISO disrupts the cell membrane. 100 and 250 µM ISO increased membrane damage in galactose-conditioned media. According to docking simulations, binding affinities of ISO to ETC complexes are in descending order: Complex IV > Complex I > Complex III > Complex II. Inhibition of complex IV by ISO inhibits the transfer of electrons from cytochrome c to oxygen, and the proton gradient collapses. The present study proposed that ISO leads to mitochondrial dysfunction via inhibition of the ETC.*
Keywords: *Anticancer, crabtree effect, electron transport chain, isoimperatorin, mitochondrial dysfunction*

ÖZ

Amaç: *Çalışma kapsamındaki ilk amaç, glukoz veya galaktoz içeren besiyerlerinde İzoimperatorin'e (İZO) maruz kalmış HepG2 hücrelerindeki mitokondrinin rolünü Crabtree etkisi nedeni ile araştırmaktır. İkinci amaç, mitokondriyal disfonksiyonun ortaya çıkmasında rolü olabilecek olan İZO ve elektron transfer zinciri arasındaki (ETZ) etkileşimi öngörmektir.*
Gereç ve Yöntem: *Glukoz veya galaktoz içeren besiyerlerinde İZO (12.5, 25, 50, 100 ve 250 µM) ile inkübe edilen HepG2 hücrelerin canlılığı ve membran hasarı MTT ve LDH sızma deneyleri ile gerçekleştirilmiştir. İZO'nun ETZ kompleksleri üzerine olan afinitesi moleküler kenetleme çalışması ile analiz edilmiştir.*
Sonuç ve Tartışma: *MTT deneyi sonuçlarına göre glukoz içeren besiyerinde 250 µM İZO sitotoksik etki gösterirken, galaktoz içeren besiyerinde 25 µM ve daha yüksek konsantrasyonlar hücre canlılığını azaltmıştır. Glukoz içeren besiyerinde gerçekleştirilen membran hasarı deneyi, 250 uM İZO'nun membran bütünlüğünü bozduğunu göstermiştir. Galaktoz içeren besiyerinde 100 ve 250 uM İSO membran hasarını artırmıştır. Moleküler kenetlenme çalışma sonuçlarına göre İZO'nun ETZ komplekleri üzerine olan afinitesi Kompleks IV > Kompleks I > Kompleks III > Kompleks II şeklindedir. İZO, elektronun sitokrom C'den oksijene aktarılmasını engelleyerek kompleks IV'ün inhibisyonunu yapmakta ve proton gradiyentinin azalmasına neden olmaktadır. Elde edilen sonuçlar, İZO'nun gerçekleştirdiği ETZ inhibisyonunun mitokondriyal disfonksiyona neden olabileceğini göstermektedir.*
Anahtar Kelimeler: *Antikanser, crabtree etkisi, elektron transfer zinciri, izoimperatorin, mitokondriyal disfonksiyon*

INTRODUCTION

Cancer is a global public health challenge. It ranks second in death cases after cardiovascular disorders in nations with high life quality, which means that cancer is an obstacle to increasing human life expectancy [1]. One of the most common cancer treatments is chemotherapy. Increasing cancer incidence and mortality has encouraged researchers to focus on novel strategies. Even though drug development studies for cancer treatment have gradually increased recently, there is no precise and successful treatment for all types of cancer. Original molecules and novel molecular pathways must be studied to cope with cancer treatment challenges such as resistance or drug-induced adverse effects. Taken together, novel mechanisms for marketed chemicals showing anticancer activity must also be defined *in vitro*, as new targets might contribute to developing more effective and safer anticancer molecules [2,3].

Mitochondria maintain many critical cellular processes. It plays a central role in energy homeostasis in normal cells, producing more than 90% of adenosine triphosphate (ATP). Nevertheless, cancer cells produce approximately 50-60% of adenosine triphosphate (ATP) in the cytosol with increased glucose uptake and lactate production, called Crabtree or Warburg effects [4]. *In vitro* anticancer studies are performed with immortalized cancer cells using high glucose-conditioned media to illuminate new pathways. However, mechanistic studies investigating the role of mitochondria cannot reflect reliable results in cancer cells cultured in high glucose-conditioned media due to the Crabtree

effect, as the mitochondria are not sufficiently active for energy homeostasis in cancer cells in high glucose-conditioned media. Marroquin et al. (2007) proposed a model of HepG2 cells by replacing glucose with galactose in cell culture media. This model forces HepG2 cells to produce ATP by shifting from the cytosol to mitochondria, consequently making HepG2 cells vulnerable to mitochondrial toxicity [5]. Several studies adopted this model to investigate mitochondrial dysfunction in several cell types [6-9]. When taking into account that mitochondria play an essential role in biomass synthesis, including fatty acids, amino acids, and nucleotides, which are required for the growth and proliferation of cancer cells, it is noteworthy for scientists to investigate the interactions of anticancer molecules with possible regions or structures in mitochondria by using HepG2 cells vulnerable to mitochondrial toxicity [8-10].

Phytochemicals and their synthetic derivatives have been increasingly used to treat and manage diseases. Several phytochemicals have been identified and characterized to date in order to suppress cancer progression and development [11,12]. Isoimperatorin (ISO) is a secondary plant metabolite belonging to natural furanocoumarins. It is one of the most abundant compounds in the Apiaceae family, which includes Angelica, Notopterygium, Peucedanum, Ferula, Ferulago, and Prangos species [13-15].

Previous studies reported that ISO shows various pharmacological activities, including antitumor, anti-inflammatory, antibacterial, anti-hypertensive, analgesic, and antiviral properties. ISO has been commonly used in traditional Chinese medicine (TDC) due to its various biological activities [16-19]. ISO was reported to have anticancer activities *in vitro* [20,21]. However, the mitochondrial pathway of ISO-induced anticancer activity using human hepatocellular carcinoma (HepG2) cells vulnerable to mitochondrial toxicants in different conditioned media remained ambiguous. Therefore, the main goal of the present study is twofold; i) to assess the role of mitochondria in HepG2 cells exposed to ISO in either glucose- or galactose-conditioned media ii) to predict the possible targets for ISO in mitochondria by molecular docking studies.

MATERIAL AND METHOD

Materials and Cell Culture

All chemicals and compounds were purchased from Sigma-Aldrich (Darmstadt, Germany) except for cell culture reagents. All cell culture reagents and supplements were obtained from Thermo-Fisher Scientific (Loughborough, UK). HepG2 cells were cultured under high glucose and galactose-conditioned media, as described previously [5].

Isolation and Characterization of Isoimperatorin

ISO was isolated and identified from endemic *Prangos heyniae* H.Duman & M.F.Watson in our previous studies [22,23]. The plant roots were collected from Konya province (1580 m altitude) in Türkiye on 3 June 2016. The collected plant was authenticated by authors and deposited in Ege University. Air-dried roots of the plant (399g) were extracted with *n*-hexane (3×3l), chloroform (3×3l), and methanol (3×3l), sequentially in a sonicator (Bandelin-Sonorex RK-514-BH, Germany) at room temperature. After filtration and evaporation (Heidolph Laborota-4000, Germany) of three extracts, *n*-hexane (25g), chloroform (9g), and methanol extracts were yielded. Chloroform extract was suspended in MeOH (250 ml) at 4°C for 24 h in oreder to precipitate the compounds. The mix was filtered and separated into two fractions. The precipitated portion (630mg) was applied to a silica gel column (50g; column size, 3id×900 mm) with an isocratic elution of n-hexane/ethyl acetate (7:3, 300 ml). The compound (200mg) was purified and yielded after this process, and its structure was elucidated as ISO by 1D NMR and LC-MS [22].

Cell Viability Assay

The cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in high glucose or galactose conditioned media as described previous studies with minor modifications [5,24]. In brief, HepG2 cells (10^4 cells/well) were exposed to ISO (12.5, 25, 50, 100, and 250 μM) for 24 h at 37˚C with 5% CO_2. Final Dimethyl sulfoxide (DMSO, solvent control) and Triton X-100 (positive control) concentrations were 1%. After treatment, MTT was added for each

well, and the plate was incubated for 4 h. At the end of the incubation, formazan crystals were dissolved with DMSO, and the colour of intensity was measured by a multi-plate reader as described in our previous study [24].

Membrane Integrity Assay

Lactate dehydrogenase (LDH) enzyme exists in cytosol of mammary cells. LDH enzyme releases from the cytosol into the medium when the cell membrane is damaged. Measurement of the extracellular LDH enzyme activity is used to detect membrane damage, which means that increased LDH enzyme activity is proportional to membrane disruption [25].

Membrane damage was investigated by LDH leakage assay in HepG2 cells in high glucose or galactose-conditioned media as described previous studies with minor modifications [5,26]. In brief, HepG2 cells (10^4 cells/well) were exposed to ISO (12.5, 25, 50, 100, and 250 μM) 24 h at 37˚C with 5% CO_2. Final DMSO (solvent control) and Triton X-100 (positive control) concentrations were 1%. After treatment, LDH activity was determined by diluting media with pH 7.4 phosphate buffer (1:2) at 37 °C. Then, NADH (300 μM, final concentration), and sodium pyruvate (770 μM, final concentration) were added to the media. Absorbances of the media were measured by a multi-plate reader at 340 nm for 4 minutes as described in previous study [26].

Molecular Docking Study

Molecular docking studies were conducted using the MOE 2020 (Molecular Operating Environment 2020) program to determine the interactions of ISO with electron transfer system enzyme complexes. The crystal structures of Complex I (PDB ID: 5XTD) (Guo et al., 2017), Complex II (PDB ID: 8GS8) (Du et al., 2023), Complex III (PDB ID: 5XTE) (Guo et al., 2017), and Complex IV (PDB ID: 5Z62) (Zong et al., 2018) were obtained from the RCSB Protein Data Bank (www.rcsb.org). However, the human crystal structure of Complex V could not be used as it has not yet been defined. To prepare the enzymes for docking, they were energy-minimized, water molecules and ligands used for crystallization were removed, and the surfaces of the complexes were scanned to identify the active sites.

The 2D structure of ISO was drawn in the ChemDraw 19.1 (Perkin Elmer Informatics) program, optimized by MOE, and subjected to energy minimization using the MMFF 94x (Merck Molecular Force Field) package program.

In the docking study, the binding sites used during enzyme crystallization were selected and modeled as the binding sites for ISO. The modeling process was limited to the 30 most stable conformers with different torsion angles for ISO. The obtained data were analyzed based on the Root Mean Square Deviation (RMSD) and binding energy to interpret the results.

Statistics

Data were expressed as the mean ± SD from three experiments in triplicate. GraphPad Prism Software version 8.4.2 (San Diego, CA, USA) for Windows were used for statistical analyses. Statistical comparisons were made using the Mann-Whitney U test with a confidence level of 95%. Accordingly, significance was accepted when $p \leq 0.05$.

RESULT AND DISCUSSION

The basic purpose of anticancer therapy depends on killing cancer cells having uncontrolled and high proliferation rates. One of the most used targets in cancer cells is to inhibit mitochondrial function, as the mitochondria play a key role in maintaining genomic stability and generating building blocks by synthesizing constitutional nucleotides, amino acids, and enzymes [27]. Immortalized cancer cells cultured in glucose-conditioned media make cells to mitochondrial toxicants inconvenient for illuminating novel anticancer mechanisms due to the Crabtree effect [28,29].

In this present study, HepG2 cells were cultured in either high glucose or galactose-conditioned media to overcome the Crabtree effect and investigate the possible role of mitochondria in the anticancer

activity of ISO. Molecular docking studies were also performed to predict the binding affinities of ISO in ETC.

Cell Viability Assay

Previous studies reported that ISO led to anticancer and antiproliferative activities in various types of cancer cells, including lung, ovarian, skin, colon, gastric, breast, and glioblastoma, which indicated a wide range of IC_{50} values ranging between approximately 50 µM and 1.1 mM [10,30,31]. However, only a few studies have mentioned the mechanism of mitochondrial alterations in ISO-induced anticancer activity. Tong et al. (2017) revealed that ISO induces apoptosis in SGC-7901 cells by altering the expression levels of apoptotic proteins [22]; in contrast, another study suggested that ISO improves mitochondrial function and shows a protective effect against carbon tetrachloride-induced liver injury [32]. These mechanistic studies revealed data about mitochondrial activity; however, they estimated the Crabtree effect and did not make the cells vulnerable to mitochondrial toxicants to uncover the mechanism of mitochondrial alterations.

Cytotoxic concentrations of ISO were determined by MTT assay in HepG2 cells cultured in high glucose (Figure 1A) or galactose (Figure 1B) conditioned media. Although ISO did not cause cytotoxicity between the 12,5 and 100 µM range in glucose-conditioned media, 250 µM of ISO moderately reduced cell viability by 33% compared to the control (IC_{50}>250 µM). ISO caused dose-dependent manner cytotoxicity in galactose-conditioned media. 12.5, 25, 50, 100, and 250 µM ISO reduced cell viability approximately by 34, 42, 45, and 55%, respectively, compared to the control (IC_{50}:161 µM). Triton X-100 decreased cell viability by approximately 92% compared to control in both media (Figures 1A and 1B)

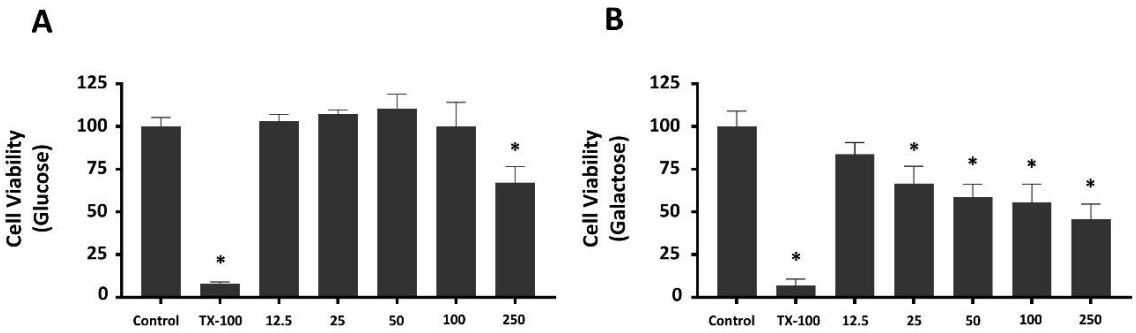

Figure 1. Cytotoxicity of ISO was determined by MTT assay in high glucose (A) or galactose (B) conditioned media in HepG2 cells after 24 hours incubation. Values are mean ± SD from three independent experiments. Experimental data were expressed percent of solvent (1% DMSO) control. (*) significantly different ($p<0.05$) than the solvent control (1% DMSO)

Membrane Integrity Assay

MTT assay indicated that replacing glucose with galactose exacerbated the cytotoxicity of ISO in HepG2 cells (Figures 1A and 1B). Then, an LDH leakage assay was also performed to observe alterations in cell membrane integrity in HepG2 cells exposed to ISO in high glucose (Figure 2A) or galactose (Figure 2B) conditioned media. ISO did not cause any alteration in membrane integrity between the range of 12.5 and 100 µM (Figure 2A); nevertheless, LDH activity statistically increased by 33% at only 250 µM ISO compared to the control in high glucose-conditioned media (IC_{50}>250 µM). Galactose-conditioned media made HepG2 cells vulnerable to ISO-induced membrane damage (Figure 2B). Although 12.5, 25, and 50 µM ISO did not alter LDH activities, 100 and 250 µM ISO caused membrane damage and increased LDH activities by 41% and 69%, respectively (IC_{50}: 195 µM). Triton X-100 increased LDH activity by 42% and 106% in glucose and galactose-conditioned media, respectively.

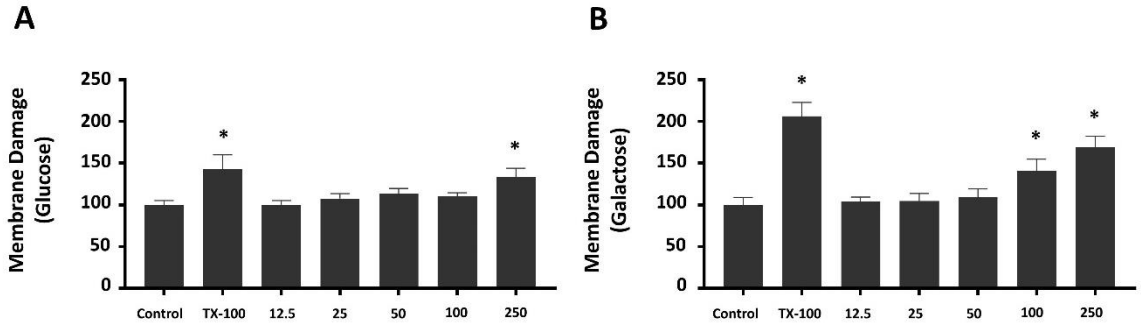

Figure 2. Membrane damage of ISO was determined by LDH leakage assay in high glucose (A) or galactose (B) conditioned media in HepG2 cells after 24 hours incubation. Values are mean ± SD from three independent experiments. Experimental data were expressed percent of solvent (1% DMSO) control. (*) significantly different ($p<0.05$) than the solvent control (1% DMSO)

MTT assay indicated that IC_{50} of glucose conditioned media (>250 μM) is at least 0.55 fold more than IC_{50} of galactose conditioned media (161 μM) (Figures 1). LDH leakage assay revealed that IC_{50} of glucose conditioned media (>250 μM) at least 0.28 fold more than IC_{50} of galactose conditioned media (195 μM) (Figures 2). Lower IC_{50} values calculated from galactose conditioned media mean that HepG2 cells are more prone and vulnerable to cytotoxicity. Given these findings we might conclude that replacing glucose with galactose uncovered the role of mitochondria in ISO-mediated anticancer activity.

Molecular Docking Studies

Mitochondria are double-membrane organelles. The outer membrane transfers ions and molecules of less than 1.5 kDa via anion channels and pores, while the inner membrane is impermeable and allows only tiny molecules via specialized transporters. The inner membrane also contains five protein complexes (I, II, III, IV, and V), which produce ATP and membrane potential required for mitochondrial homeostasis and function. ETC synthesizes constitutional nucleotides, amino acids, and enzymes such as aspartase, pyrimidine, and proline. These building blocks are essential and required for highly proliferative cells such as cancer cells. Inhibition of ETC was reported to contribute to the decrease in cancer cell growth [27,33,34]. Also, MTT and LDH leakage assays proposed that mitochondrial pathways play a primary role in ISO-induced anticancer activity (Figures 1 and 2). For this reason, molecular docking studies were also applied to investigate whether ISO shows affinity to ETC in mitochondria, which is the possible reason for mitochondrial dysfunction (Table 1 and Figure 3).

Table 1. Docking result of ISO with the complexes

Targets	Ligand = ISO			
	Binding energy (kcal/mol)	**RMSD values**	**Binding site amino acids**	**Interactions**
Complex I (5XTD)	-6.9640	1.4263	Phe 64, Gly 63, Asp 205	Aren (π)-H, Ligand exposure
Complex II (8GS8)	-6.5313	1.6468	Asn 81, Arg 512, Leu 513, Gln 516	Aren (π)-H, Aren (π)-cation, H-bond acceptor, H-bond donor, Ligand exposure
Complex III (5XTE)	-6.7658	1.1445	Ala 84, Gly 130, Tyr 131	Ligand exposure
Complex IV (5Z62)	-7.1741	1.2043	Trp 126, Tyr 129, Trp 236, His291, Val 373	Aren (π)-H, Aren (π)- Aren (π), Ligand exposure

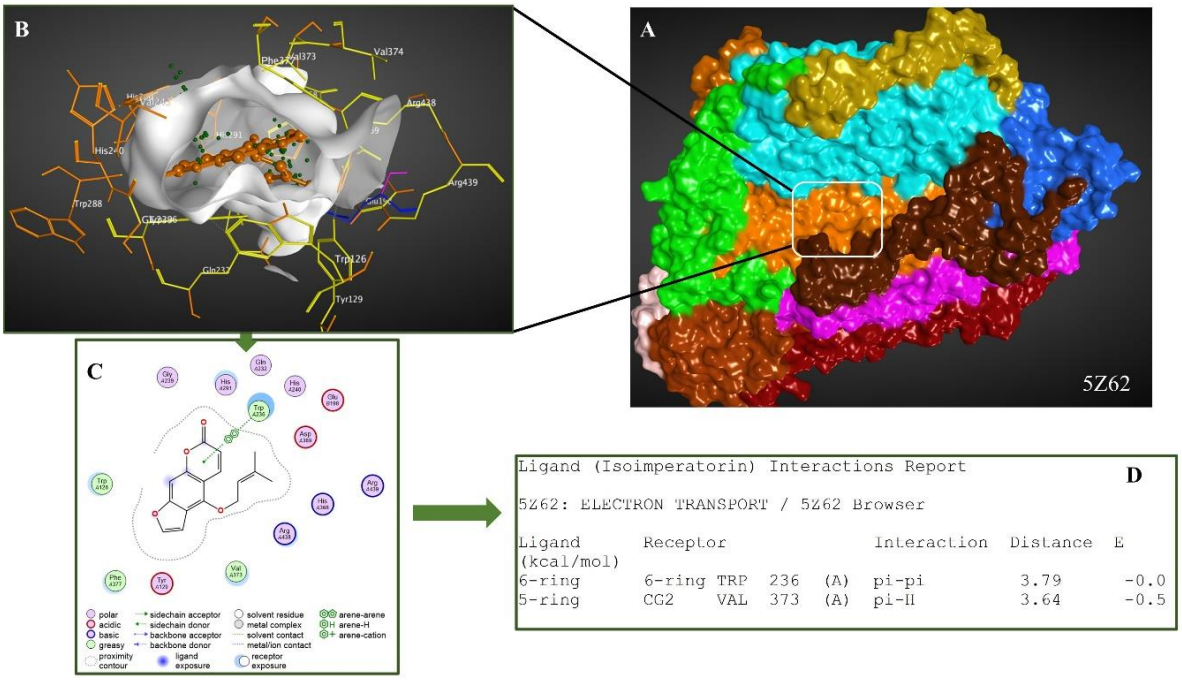

Figure 3. 2D binding pose of ISO with human cytochrome c oxidase (PDB ID: 5Z62) active site. Receptor cite (A), binding site amino acids (B), interactions (C), and ligand interaction report (D)

The binding energy in MOE 2020 shows the bonding relationship between ISO and amino acids in the enzymes. According to docking simulations, binding affinities of ISO to ETC complexes are in descending order: Complex IV > Complex I > Complex III > Complex II. Since the human three-dimensional crystal structure of complex V has not been defined, it could not be used in the docking study. ISO showed better binding with Complex IV (-7.1741 kcal/mol) than other complexes, considering the binding energy and RMSD values. It is observed that hydrophobic amino acids (tryptophan, tyrosine, valine, etc.) play a significant role in the interaction at the receptor site and increase the capacity to bond with unsaturated bonds (Table 1). The amino acids Trp 126, Tyr 129, Trp 236, and Val 373 in the receptor region of the A chain of this protein were essential in the better binding energy of ISO with complex IV (Figure 3). The function of this complex IV is to transfer electrons from cytochrome c to oxygen, creating a proton gradient. Inhibiting the complex IV by ISO might suppress ATP generation by blocking the proton gradient.

The present study assessed the role of mitochondria in ISO-induced anticancer activity in HepG2 cells vulnerable to mitochondrial toxicants. Our data suggest that ISO primarily causes anticancer activities via mitochondrial dysfunction with the highest binding energy to complex IV. Further studies need to be implemented to determine which molecular pathways play a role in ISO-induced mitochondrial dysfunction in HepG2 cells vulnerable to mitochondrial toxicants.

ACKNOWLEDGEMENTS

We gratefully thank Prof. Ayşe NALBANTSOY (Bioengineering Department, Ege University) for the laboratory facilities.

AUTHOR CONTRIBUTIONS

Concept: A.E.; Design: A.E., E.A., F.K.; Control: A.E., E.A., G.A., F.K., H.O., Ş.B.; Sources: A.E., E.A., G.A., F.K., H.O., Ş.B.; Materials: A.E., E.A., G.A., F.K., H.O., Ş.B.; Data Collection and/or Processing: A.E., E.A., G.A., F.K., H.O., Ş.B.; Analysis and/or Interpretation: A.E., E.A., G.A., F.K.,

H.O., Ş.B.; Literature Review: A.E., E.A., G.A., F.K., H.O., Ş.B.; Manuscript Writing: A.E., E.A., G.A., F.K., H.O., Ş.B.; Critical Review: A.E., E.A., G.A., F.K., H.O., Ş.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., 47(3): 845-852, 2023
Doi: 10.33483/jfpau.1315012

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ETHICAL SENSITIVITY: A COMPARATIVE ANALYSIS OF PHARMACY STUDENTS TAKING AND NOT TAKING AN ETHICS COURSE IN TERMS OF VARIOUS VARIABLES

ETİK DUYARLILIK: ÇEŞİTLİ DEĞİŞKENLER AÇISINDAN ETİK DERSİ ALAN VE ALMAYAN ECZACILIK ÖĞRENCİLERİNİN KARŞILAŞTIRMALI ANALİZİ

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ABSTRACT

Objective: *The present study aimed to assess ethical sensitivity in second, third, fourth, and fifth-year pharmacy students who have already completed the ethics course in Erzurum province, Turkey, as well as first-year pharmacy students who have not yet taken the ethics course in terms of various variables.*

Material and Method: *This study adopts a cross-sectional design to compare the ethical sensitivity of two groups of pharmacy students: those who have completed an ethics course and those who have not. This study included 476 out of 840 undergraduate pharmacy students from Erzurum province. Differences in ethical sensitivity based on demographic characteristics were analyzed using t-tests and ANOVA. The level of statistical significance was set at 0.05.*

Result and Discussion: *In the comparison between students who have taken the ethics course and those who have not, a statistically significant difference was found between female and male students who have taken the ethics course (p=0.00), indicating that gender plays a role in ethical sensitivity among students who have received ethics education. However, among students who have not taken the ethics course, no statistically significant difference was found between genders (p=0.13). No statistically significant difference was observed among students in different university classes. In the study assessing professional ethical sensitivity among pharmacy students, it was found that their level of ethical sensitivity was above average. To cultivate a heightened level of professional ethical*

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Submitted / Gönderilme : 15.06.2023
Accepted / Kabul : 20.07.2023
Published / Yayınlanma : 20.09.2023

sensitivity, it is widely believed that pharmacy education should integrate more comprehensive training focused on emphasizing the significance of professional behavior and ethical values.
Keywords: *Ethical sensitivity, pharmacist, pharmacy education*

ÖZ

Amaç: *Bu çalışmanın amacı, Erzurum ilinde etik dersini tamamlamış ikinci, üçüncü, dördüncü ve beşinci sınıf eczacılık öğrencileri ile henüz etik dersini almamış birinci sınıf eczacılık öğrencilerinin çeşitli değişkenler açısından etik duyarlılıklarını değerlendirmektir.*
Gereç ve Yöntem: *Bu çalışma, etik dersi almış ve almamış iki grup eczacılık öğrencisinin etik duyarlılıklarını karşılaştırmak için kesitsel bir tasarım benimsemiştir. Bu çalışmaya Erzurum ilindeki 840 eczacılık lisans öğrencisinden 476'sı gönüllü olarak katılmıştır. Demografik özelliklere göre etik duyarlılıktaki farklılıklar t-testi ve ANOVA kullanılarak analiz edilmiştir. İstatistiksel anlamlılık düzeyi 0.05 olarak belirlenmiştir.*
Sonuç ve Tartışma: *Etik dersi alan ve almayan öğrenciler arasında yapılan karşılaştırmada, etik dersi alan kız ve erkek öğrenciler arasında istatistiksel olarak anlamlı bir fark bulunmuştur (p=0.00), bu da etik eğitimi alan öğrenciler arasında cinsiyetin etik duyarlılık üzerinde rol oynadığını göstermektedir. Ancak etik dersi almayan öğrenciler arasında cinsiyetler arasında istatistiksel olarak anlamlı bir fark bulunmamıştır (p=0,13). Farklı üniversite sınıflarındaki öğrenciler arasında da istatistiksel olarak anlamlı bir fark gözlenmemiştir. Eczacılık öğrencilerinin mesleki etik duyarlılıklarının değerlendirildiği çalışmada, öğrencilerin etik duyarlılık düzeylerinin ortalamanın üzerinde olduğu tespit edilmiştir. Mesleki etik duyarlılığı artırmak için, eczacılık eğitiminin, mesleki davranışın ve etik değerlerin önemini vurgulamaya yönelik daha kapsamlı bir eğitimi içermesi gerektiği düşünülmektedir.*
Anahtar Kelimeler: *Eczacı, eczacılık eğitimi, etik duyarlılık*

INTRODUCTION

Ethical sensitivity is a crucial component of ethical decision making, serving as an individual's disposition to guide them in making ethical choices [1]. Individuals with high ethical sensitivity demonstrate strong ethical judgment. Conversely, low ethical sensitivity can lead to unethical behavior, having negative consequences for both individuals and the workplace [2].

Pharmacists, due to their involvement in patient care, often encounter various ethical dilemmas that require different judgments. These can range from simple issues like selecting the most suitable product for a patient to complex ethical conflicts, such as balancing personal/professional values and legal requirements when faced with a patient's request for a controlled drug without a prescription but in a dire situation [3]. To address these challenges, it has been suggested that pharmacy students should receive comprehensive ethics training, including social, bio, and environmental ethics, both before and after long-term placements, especially in hospital and pharmacy settings [4]. The National Pharmacy Core Education Programme states that pharmacists should demonstrate valid professional and ethical behavior, protect the private life and privacy of patients or beneficiaries, have adequate knowledge of issues related to drugs/medical products, and practice patient-oriented, professional, and ethical practices. They should be able to apply their knowledge in these areas to solve pharmacy problems and possess a sense of professional ethics and responsibility [5]. At our faculty, ethical issues are also taught in the first semester as part of the "History and Deontology of Pharmacy" course. A recent study conducted in Jordan in 2022 provided ethics training to pharmacy students and evaluated their progress using questionnaires and focus group interviews. The findings revealed that students' self-confidence increased after the ethics training, leading to more decisive actions in ethical dilemmas [6]. Numerous studies have emphasized the potential of training programs to enhance ethical sensitivity among healthcare professionals, including pharmacists [7-9]. Very few studies evaluating sensitivity in pharmacy students have been found in the literature [10-11]. No study specifically addressing this topic has been conducted in Turkey. Consequently, to address this gap in the literature, the present study aimed to assess ethical sensitivity in second, third, fourth, and fifth-year pharmacy students who have completed the ethics course in Erzurum province, Turkey, as well as first-year pharmacy students who have not yet taken the ethics course.

MATERIAL AND METHOD

Design

The present study employed a cross-sectional descriptive questionnaire survey to assess the level of professional ethical sensitivity among pharmacy students.

Participants and Data Collection

This study adopts a cross-sectional design to compare the ethical sensitivity of two groups of pharmacy students: those who have completed an ethics course and those who have not. This study included 476 out of 840 undergraduate pharmacy students from Erzurum province. The required sample size for analysis of variance (ANOVA) and t test was determined using G*Power 3.1.9.6 [12], indicating a minimum of 474 participants for an effect size of 0.25, a significance level of 0.05, and 95% power. Thus, our sample size was considered more than adequate. Data were gathered during the middle of the second semester at Erzurum Atatürk University, Faculty of Pharmacy, between April and May 2023. The data collection process involved the distribution of questionnaires via Google Forms to a total of 840 students. Out of these, 477 students successfully completed the questionnaire. One student's questionnaire was excluded, leaving a total of 476 students' responses for data analysis.

Instruments

The questionnaire used in this study was divided into two sections: demographic characteristics and a questionnaire assessing ethical sensitivity.

Demographic Characteristics: The demographic characteristics section collected information on four academic variables: age, gender, university class, and whether the participants were taking an ethics course or not.

Professional Ethical Sensitivity: The measurement of professional ethical sensitivity was conducted using a questionnaire developed by Alaca and Aydınlı Kulak [13]. The questionnaire was designed in accordance with Turkish pharmacy legislation and deontology regulations. Prior to implementation, the questionnaire's reliability and validity were tested among pharmacy students, and approval from the developers was obtained. The questionnaire included a total of 10 items, and participants were asked to rate each item on a Likert scale ranging from 1 to 5 (1: “can be done”; 5: “should not be done”). The validity coefficient of the questionnaire was determined to be 0.868. In the development study, the internal consistency reliability of the 10 scales, measured by Cronbach's alpha coefficient, was found to be 0.87. In this current study, the Cronbach's alpha coefficient was found to be 0.72. Statistical inference in this study utilized a statistical significance level of 0.05 to minimize the likelihood of Type I error.

Data Analyses

The gathered data were subjected to analysis using the SPSS 26.0 software. Before conducting the analysis, the assumption of normality was assessed. and they were considered to meet the assumption of normality. Descriptive statistics, such as percentages, frequencies, ranges (min-max), means and standard deviations (SD) were employed to provide a comprehensive description of the demographic characteristics of the participants. To examine professional ethical sensitivity, mean and standard deviation were calculated on a scale ranging from 1 to 5. Differences in ethical sensitivity based on demographic characteristics were analyzed using t-tests and ANOVA. The level of statistical significance was determined at 0.05.

RESULT AND DISCUSSION

The age range of the participants was between 18 and 32 years, and the average age was calculated to be 22.4 years. Among the participants, 75% were female. In terms of academic classification, 27.1% were first-year students (i.e., students who had not taken an ethics course), 16.2% were second-year students, 17.0% were third-year students, 19.5% were fourth-year students, and 20.2% were fifth-year students (see Table 1).

Table 1. Demographic characteristics of participants (N = 476)

Characteristics	Taking an Ethics Course	Category	N (%) or mean±SD (range)
Age (years)			22.4 +1.7 (18-32)
Gender	Yes	Male	79 (22.8)
		Female	268 (77.2)
	No	Male	40 (41.0)
		Female	89 (69.0)
	Total	Male	119 (25.0)
		Female	357 (75.0)
University Year		First	129 (27.1)
		Second	77 (16.2)
		Third	81 (17.0)
		Fourth	93 (19.5)
		Fifth	96 (20.2)

* SD: standard deviation

The average score for professional ethical sensitivity was found to be 3.77±0.59 for students who had taken the ethics course, while it was 3.16±0.46 for students who had not yet taken it. Statistical analysis indicated that there was a statistically significant difference between the two groups for most items, excluding items 2, 8, and 9. These findings suggest that students who had taken the ethics course generally demonstrated a higher level of professional ethical sensitivity compared to those who had not taken the course (see Table 2 for detailed results).

Table 2. Variances in professional ethics awareness based on the inclusion of an ethics course (N=476)

Items	Taking an Ethic Course	N	Professional Ethics Awareness Mean (SD)	p
1. Pharmacy technicians can suggest drugs to patients.	Yes	347	3.80 (1.09)	.000*
	No	129	2.05 (0.98)	
2. In urgent cases, I can dispense prescription drugs without a prescription.	Yes	347	3.54 (1.21)	.876
	No	129	3.56 (1.15)	
3. A pharmacist's constant presence in the pharmacy is unnecessary.	Yes	347	4.04 (1.17)	.038*
	No	129	3.79 (1.18)	
4. Sharing patient information with demanding companies and individuals (except in legal cases) is frowned upon.	Yes	347	4.32 (1.23)	.000*
	No	129	2.55 (1.33)	
5. I can deliver drugs to patients without consulting a doctor, even if I suspect there is an error in the prescription.	Yes	347	4.48 (1.00)	.000*
	No	129	1.76 (1.24)	
6. It's acceptable for me to recommend more expensive alternative drugs.	Yes	347	3.67 (1.30)	.000*
	No	129	4.42 (1.02)	
7. Promotional activities in the pharmacy are possible in today's competitive work environment.	Yes	347	3.06 (1.24)	.000*
	No	129	3.95 (1.21)	

Table 2 (*continue*). Variances in professional ethics awareness based on the inclusion of an ethics course (N=476)

Items	Taking an Ethic Course	N	Professional Ethics Awareness Mean (SD)	p
8. I can recommend non-medical health products to patients in addition to drug.	Yes	347	3.24 (1.18)	.976
	No	129	3.23 (1.23)	
9. Non-medical product advertisements can be used in the pharmacy.	Yes	347	3.25 (1.35)	.099
	No	129	3.47 (1.24)	
10. Selling non-medical health products on social media is appropriate.	Yes	347	4.29 (1.10)	.000*
	No	129	2.84 (1.28)	
Total	Yes	347	3.77 (0.59)	.000*
	No	129	3.16 (0.46)	

SD: standard deviation, * p <.05

In the comparison between students who have taken the ethics course and those who have not, it was observed that female students (3.86±0.55 and 3.20±0.41, respectively) tend to have a higher level of professional ethical sensitivity compared to male students (3.46±0.61 and 3.07±0.56, respectively). A statistically significant difference was found between female and male students who have taken the ethics course (p=0.00), indicating that gender plays a role in ethical sensitivity among students who have received ethics education. However, among students who have not taken the ethics course, no statistically significant difference was found between genders (p=0.13).

Furthermore, within the group of students who have taken the ethics course, it was found that 3rd-year students exhibited a higher level of professional ethical sensitivity (3.84±0.63). However, no statistically significant difference was observed among students in different university classes, indicating that the impact of university year on ethical sensitivity may be limited (see Table 3 for detailed results).

Table 3. Difference in professional ethics awareness by gender and university year (N=476)

Characteristics	Taking an Ethic Course	Category	Professioanal Ethics Awareness Mean (SD)	p
Gender	Yes	Male	3.46 (0.61)	0.00*
		Female	3.86 (0.55)	
	No	Male	3.07 (0.56)	0.13
		Female	3.20 (0.41)	
University Year	Yes	Second	3.67 (0.54)	0.33
		Third	3.84 (0.63)	
		Fourth	3.79 (0.63)	
		Fifth	3.76 (0.54)	

SD: standard deviation, * p <.05

In a study conducted by Jagger in 2011, it was found that individuals with a low level of ethical sensitivity may experience significant challenges in the development of moral judgment. The research emphasized the negative consequences associated with insufficient ethical sensitivity and its potential impact on an individual's moral decision-making abilities. The study highlights that the primary objective of any ethics course should be to elevate students' ethical sensitivity levels [14]. Contrary to the findings of Kırılmaz et al., who reported no change in the ethical sensitivity of healthcare professionals following ethics training, our study found that ethics training during undergraduate education significantly improved ethical sensitivity levels [15]. This suggests the importance of integrating ethics education into undergraduate programs rather than relying solely on in-service training. The influence of ethics education on ethical sensitivity is emphasized in the study by

Tukamuhabwa et al. [16].

In our study, we observed a significant difference in 7 out of 10 items between students who have already taken ethics courses and those who had not yet taken them. Among the students who took the ethics course, only a small number of items scored below 4 out of 5, indicating a generally high level of professional ethical sensitivity. These items included: pharmacy technicians recommending drugs to patients, delivering prescription drug without a prescription in urgent situations, and recommending more expensive alternative drugs. A study conducted with pharmacy students in Jordan in 2022 involved ethics training and the evaluation of students through questionnaires and focus group interviews before and after the training. Thematic analysis of the data yielded three key themes: the importance of ethics education in the field of pharmacy, the perceived influence of ethics courses on pharmacy practice, and recommendations for enhancing the content of these courses. Following ethics education, students consistently emphasized the necessity and effectiveness of this education [6]. According to the findings of a study conducted by Alaca and Aydınlı Kulak, the majority of participants who received ethics/deontology training expressed positive opinions regarding the importance of ethical rules and the necessity of education. Only a small number of participants held negative opinions. The study concluded that the overall ethical sensitivity of pharmacy students is significantly high. It further supports the inclusion of the deontology course in the curriculum, highlighting its benefits for pharmacy candidates [13].

It was observed that 3rd-year students (3.84±0.63) had a higher level of professional ethical sensitivity compared to other university years. This may be attributed to the fact that they had already completed their first pharmacy internship before entering the 3rd year. However, a decrease in ethical sensitivity levels was observed in the final year (3.76±0.54). A review of ethics education in the final year may be beneficial in improving this situation.

Additionally, this study found that female students tended to have higher levels of professional ethical sensitivity compared to male students. This difference could be attributed to the higher representation of female students in the pharmacy program, as observed in this study. Their larger numbers may contribute to a decreased likelihood of engaging in misconduct.

Overall, this study investigated the professional ethical sensitivity of pharmacy students based on variables such as gender, university year, and taking an ethics course. The results provide evidence-based data supporting the need for implementing ethics education in the pharmacy curriculum.

Two major limitations of this study should be taken into account. Firstly, the study's sample was limited to pharmacy students from Erzurum province in Turkey, which may restrict the generalizability of the findings to pharmacy students in other regions. Secondly, the study relied solely on a two-part self-report questionnaire, which did not allow for a detailed analysis of students' professional ethical sensitivity. Although the questionnaire had face validity, no additional validity questionnaire was administered. Qualitative methods offer the advantage of providing a more in-depth understanding of the various contexts of professional ethical sensitivity. Therefore, future research should consider larger sample sizes and intervention studies aimed at enhancing the level of professional ethical sensitivity. However, by identifying the level of professional ethical sensitivity among pharmacy students and the factors associated with it, this study provides valuable baseline data for the development of programs aimed at improving professional ethical sensitivity.

In the study assessing professional ethical sensitivity among pharmacy students, it was found that their level of ethical sensitivity was above average. To enhance their professional ethical sensitivity, it is important to provide more education on the significance of professional behavior within the classroom and its implications for future situations. Notably, differences in professional ethical sensitivity were observed between students who had taken ethics courses and those who had not. There are studies investigating ethical sensitivity in nursing and medicine [17-19], which are other health disciplines, but there is a limited number of studies in the literature that highlight the importance of ethics education specifically for pharmacy students, particularly in undergraduate programs. In light of this study, it is essential to increase the number of studies examining ethical sensitivity among pharmacy students and evaluating the effectiveness of ethics education in this field. For future research, it is crucial to consider factors that might have been overlooked in the current study, which could influence professional ethical sensitivity. Additionally, conducting comparative research between pharmacy students and students

from other disciplines would be valuable. Additionally, it is recommended to conduct a comparative study to investigate the differences in ethical sensitivity between pharmacy students and licensed pharmacists. Such research would contribute to a deeper understanding of how ethical sensitivity develops and evolves throughout the professional journey, providing valuable insights for educational programs and professional development in the field of pharmacy.

AUTHOR CONTRIBUTIONS

Concept: E.U.D.; Design: E.U.D., R.E.; Control: E.U.D., R.E.; Sources: E.U.D., R.E.; Materials: E.U.D., R.E.; Data Collection and/or Processing: E.U.D., R.E.; Analysis and/or Interpretation: E.U.D.; Literature Review: E.U.D., R.E.; Manuscript Writing: E.U.D., R.E.; Critical Review: E.U.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 study received ethical approval from the Clinical Research Ethics Committee of the Atatürk University Faculty of Medicine on 30 March 2023, with reference number B.30.2.ATA.0.01.00/223.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 853-863, 2023
Doi: 10.33483/jfpau.1253561

ORIGINAL ARTICLE / ÖZGÜN MAKALE

EFFECTS OF DRYING TEMPERATURE ON CURCUMIN AND PIPERINE DISSOLUTION AND THE RELEASE KINETICS OF SOLID DISPERSION-BASED MICROPARTICLES: A PRELIMINARY STUDY

KURUTMA SICAKLIĞININ KURKUMİN VE PİPERİN ÇÖZÜNMESİNE VE KATI DİSPERSİYON BAZLI MİKROPARTİKÜLLERİN SALINIM KİNETİĞİNE ETKİLERİ: BİR ÖN ÇALIŞMA

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ABSTRACT

Objective: *One of the major challenges in developing curcumin as a pharmaceutical agent is its low bioavailability after oral administration. Co-administration of curcumin-piperine combined with employing solid dispersions (SD) approach has been shown to enhance curcumin dissolution and bioavailability. Understanding the influence of the processing temperature during spray drying is crucial in SDs preparations; the purpose of this study is to inquire the effect of inlet temperature spray-dryer on dissolution behavior and the best-fit kinetic model of dissolution itself.*

Material and Method: *The SD powder was prepared using a spray-drying method by varying the inlet temperature (105°C; 115°C; 125°C) and involved polyvinyl alcohol (PVA) as a carrier. The SD were prepared at 30% Curcuma longa and 10% Piper nigrum extracts. Yield (%) of the dried powder resulted from the spray drying process was monitored, and dissolution behavior of curcumin and piperine were analyzed using a dissolution efficiency (DE) value. Furthermore, mathematical model describing the release mechanism of curcumin and piperine from the dissolution were evaluated using a DDSolver software.*

Result and Discussion: *The variation of drying temperature on the spray dryer affects the dissolution behavior and the % yield of the PVA-based SD containing C. longa and P. nigrum extract. The most ideal mathematical model of kinetic release for curcumin and piperine were the Quadratic model, indicating that the mechanism of dissolution is diffusion through a gap between the PVA particle and the surrounding medium.*

Keywords: *Curcuma longa, DDSolver, dissolution, Piper nigrum, solid dispersion*

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Submitted / Gönderilme : 20.02.2023
Accepted / Kabul : 25.07.2023
Published / Yayınlanma : 20.09.2023

ÖZ

Amaç: *Kurkuminin farmasötik bir ajan olarak geliştirilmesindeki en büyük zorluklardan biri, oral uygulamadan sonra düşük biyoyararlanımıdır. Kurkumin-piperin'in katı dispersiyon (KD) yaklaşımı kullanılarak birlikte uygulanmasının kurkumin çözünmesini ve biyoyararlanımını artırdığı gösterilmiştir. Püskürtmeli kurutma sırasında işlem sıcaklığının etkisinin anlaşılması, KD preparatlarında çok önemlidir; bu çalışmanın amacı, püskürtmeli kurutucunun giriş sıcaklığının çözünme davranışı ve çözünmenin en uygun kinetik modeli üzerindeki etkisini araştırmaktır.*

Gereç ve Yöntem: *KD tozu, giriş sıcaklığı değiştirilerek (105°C; 115°C; 125°C) ve taşıyıcı olarak polivinil alkol (PVA) kullanılarak püskürtmeli kurutma yöntemi ile hazırlanmıştır. KD, %30 Curcuma longa ve %10 Piper nigrum ekstraktları kullanılarak hazırlanmıştır. Püskürtmeli kurutma işleminden elde edilen kurutulmuş tozun verimi (%) izlenmiş ve kurkumin ve piperinin çözünme davranışı, çözünme etkinliği (ÇE) değeri kullanılarak analiz edilmiştir. Ayrıca, kurkumin ve piperinin çözünmeden salınım mekanizmasını tanımlayan matematiksel model bir DDSolver yazılımı kullanılarak değerlendirilmiştir.*

Sonuç ve Tartışma: *Püskürtmeli kurutucuda kurutma sıcaklığının değişimi, C. longa ve P. nigrum ekstresi içeren PVA bazlı KD'nin çözünme davranışını ve % verimini etkilemektedir. Kurkumin ve piperin için kinetik salınımın en ideal matematiksel modeli, çözünme mekanizmasının PVA partikülü ile çevreleyen ortam arasındaki bir boşluktan difüzyon olduğunu gösteren Kuadratik modeldir.*

Anahtar Kelimeler: *Curcuma longa, çözünme, DDSolver, katı dispersiyon, Piper nigrum*

INTRODUCTION

Herbal secondary metabolites have proved to be valuable sources that play an essential role in Indonesian traditional medicine for maintaining health and curing various diseases. In the community, herbal preparation is often found as combining two or more plant extracts for more health benefits [1]. One of the natural polyphenols found in the rhizome of *Curcuma longa* Linn is curcuminoids, with curcumin being the most prominent component among the curcuminoids. The polyphenolic compound curcumin plays an essential role as an antioxidant with several pharmacological activities, such as anti-inflammation and anticancer properties. Although curcumin has many beneficial properties, the clinical application of curcuminoids as pharmaceutical agents is limited due to their low bioavailability after oral administration. Curcumin is classified as Biopharmaceutics Classification System (BCS) II which is less soluble in water (11 ng/ml) but has high membrane permeability. The low bioavailability of curcumin is due to its sensitivity to phase II metabolism in the gastrointestinal tract, where it is known to be a substrate for uridine 5′-diphosphate-glucuronosyltransferase (UGT) enzymes, presenting a significant barrier to its development as an active pharmaceutical ingredient [2,3].

One way to overcome low bioavailability is the use of bioenhancer. Piperine, the main active ingredient of P. nigrum, showed effective results in increased bioavailability of curcumin in combination dosage [4,5]. Co-administration of piperine with curcumin (1:100) enhanced serum curcumin concentration by 154% for 1-2 hours after the onset. At the dosages used in the study, piperine appears to increase plasma concentrations, absorption rate, and curcumin bioavailability in rats and human subjects with no adverse effects [3]. Another study discovered that when curcumin was co-administered with piperine, the absorption was increased and remained in the body tissues for significantly longer (maximum 48 hours) [6]. However, piperine is also classified as BCS II because of its poor water solubility (40 mg/L) [4]. In contemplation of overcoming the poor aqueous solubility the SD method of *C. longa* and *P. nigrum* extracts is suggested.

Spray drying is a common SD method for increasing the dissolution rate by evaporating liquid into microscopic droplets. This technique uses atomization in hot water to remove the solvent from the dispersion of the target compound in the matrix solution, resulting in a powder. The final product quality and drying yield of spray-dried product is affected by manufacturing parameters such as feed flow rate, inlet and outlet air temperatures, atomization speed or pressure, feed concentration, etc [7]. Inlet temperature is the primary variable besides the feed flow rate in spray drying that needs to be optimized [8]. Several studies have published the effect of operating parameters and drying conditions on the physical properties of spray-dried powders, such as inlet drying yield, moisture content (MC) [9-12],

and wettability [9,11,12]. Neither report studied the impacts of the spray-drying operating parameters on the dissolution rate of spray-dried powder properties.

Prior research on the bioavailability of curcumin encapsulation in different ratios with varied carriers using SD has been conducted by Hu et al. [13]. Meanwhile, Wang et al. [14] investigated the bioavailability of curcumin SD co-formed with piperine without an excipient/carrier. Meanwhile, other studies, including the investigations by Jumah et al. [15], Kumar and Muzaffar [16], and Fujita et al. [17] have observed the effects of spray-drying method operating parameters on the powder characteristics. Nonetheless, the effect of inlet spray-drying temperature on the functional properties of curcumin piperine encapsulation using PVA has not yet been investigated. Hence, in this study inlet temperatures varied from 105°C to 125°C were applied to dry the PVA-based SD containing *C. longa* and *P. nigrum* extract.

In order to estimate curcumin and piperine absorption, it is also necessary to discover the kinetics and mechanism of dissolution. To determine the mechanism of drug release, various mathematical models for assessing dissolution profiles have been proposed. A theoretical investigation of the process can produce mathematical models of a dissolution profile, but due to the variety of dosage forms and its complexity, no theoretical base exists in most situations. As a result, empirical models must be used to fit dissolution data [18,19]. For that reason, a quantitative assessment of dissolution profile character is required.

DD Solver is a valuable software that involves a non-linear regression approach to perform kinetic analysis of dissolution profile [20]. DDSolver can be used as a predominant tool for monitoring drug product reliability and stability, as well as a quick and low-cost technique for predicting *in vivo* drug absorption. To the best of our knowledge, the application of mathematical models in evaluating the curcumin piperine release from SD is limited. Therefore, in addition to investigating the temperature of the inlet spray-drying on the functional properties of curcumin piperine, this study also presents empirical mathematical models to simulate and predict *in vivo* drug absorption of SD curcumin piperine.

MATERIAL AND METHOD

Material

C. longa extract of 97.56% curcuminoid content was given by PT. Phytochemindo Reksa, Bogor, Indonesia. *P. nigrum* extract was given by Dr.rer.nat Yosi Bayu Murti, Faculty of Pharmacy, Universitas Gadjah Mada Yogyakarta, Indonesia. The piperine standard (Sigma Aldrich) and the validated reversed phase High-Performance Liquid Chromatography (RP-HPLC) method were used to extract and further define the sample (validity recovery 91.14% with >0,999 correlation coefficient) [21]. PVA was donated by PT Konimex Solo, Central Java, Indonesia. Ethanol 96%, methanol, Sodium dihydrogen phosphate, and Sodium Lauryl Sulfate (SLS) were purchased from Merck, Darmstadt, Germany. Dissolution medium consisting of sodium phosphate buffer of pH 6.0 and Milli-Q water were prepared in the laboratory.

Preparation of Spray Dried Curcumin-Piperine

The SD of *C. longa* and *P. nigrum* extract were prepared by spray drying using PVA as a carrier. The SD contained 30% w/w *C. longa* extract; 10% w/w *P. nigrum* extract: 60% w/w PVA. In brief, *C. longa*, and 0.6 mg of *P. nigrum* extracts were diluted in 600 ml ethanol, and 3.6 mg PVA was diluted in 20 ml hot water (80-90°C). The resulting solution was loaded through a two-way nozzle into a BUCHI B-290 mini spray dryer installed with a B-290 dehumidifier under the following operating conditions: feed pump rate of 8%, aspirator rate of 100%, nozzle cleaner of 2%, and inlet temperature varied 105, 115, and 125° (not exceed 180°C) [22,23]. The spray-dried powder obtained was precisely weighed for drying yield calculation (33.50%; 33.76%; 37.01%) and placed in a desiccator for further evaluation.

Dissolution Test

The dissolution test was performed in 900 ml of 0.5 w/v % SLS (0.5 gram of SLS is used to make up a total volume of 100 ml) in a 20 mM phosphate buffer solution with a pH of 6 (USP Apparatus II). The study was conducted for 120 minutes under a stirring speed of 75 rpm (37 ± 0.5ºC). To maintain

the sink condition, at predetermined time intervals, the sample (5.0 ml) was removed and replaced with a fresh dissolution medium at the same temperature.

Curcumin and piperine concentrations in dissolution samples were determined using validated spectrophotometry and Vierordt's method for simultaneous determination of curcumin and piperine (equations 1 and 2) [24]. The compound absorptivity for each wavelength was determined by plotting the absorbance obtained at the respective wavelength into the calibration equation of y = 0.1606x + 0.0045 (curcumin) and y = 0.09x – 0.0088 (piperine) (piperine).
Simultaneous Equation (Vierordt's Method):

$$Cc = \frac{(A2.ap1)-(A1.ap2)}{(ac2.ap1)-(ac1.ap2)} \quad (1)$$

$$Cp = \frac{(A1.ac2)-(A2.ac1)}{(ac2.ap1)-(ac1.ap2)} \quad (2)$$

Cc: Concentration of curcumin
Cp: Concentration of Piperine
A1: Absorbance measured at wavelength 1
A2: Absorbance measured at wavelength 2
Ac1: Curcumin absorbtivity at wavelength 1 in absorbance/(g/100ml)
Ac2: Curcumin absorbtivity at wavelength 2 in absorbance/(g/100ml)
Ap1: Piperine absorbtivity at wavelength 1 in absorbance/(g/100ml)
Ap2: Piperine absorbtivity at wavelength 2 in absorbance/(g/100ml)

The curcumin-piperine concentrations of the samples were measured using a verified method UV spectrophotometer at 430,5 and 344,4 nm. The dissolution data were expressed as a percentage (%) dissolved and dissolution efficiency at 120 min (DE120).

$$DE_t = \int_0^t \left(\frac{Ydt}{Y100t}\right) x100\%)$$

Statistical Analysis

All experiments were experiments were performed in triplicate, and statistical tests were carried out using one-way ANOVA Test for more than two data (obtained to describe the closeness of dissolution profiles). Statistically significant data was accepted if the p-value was less than 0.05. *In-vitro* dissolution data were fitted to the mathematical kinetics model in DD Solver with (1) statistical parameters-based evaluation of the release kinetics model: $R^2_{adjusted}$, Akaike Information Criterion (AIC), and Model Selection Criterion (MSC).

RESULT AND DISCUSSION

Drying Yield

Drying yield is the most important criterion when evaluating the viability of the spray-drying process in SD methodology. Table 1 shows the drying yields for spray-dried curcumin-piperine at different temperature of the inlet. The drying yield was increased along with the increasing temperature, and the highest yield was attained when the inlet temperature reached 125°C. In a study, the authors observed that increasing inlet temperature from 130°C–180°C led to increased process yield of microsized maltodextrin (MDX) (6.75 – 40.25%) [25]. Our findings were similar to this result. Another study reported an increase in the drying yield of waxy rice starch, from 74.83% to 88.66%, when the temperature on inlet spray drier was elevated from 40°C to 80°C. However, because of the sticking problem, the drying yield of waxy rice starch decreased at 100°C, indicating that when the drying temperature exceeds the gelatinization onset temperature, the droplets become drier and stick to the cyclone wall [26,27]. The inlet temperature positively affects the drying yield, with higher inlet air

temperatures increasing the effectiveness of mas and heat transfer processes while reducing the risk of inadequate drying particles hitting and forming crust on the drying chamber wall [28].

Table 1. Drying yield of spray-dried curcumin-piperine at different temperatures

	Formula 1 (105°C)	Formula 2 (115°C)	Formula 3 (125°C)
Curcumin (30%)	2.3995 g	1.8002 g	1.8001 g
Piperine (10%)	0.7998 g	0.6001 g	0.6000 g
PVA (60%)	4.8003 g	3.6001 g	3.6001 g
Total weight	7.9996 g	6.0004 g	6.0002 g
Yield calculation	33.50%	33.76%	37.01%

In-Vitro Dissolution Study

An *in-vitro* dissolution is an essential approach in the pharmaceutical sector for drug development and estimating a drug product's *in vivo* performance as a quality control test. A dissolution study can be used in place of determining bioequivalence (biowaiver). An *in-vitro* dissolution study also can be used to identify pharmaceutical products' long-term stability and shelf life [29].

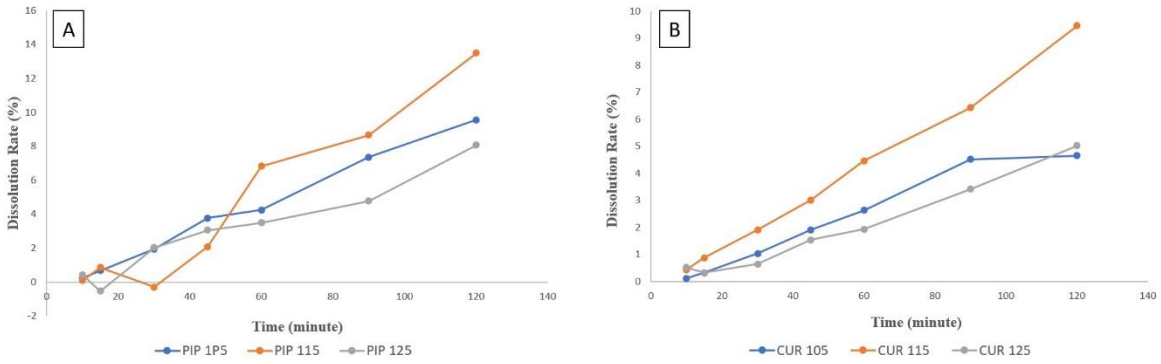

Figure 1. The dissolution rate of Curcumin (A); and Piperine (B) in sodium phosphate buffer (pH 6.0) at temperature 37±0.5°C

The dissolution profiles represented as percentage dissolution rate versus time dilution of SD at various inlet temperatures are illustrated in Figure 1. The percent dissolution value was also calculated as the Area Under the Curve (AUC) and Dissolution Efficiency (DE) values at 120 minutes. Figure 2 depicts the DE120 values of curcumin and piperine which were calculated to compare the dissolution profile. The data from the three formulas were found to be normally distributed by the normality test results, hence the ANOVA test was carried out. The ANOVA test revealed that piperine and curcumin had significantly different dissolution rates amongst the SD formulations obtained ($p>0.05$). The dissolution rate of curcumin and piperine increased from 105°C to 115°C, but then slightly decreased when the temperature of the inlet was raised to 125°C. The slight decreased dissolution rate obtained at 125°C could be explained by the crust formation which prevents water penetrasion during the dissolution study. The crust was formed to due rapid moisture evaporation occurring at 125°C drying temperature [16,30]. Thereby, the crust formation during drying in a spray dryer can be prevented by conducting it at lower temperatures. However, if the temperature of the inlet is defectively lower, the particle will retain moisture for a longer period of time and shrinks resulting in a smaller particle size which can affect the dissolution rate [17]. Shi et al. [10] reported similar dissolution behaviors in the

dried watermelon SD which was processed at varied inlet temperatures from 120°C to 150°C. The particle size decreased from 21.64 nm (120°C drying temperature) to 13.44 nm (140°C drying temperature). Then, the particle size increased to 21.21 nm when the inlet temperature was raised to 150°C, resulting in a lower dissolution rate. Another study by Santhalakshmy et al. [11] obtained similar results, analyzing the production of spray-dried jamun fruit juice powder at 140°C to 160°C. Furthermore, Figure 1 was shown that the highest dissolution rate only reached 13.49% ± 4.07% at 115°C. This is most likely due to the PVA carrier. The dissolution results in the three formulas where the remaining capsules are not dissolved at 120 minutes show that PVA can form a gel layer, making it difficult to dissolve. The formation of the gel layer causes the diffusion layer to thicken, affected in delaying dissolution [31]. Water molecules are prevented from penetrating through the particles due to the surface layer. By decreasing the particle's wettability, the dissolution powder and transfer rate provided an enormous force for evaporation, allowing powders with lower water content to be formed [32,33]. In addition, due to the formation of the gel layer, the PVA carrier also increased the viscosity of a preparation which affected the course of dissolution. Viscosity is inversely proportional to dissolution rate, so as viscosity increases, the dissolution profile in a medium will presumably decrease [34,35].

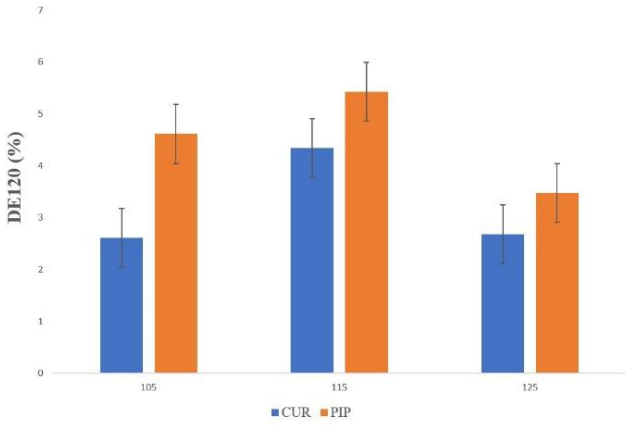

Figure 2. Dissolution Efficiency (DE120) of curcumin and piperine at 120 minutes

Dissolution Study of Curcumin-Piperine Kinetic Release using DDSolver

Mathematical models of kinetics drug release are useful for predicting the exact transport mechanism that affects a drug's *in vivo* dissolution profile [34]. Dissolution profiles of curcumin-piperine in various inlet temperatures were analyzed using drug release kinetic models. The obtained dissolution profiles of curcumin-piperine were fitted onto several mathematical models conducted by a non-linear regression approach using the DDSolver. The DDSolver was used to plot the dissolution profile data, which included the dissolution time (minutes) and the percentage of drug dissolved.

DDSolver offers a few statistical metrics to assess the dissolution model, including adjusted coefficient of determination, correlation coefficient, and coefficient determination. The most well-known and extensively used of these criteria for identifying *in vitro* drug release data modeling are $R^2_{adjusted}$, AIC, and MSC [36]. The $R^2_{adjusted}$ was considered the most acceptable parameter to compare the dissolution models [19]. As shown in Table 2, the result revealed that the prepared curcumin and piperin SD at all temperatures exhibited Quadratic model tendencies, with $R^2_{adjusted}$ values of 0.98427, 0.98787, and 0.95503 for curcumin and 0.97992, 0.96202, and 0.87643 for piperin. However, the results showed a high similarity between models at different temperatures. As a result, other statistical criteria, AIC and MSC were applied using DDSolver.

Table 2. Results of statistical parameters to describe the release of SD curcumin and piperine by each model

SD	Model	Dissolution Model Parameters (mean)					
		$R^2_{adjusted}$		MSC		AIC	
		Curcumin	Piperine	Curcumin	Piperine	Curcumin	Piperine
105	Zero-order	0.95864	0.95839	2.79162	2.91618	2.55799	11.57415
	First-order	0.95912	0.95643	2.79969	2.81064	2.49343	12.41852
	Higuchi	0.79417	0.77688	1.18165	1.11619	15.43776	25.97409
	Hixson-Crowell	0.95899	0.95716	2.79756	2.84498	2.51046	12.14375
	Hopfenberg	0.95235	0.95275	2.55256	2.68150	4.47048	13.45165
	Weibull	0.86903	0.92924	1.50097	3.01295	12.88314	10.80005
	Quadratic	0.98427	0.97992	2.64386	4.03150	3.74001	2.65158
	Korsmeyer-Peppas	0.84935	0.91540	1.53594	2.24456	12.60343	16.94712
	Gompertz	0.96055	0.96712	2.79172	3.32432	2.55720	8.30906
115	Zero-order	0.95973	0.91568	2.92895	2.07868	3.45045	13.89547
	First-order	0.95625	0.91392	2.83752	2.05761	4.18193	14.06399
	Higuchi	0.73873	0.77688	0.96011	1.11619	19.20115	25.97409
	Hixson-Crowell	0.95743	0.91454	2.86762	2.06493	3.94110	14.00548
	Hopfenberg	0.95302	0.90180	2.67895	1.83021	5.45045	15.88320
	Weibull	0.92859	0.88359	2.26581	1.60994	8.75560	17.64536
	Quadratic	0.98787	0.96202	4.05118	2.95320	5.52737	14.89926
	Korsmeyer-Peppas	0.96224	0.91540	2.96048	2.24456	3.19825	16.94712
	Gompertz	0.92975	0.90005	2.22296	1.81909	9.09839	15.97214
125	Zero-order	0.88825	0.84292	1.88969	1.54995	18.23976	27.37747
	First-order	0.88563	0.83605	1.85928	1.49783	18.48306	27.79443
	Higuchi	0.70210	0.63066	0.82793	0.64051	26.73386	34.65304
	Hixson-Crowell	0.88657	0.83848	1.86973	1.51559	18.39942	27.65239
	Hopfenberg	0.86974	0.81676	1.64046	1.30003	20.23357	29.37687
	Weibull	0.78403	0.73191	1.06917	0.86727	24.80395	32.83897
	Quadratic	0.95503	0.87643	2.50284	2.39663	13.33454	20.60408
	Korsmeyer-Peppas	0.61860	0.80884	0.60494	1.35567	28.51776	28.93170
	Gompertz	0.87629	0.82748	2.43229	1.43095	13.89894	28.32953

The ideal model is the one with the lowest AIC value, while the most accurate model has the highest MSC value. MSC is a modified opposite form of the AIC that has been validated to be independent of the underlying point scaling. The appropriate MSC value is greater than 2 or 3 [19,36]. Thus, the MSC and AIC values for all the kinetic dissolution models of varied temperatures were evaluated. From the kinetic model parameters as depicted in Table 2, the quadratic model is the most appropriate model to explain the phenomenon of curcumin piperine SD dissolution reverse to the result of AIC, MSC, and $R^2_{adjusted}$ value. Curve fitting results also indicated that the quadratic model is the best model for explaining the behavior of curcumin and piperine dissolution profiles at all inlet temperatures (Figure 3).

The Quadratic model is based on this equation $F = 100 (k_1.t^2 + k_2.t)$ [36]. A study by Delfour and Garon explained how a quadratic model could be applied in the case of this study. It was revealed that the quadratic model can be applied not only in time-dependent or nonlinear diffusion but also through a circumstance of the polymer-medium interface [37]. From those findings, the dissolution kinetic of curcumin-piperine from the PVA based microparticle containing *C. longa* and *P. nigrum* extracts in this study can be assumed that the PVA polymer might form tiny holes or cracks at which the drug curcumin-piperine could diffuse through the gap between the PVA particle and the medium.

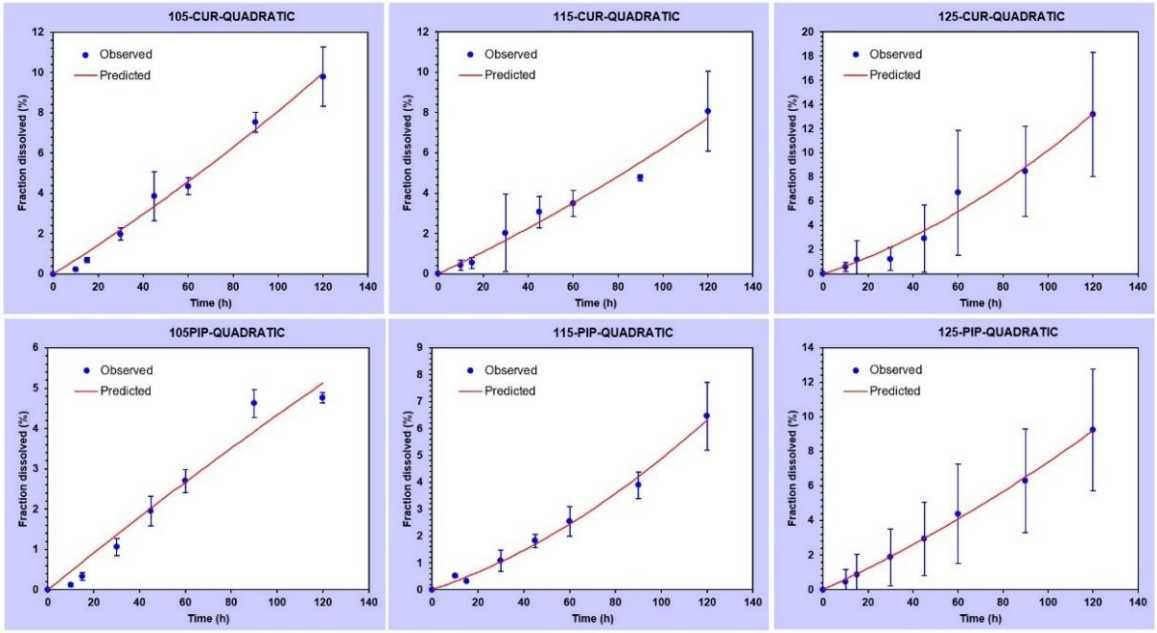

Figure 3. The most precise model prediction of the kinetic release of curcumin (CUR) and piperine (PIP)

Understanding the mechanism of oral drug absorption is important for efficacy and safety. Drug disintegration and dissolution, degradation, stomach emptying, intestinal transit, intestinal permeation and transport, intestinal metabolism, and hepatic metabolism are all possible phases in oral drug absorption. Dosage form, physicochemical and biological properties of the active pharmaceutical ingredient, and gastrointestinal (GI) tract physiology are all factors that may influence the rate and extent of drug absorption [37]. The kinetic modeling dissolution is known to be essential for estimating the absorption process. The absorption process itself is also a major challenge to control and maintain in oral administration. However, dissolution kinetic modeling cannot always illustrate the complex relationship between formulation attributes and oral absorption *in vivo*. The absorption modeling method is also considered necessary to investigate the effect of formulation attributes on oral absorption [19]. A study by Stillhart et al. [38] observed the beneficial effect of combining absorption modeling and in vitro dissolution tests of Basmisanil rather than only dissolution test to identify the rate-limiting processes in oral drug absorption. Therefore, predictive absorption modeling is required for further evaluation in future studies to demonstrate the compatibility between dissolution testing and the absorption process for finding the best strategy of formulation development.

ACKNOWLEDGEMENTS

The research was supported by Sanata Dharma University of Center for Research and Community Services under contract number 007 Penel./LPPM-USD/II/2022.

AUTHOR CONTRIBUTIONS

Concept: D.S.; Design: D.S.; Control: D.S.; Sources: D.S.; Materials: D.S.; Data Collection and/or Processing: M.O.T.D.; Analysis and/or Interpretation: M.O.T.D.; Literature Review: M.O.T.D.; Manuscript Writing: M.O.T.D.; Critical Review: D.S.; 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, 47(3): 864-882, 2023
Doi: 10.33483/jfpau.1309030

ÖZGÜN MAKALE /ORIGINAL ARTICLE

TÜRKİYE'NİN BİYOAKTİF TOHUMLU BİTKİLERİ VERİTABANI

BIOACTIVE SEED PLANTS DATABASE OF TURKEY

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ÖZ

Amaç: *Bugüne kadar, tohumlu bitkilerin kimyasal içerikleri ve biyoaktiviteleri üzerine pek çok çalışma yapılmış olup, halen de yapılmaya devam edilmektedir. Ancak bu çalışmalar, farklı erişim kaynaklarında dağınık halde bulunmaktadır ve bu sebeple bilgiye erişim geç ya da eksik olabilmektedir. Bu nedenle bu çalışmada, Türkiye Florası tohumlu bitkileri üzerinde gerçekleştirilen biyoaktivite araştırmalarını kapsayan uzaktan erişilebilir, Türkçe ve İngilizce bir veritabanı oluşturularak bilgilerin sistematik olarak depolanması amaçlanmıştır.*

Gereç ve Yöntem: *Çalışmanın ilk aşamasında Türkiye Florası tohumlu bitkileri üzerinde yapılmış ve 1928-2018 yılları arasında yayınlanmış biyoaktivite araştırmalarına ulaşılarak, bunlar incelenmiştir ve bir bibliyografya hazırlanmıştır. Çalışmanın ikinci aşamasında ise biyoaktivite çalışmalarının içeriğinde yer alan bilgilerin aktarılabilmesi için web tabanlı veritabanı yönetim sistemi geliştirilerek elde edilen literatürün ve içeriklerinin veritabanına kaydı yapılmıştır. Son olarak da veritabanına kaydedilen içeriklerin analizleri gerçekleştirilmiştir.*

Sonuç ve Tartışma: *Yapılan taramalar sonucunda 1307 literatür bibliyografyaya dahil edilirken, bunların 1088'inin veritabanına kaydı uygun bulunmuş ve toplam 49.486 satır veri girişi yapılmıştır. Veriler detaylı incelendiğinde, 107 familyaya ait 430 cins ve 1594 taksonun biyoaktivite çalışmalarının kayıtlı olduğu görülmüştür. En çok taksonun yer aldığı familyalar ise Lamiaceae (307 takson), Asteraceae (271 takson) ve Fabaceae (125 takson) olarak belirlenmiştir. Veritabanına, 115 farklı aktivitenin kaydı yapılmıştır. En fazla sayıda taksonun; antimikrobiyal aktivite (962 takson), antioksidan aktivite (950 takson), sitotoksik aktivite (220 takson), antienflamatuar aktivite (160 takson) ve analjezik-antinosiseptif aktivite (113 takson) açısından araştırıldığı görülmektedir. En fazla biyoaktivite çalışmalarının yapıldığı türler ise Urtica dioica (18 aktivite), Hypericum perforatum (17 aktivite) ve Cistus laurifolius (16 aktivite) olarak tespit*

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Gönderilme / Submitted : 02.06.2023
Kabul / Accepted : 28.07.2023
Yayınlanma / Published : 20.09.2023

edilmiştir. Lokalite kayıtları incelendiğinde en fazla taksonun İç Anadolu Bölgesinden, en az taksonun ise Güneydoğu Anadolu Bölgesinden temin edildiği saptanmıştır.
Anahtar Kelimeler: *Biyoaktivite, tıbbi bitki, Türkiye Florası, veritabanı*

ABSTRACT

Objective: *To date, many studies have been conducted on the chemical content and bioactivity of seed plants and are still being conducted. However, these studies are scattered in different access sources and therefore access to information may be late or incomplete. Therefore, in this study, we performed literature on seed plants of the Flora of Turkey of the research bioactivity containing information as can be queried remotely accessible, systematic storage of information by creating a database of Turkish and English and is intended to ensure that information.*
Material and Method: *At the first stage of the study, bioactivity studies conducted on seed plants of the Flora of Turkey and published between 1928-2018 were accessed, deciphered and a bibliography was prepared. In the second stage of the study, a web-based database management system was developed in order to transfer the information contained in the content of bioactivity studies, the literature was obtained, and its contents were recorded in the database. Finally, the analysis of the contents recorded in the database was carried out.*
Result and Discussion: *As a result of the scans, 1307 literature was included in the bibliography, while 1088 of them were found suitable for registration in the database, and a total of 49.486 lines of data were entered. When the data were examined in detail, it was seen that bioactivity studies of 430 genera and 1594 taxa belonging to 107 families were recorded. The families with the most taxa were determined as Lamiaceae (307 taxa), Asteraceae (271 taxa) and Fabaceae (125 taxa). 115 different activities were recorded in the database. The largest number of taxa; antimicrobial activity (962 taxa), antioxidant activity (950 taxa), cytotoxic activity (220 taxa), anti-inflammatory activity (160 taxa) and analgesic-antinociceptive activity (113 taxa) are investigated in terms of. The species with the most bioactivity studies were determined as Urtica dioica (18 activities), Hypericum perforatum (17 activities) and Cistus laurifolius (16 activities). When the locality records were examined, it was determined that the most taxa were obtained from the Central Anatolia Region and the least taxa were obtained from the Southeastern Anatolia Region.*
Keywords: *Bioactivity, database, Flora of Turkey, medicinal plant*

GİRİŞ

Dünya Sağlık Örgütü'nün verilerine göre, dünya nüfusunun %80'i hastalıkların tedavisinde bitkilerden yararlanmaktadır. Gelişmekte olan ülkelerde bu oranın daha yüksek olduğu görülmektedir. Amerika Birleşik Devletleri ve Avrupa ülkelerinde nüfusun üçte birinin, bitkileri tıbbi amaçlı kullandığı bilinmektedir [1]. Hiç şüphesiz ki her dönem ve disiplin kendisi için değerli bilgileri kaydetmek, korumak ve kullanmak konusunda çaba harcamıştır. Günümüzde bu çabaya bilgisayarlar, yazılımlar, veri tabanları, dijital görüntüleme ve tarama araçları eşlik etmektedir [2].

Bitkilerin tıbbi amaçla kullanımı antik çağlara dayanmaktadır. Yontmataş (Paleolitik Çağ) devrinden beri (M.Ö. 50.000-7.000 yılları) Anadolu'da yaşamakta olan "Anadolu insanı", çevresindeki bitkileri gıda ve yakacak olarak veya silah ve mesken yapımı için kullanmış, zaman içerisinde bitkilerden ilaç hazırlamayı da öğrenmiştir [3]. Ninova Kitaplığı'nda bulunan ve M.Ö. 3000 yıllarına uzanan tabletlere göre bu dönemde 250 civarı bitkisel drogun varlığı, M.Ö. 2500 dolaylarında İmparator Shen Nung tarafından yazılan "Pen T'Sao" adlı eserde ise 365 bitkisel kökenli ilacın varlığı bilinmektedir. Benzer şekilde Hint kutsal kitapları Veda'larda da bitkilerle yapılan tedavilerden bahsedilmektedir [3-5]. M.Ö. 1550 yıllarına ait olan ve 110 sayfa, 2289 satırdan oluşan Ebers Papirüsü'nün de 700 kadar bitkisel, hayvansal ve mineral kaynaklı drog ile 800'den fazla reçete içerdiği bilinmektedir. Hitit devletinin başkenti Hattuşaş'da bulunan tabletler de Hitit Tıbbı hakkında bilgi vermektedir [3-5]. Theophrast'ın (M.Ö. 371-287) "De Causis Plantarium" ve "De Historia Plantarium" adlı kitapları, Celsus'un (M.Ö. 25- M.S. 50) "De re Medica", Dioscorides'in (M.S. 40-90) "De Materia Medica", İbn-i Sina'nın (M.S. 980-1037) "El-Kanun fi't-Tıb" ve İbn-i Baytar'ın (M.S. 1197- 1248) "Kitab el-Cami'fi el-Adviyye el-Müfredah" adlı eserleri de tedavide yararlanılan tıbbi bitkiler ve droglar hakkında bilgiler içermektedir [4,5].

Anadolu'da ise tıbbi bitkiler üzerinde yapılan araştırmalar 16. yüzyılın ikinci yarısından sonra

başlamıştır. Bunlar arasında P. Belon, P. De Tournefort ve G.A. Olivier'in seyahatnameleri büyük önem taşımaktadır. Bu araştırmacıların Anadolu bitkileri hakkında verdiği bilgiler diğer araştırmacıların dikkatini çekmiştir ve bitki örnekleri toplamak amacıyla Anadolu'ya yapılan bilimsel geziler artmıştır. Osmanlı İmparatorluğu döneminde Anadolu tıbbi bitkileri ile ilgili yapılan yayın ve araştırmalar sınırlıyken Cumhuriyet dönemi ile araştırmalarda artış olmuştur [3]. Özellikle 19. yüzyılın başlarından itibaren, bitkilere ait çeşitli metabolitlerin izolasyonu ile bu alandaki çalışmalar da hız kazanmıştır [4,5].

Dünyada bitki veritabanları 1970'lerin sonlarından beri hazırlanmaktadır. Biyoaktivite çalışmalarına ilişkin bulguların veritabanları çatısında toplanmasına yönelik çalışmalara göz atılacak olursa; bunlardan biri olan NPACT (Naturally Occurring Plant-based Anti-cancer Compound Activity Target Database), antikanser aktivite gösteren bitkisel kaynaklı doğal bileşikleri içeren bir veritabanıdır. Her kayıt için ID numarası, IUPAC kurallarına uygun olarak gösterilen molekül adı, bu moleküllerin yapısı ve özellikleri, hangi kanser türü üzerinde etkili olduğu, kullanılan hücre hatları, inhibitör değerleri (IC_{50}, ED_{50}, EC_{50}, GI_{50}), ticari tedarikçileri ve bileşiklerin ilaç benzerliği gibi bilgiler yer almaktadır. Veritabanı, https://webs.iiitd.edu.in/raghava/npact/ adresi üzerinden hizmet vermektedir [6].

TIPdb (A Database of Taiwan Indigenous Plants) ise, Tayvan'a özgü bitkilerden elde edilen antikanser, antiplatelet ve antitüberküloz fitokimyasallara ait verileri içeren bir veritabanıdır. Veritabanında, ilgili aktivitelerin görüldüğü bitkiler ve bunların taksonomik bilgileri, kullanılan kısımları, kimyasal içerikleri ve bu bileşenlerin 2D ve 3D yapıları, dahil olduğu kimyasal sınıfı gibi bilgiler yer almaktadır. Veritabanına, https://cwtung.kmu.edu.tw/tipdb/ adresi üzerinden erişim sağlanabilmektedir [7].

Doğal ürünlerin antienflamatuar aktivitesinin değerlendirilmesi amacıyla tasarlanan bir veritabanı olan InflamNat (Inflammatory Natural Products Database), fizikokimyasal özelliklerinin, hücre bazlı antienflamatuar biyoaktivitesinin ve eğer mevcutsa moleküler hedeflerinin kayda alındığı doğal bileşikleri içermektedir ve http://www.inflamnat.com/#/main/home adresi üzerinden hizmet vermektedir [8].

InPACdb (Indian Plant Anticancer Compounds Database), Hint Farmakopesinde yer alan ve antikanser fitokimyasallar içeren bitkilere ait verilerin yer aldığı bir veritabanıdır. Bitkinin görünüşü, bitkiye ait bilimsel ve yerel isimlendirme, moleküllerin 3D yapıları ve diğer stereokimyasal özellikleri, kimyasal tanımlamalar, kanser türü, moleküler hedef gibi bilgiler, veritabanında bulunmaktadır [8,9].

Yine antikanser aktivite gösteren bitkilerin, deniz organizmalarının ve mantarların yer aldığı ve halihazırda http://silver.sejong.ac.kr/npcare/ adresinden erişilebilen NPCARE (Natural Products Care) veritabanında; biyolojik kaynağın bilimsel adı, etki gösterdiği kanser türü, antikanser aktivitenin gösterilmesi için kullanılan hücre hatları, PubChem ID ve hedef gen/protein gibi veriler yer almaktadır [8,10].

Antimikobakteriyel fitokimyasallara ait verileri içermekte olan BioPhytMol'de; küresel floraya ait biyolojik kaynaklar ve coğrafi kökenleri, moleküllerin 2D ve 3D yapıları gibi bilgiler yer almaktadır [8,11].

MAPS Veritabanı (Medicinal Plants Activities, Phytochemicals and Structural Database) ise Pakistan'ın tıbbi bitkilerinin yanı sıra farklı literatürde veya veritabanlarında yer alan bilgileri de sunmaktadır. Veritabanında; bitki adı, aktivite, hedef, literatür referansı, kimyasal bileşikler ve yapıları gibi bilgiler yer almaktadır [12].

Türkiye'de ise ilk veritabanı çalışmaları Babaç ve arkadaşları tarafından 1985 yılında başlatılmış olup, "Elazığ Yöresi Leguminosae (Vicieae) Bitkileri Veritabanı" geliştirilmiştir. Bu çalışmayı takiben 1987 yılında "Malatya-Pötürge Yöresi Floristik Veritabanı" ve Elazığ ilinin tıbbi ve endüstriyel bitkilerini içeren bir veritabanı hazırlanmıştır. 1988 yılında ise yine Babaç tarafından "Türkiye Leguminosae Bitkileri Veritabanı" hazırlanmıştır [13].

1995 yılında yine Babaç ve arkadaşları tarafından ilk ulusal bitki veritabanı olan ve 21 veri alanı ile yaklaşık 10.000 takson içeren, "Türkiye Bitkileri Veritabanı" (TÜBVET) ardından da, Türkiye Bilimsel ve Teknolojik Araştırma Kurumu (TÜBİTAK) ve Devlet Planlama Teşkilatı (DPT)'nın da desteği ile 21 üniversitede bulunan 23 herbaryumdaki 80.000 kadar örneğin verilerinin kaydedildiği, "Türkiye Herbaryumları Merkezi Veritabanı" (TÜRKHERB) oluşturulmuştur [13].

2001 yılında, "Türkiye Bitkileri Veri Servisi (TÜBİVES)" geliştirilmiştir ve bugün http://www.tubives.com/ adresinden hizmet vermeye devam etmektedir. TÜBİVES'te; Davis'in

Türkiye ve Doğu Ege Adaları Florası'nda yer alan bitkiler ve bunların taksonomik basamakları, coğrafi dağılımları, habitatları, yerel isimleri, çiçeklenme dönemleri, endemizm durumları, illere göre flora listeleri gibi veriler de sunulmaktadır [13].

2017 yılında tamamlanan ve T.C. Tarım ve Orman Bakanlığı Doğa Koruma ve Milli Parklar Genel Müdürlüğü Biyolojik Çeşitlilik Daire Başkanlığı tarafından yürütülmekte olan "Nuh'un Gemisi Ulusal Biyolojik Çeşitlilik Veri Tabanı", ülkemiz biyolojik çeşitliliğine ait verilerin bir araya getirildiği, izlendiği ve sorgulandığı, ulusal çaptaki en büyük veritabanıdır ve günümüzde http://www.nuhungemisi.gov.tr/ adresinden hizmet vermektedir (T.C. Tarım ve Orman Bakanlığı, 20 23).

Ülkemiz florasının güncellenmesi ve Türkçe olarak hazırlanması kapsamında yayınlanmaya başlanan "Resimli Türkiye Florası"nın da elektronik versiyonu oluşturulmakta ve https://www.turkiyeflorasi.org.tr/ adresinden erişim sağlanabilmektedir (Resimli Türkiye Florası Elektronik Versiyonu, 2022).

Ayrıca "Türkiye Endemik Bitkileri Veritabanı", "Türkiye'nin Sporlu Bitkileri Veritabanı (TURKKIRP)", "Türkiye Labiatae'leri Veritabanı (TULAB)", "Türkiye'nin İletim Demetli Bitkiler Veritabanı (NOMVET)", "Türkiye Tatlı Su Algleri Veritabanı (ALGVET)", "Türkiye Algleri Veritabanı", "Türkiye Etnobotanik Araştırmaları Veritabanı (TEBVET)", "Türk Halk İlaçları Bilgi Bankası (TUHİB)" gibi çeşitli veritabanı projeleri de mevcuttur [2,13].

Bunlara ek olarak ekibimiz tarafından; Türkiye'nin ilk sanal herbaryumu olan "IZEF Herbaryum 1.0" (2002), "Türkiye ve Doğu Ege Adaları Florası"nda kayıtlı taksonları içeren "Türkiye Florası 1.0 (ISBN: 975-97964-3-0)", Türkiye Florası'nda yer alan taksonlara ait lokalite verilerine dayanan Google Earth tabanlı "Türkiye Fitocoğrafya Haritası" (2010), "FFD Monografları Veritabanı (ISBN: 978-975-567-075-1)" (2012), "Türkiye Tıbbi Bitkileri Bibliyografyası Veritabanı" (2012), "Türkiye'nin Etnofarmasötik Botanik Kullanımı Bulunan Bitkileri Veritabanı" (2012), "Türkiye Florası 2.0" (2016), "Prof. Dr. Asuman Baytop İngilizce-Türkçe Botanik Terimler Sözlüğü 1.0" (2016), "Türkiye'nin Gıda Bitkileri Veritabanı" (2016), "Türkiye Uçucu Yağ Bitkileri Veritabanı" (2018), "Türkiye'nin Etnobotanik Veritabanı" (2020) ve "Türkiye'nin Bitki Toksisitesi Veritabanı" (2021) çalışmaları gerçekleştirilmiştir [2,14-17].

Biyoaktif bitkiler ile ilgili bilgiler, potansiyel ilaç hammaddesi araştırmaları açısından oldukça önemlidir. 154 familyaya ait 1220 cins, 9753 tür ve 11.707 takson ile temsil edilen zengin bir floraya sahip olan ülkemizde de bugüne kadar bitkilerin kimyasal içerikleri ve biyoaktiviteleri üzerine pek çok çalışma yapılmıştır ve halen de yapılmaya devam edilmektedir [18-22]. Ancak bu çalışmalar, internet ortamında ya da çeşitli kütüphanelerde dağınık halde bulunmaktadır ve bu sebeple araştırmacıların bilgiye erişimi hızlı olamamaktadır. Bu nedenle çalışmaların içeriklerinin sorgulanabileceği, bilgilerin sistematik olarak depolandığı ve hızlı bir şekilde bilgiye erişilebilen veritabanlarına gereksinim duyulmaktadır.

Günümüzde bilgiye erişim ve bilginin yönetilmesi alanında sayısallaşmanın kaçınılmaz olduğu bir dönüşüm yaşanmaktadır. Tam da bu nedenle Türkiye'nin biyoaktif bitkileri konusunda, klasik veri kaynaklarından farklı olarak uzaktan erişime, sorgulamaya ve yönetime uygun, sayısallaşmış ilk bilgi kaynağının hazırlanması hedeflenmiştir.

Bu çalışma ile Türkiye Florası'nda doğal yayılış gösteren tohumlu bitkiler hakkında, 1928-2018 yılları arasında yapılmış biyoaktivite araştırmalarını kapsayan literatür, lokalite, tespit edilen bileşikler, ekstraksiyon tekniği, saptanan biyoaktiviteler ve bu biyoaktivitelerin araştırma yöntemleri ile araştırmanın özeti gibi bilgileri içeren, çapraz olarak sorgulanabilir, uzaktan erişilebilir, Türkçe ve İngilizce bir veritabanının oluşturulması amaçlanmıştır. Tüm veriler makalenin yayınlanmasını takiben http://izef. ege.edu.tr adresinden ücretsiz olarak kullanıcıların hizmetine sunulacaktır.

GEREÇ VE YÖNTEM

Çalışmanın Modeli ve Kapsamı

"Türkiye'nin Biyoaktif Bitkileri Veritabanı" çalışması içerdiği bilgiler açısından bir derleme olmakla birlikte, doğru bilgiye hızlı ve etkin bir şekilde erişimi hedefleyen bir veritabanı olması sebebiyle özgün bir niteliğe de sahiptir. Çalışmamızda, taramalar sonucu elde ettiğimiz literatüre ek

olarak, veritabanını hazırlamak ve veri girişi için yazılımlar ve bilgisayar kullanılmıştır. Çalışma 3 temel aşamadan oluşmaktadır: Çalışmanın ilk aşamasında, Latin harflerinin kabul edildiği tarih olan 1 Kasım 1928'den, çalışmamızın başladığı 2018 yılına kadar Türkiye Florası tohumlu bitkileri üzerinde yapılmış çalışmalar taranmış ve taramalar sonucunda, belirlediğimiz kabul kriterlerine uygun olan çalışmalar ile "Türkiye'nin Biyoaktif Bitkileri Bibliyografyası" oluşturulmuştur. Sonraki aşamada, literatür içeriğinde yer alan bilgilerin aktarılabilmesi için bir veritabanı yönetim sistemi geliştirilmiştir. Çalışmanın son aşamasında ise literatür verileri ve literatür künye bilgileri veritabanına kaydedilmiştir.

Biyoaktivite Çalışmalarının Taranması

Bu amaçla; "Flora of Turkey and The East Aegean Islands (Türkiye ve Doğu Ege Adaları Florası)" adlı eserin 11. cildinde yer alan "Kimyasal İçerikler (Chemical Contents)" bölümünün kaynakçası ve "Farmakognozi ve Fitoterapi Derneği"nin web sitesinde yer alan "Farmakognozik Yayınlar" bölümü kullanılmıştır. Ayrıca "Web of Science" veritabanında "Turkey, plant, activity, bioactivity" anahtar kelimeleri kullanılarak tarama yapılmıştır. Tüm bu taramalar sonucunda elde edilen literatür, detaylı şekilde incelenerek bibliyografyaya ve veritabanına dahil edilmesi uygun bulunanlar belirlenmiştir.

Ulaşılan Verilerin Tasnif Edilmesi ve Listelenmesi

Türkiye'nin Biyoaktif Bitkileri Bibliyografyasının hazırlanmasında, modifiye edilmiş Amerikan Psikoloji Birliği (APA) 6. sürüm alıntı stili kullanılmıştır. Bibliyografik künyeler, yazarların soyadına göre alfabetik şekilde listelenmiştir. Ulaşılan literatürün bibliyografyaya dahil edilip edilmemesi ile ilgili bazı kriterler belirlenmiştir (Tablo 1).

Tablo 1. Yayınların bibliyografyaya dahil edilme ve edilmeme kriterleri

Dahil Edilme Kriterleri
Latin harflerinin kabul edildiği tarih olan 1 Kasım 1928'den çalışmamızın başlangıç tarihi olan 2018 yılına kadar yayınlamış ve Türkiye Florası tohumlu bitkileri ile gerçekleştirilmiş biyoaktivite çalışmaları.
İngilizce ya da Türkçe dillerinde yayınlanmış çalışmalar ya da Türkçe veya İngilizce özet içeren çalışmalar.
Pek çok literatür bulunması sebebiyle Türkiye Florası tohumlu bitkileriyle gerçekleştirilen Kıbrıs kaynaklı çalışmalar.
Dahil Edilmeme Kriterleri
Türkiye Florası'na ait olmayan bitkiler üzerine yapılan biyoaktivite çalışmaları.
Türkiye Florası bitkisi olmasına rağmen Türkiye Cumhuriyeti sınırları dışından (Kıbrıs hariç) temin edilmiş bitkilerle yapılan biyoaktivite çalışmaları.
Anabilim dalımızda 2018 yılında gerçekleştirilmiş olan ve günümüzde güncelleme çalışmaları devam eden "Türkiye Uçucuyağ Bitkileri Veritabanı" adlı projenin bulunması nedeniyle uçucuyağlar üzerine yapılan biyoaktivite çalışmaları. Tohumlu bitki olmamaları nedeniyle mantar, liken, alg, karayasonları ve eğreltiler ile yapılan biyoaktivite çalışmaları.
Bitkinin yalnızca Türkçe veya yalnızca İngilizce adının yer aldığı çalışmalar.
Bitkinin Latince cins adının belli olmadığı çalışmalar.
Telif hakları açısından etik bulmamamız ayrıca yazarlar ile ilgili yayınevleri ve kurumların yayın haklarına yönelik hassasiyetimiz nedeniyle kitaplar, lisansüstü tezleri ve bitirme tezleri.
Veri tekrarı olmaması amacıyla derleme çalışmalar (bunlara bibliyografyada yer verilmiştir).
Taranan kaynaklardan ulaşılan bildirileri özetleri (bunlara bibliyografyada yer verilmiştir).

Veritabanının Oluşturulması

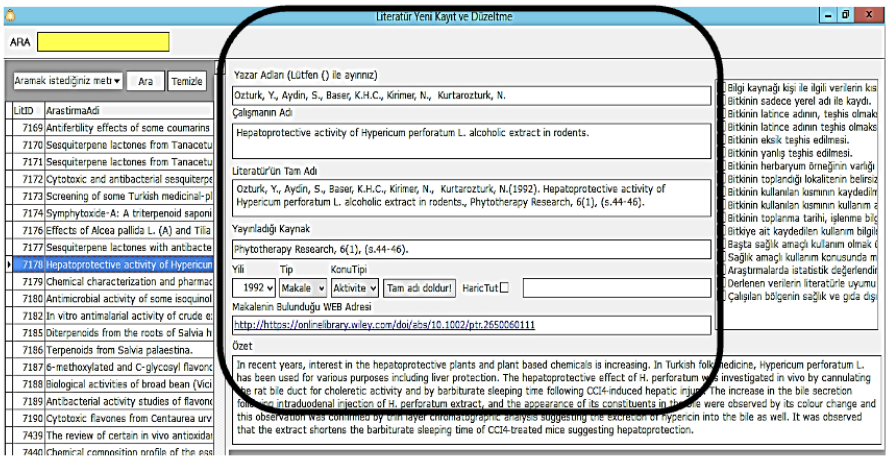
Veritabanının oluşturulmasında önceki yıllarda Öztürk ve Ege tarafından hazırlanmış olan

"Türkiye Florası 2.0" veritabanı temel alınmıştır. İlişkisel veritabanı yönetim sistemine uygun olarak "SQL Server 2014" ile oluşturulan veritabanında, programlama dili olarak "Delphi-Embarcadero RAD Studio 10" kullanılmıştır. Görsel öğeler ve kullanıcı arayüzü "DevExpress Visual Components Library (VCL) for Delphi" kullanılarak hazırlanmıştır. Veritabanına aktarılan bilgilerin internette yayınlanması için ise "Windows Server 2019" kullanılacaktır.

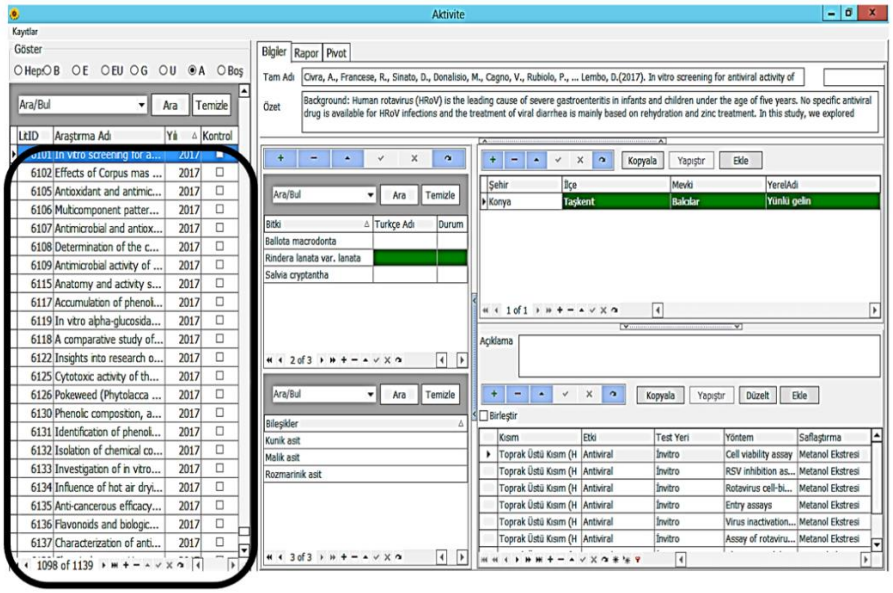
Literatür Girişi

Bibliyografyada yer alan literatüre ait künye bilgileri (yazar adları, çalışma adı, yayınlandığı kaynak, yayın yılı) veritabanına aktarılmış ve her bir literatür için bir kod numarası (ID) verilmiştir. Her literatüre ait "özet" bilgisi ve literatürün yer aldığı web adresi de veritabanına aktarılmıştır (Şekil 1). Bu işlemler sonucunda "Türkiye Sayısal Biyoaktivite Bibliyografyası" oluşturulmuştur (Şekil 2).

Literatür verileri veritabanına aktarılırken öncelikle "Türkiye Sayısal Biyoaktivite Bibliyografyası" bilgi paketinden, verileri girilecek literatür seçilmiştir. Daha sonra sırasıyla bitki adı, lokalite, kimyasal içerik, kullanılan kısım, etki, test yeri, yöntem ve ekstraksiyon bilgi paketleri doldurularak veri aktarımı tamamlanmıştır.



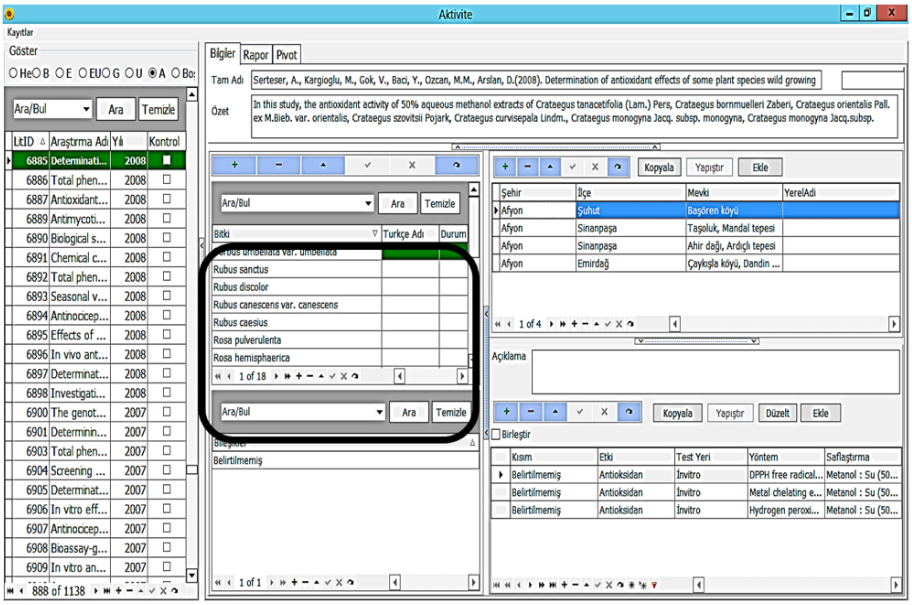
Şekil 1. Literatür bilgilerinin kaydedilmesi



Şekil 2. Türkiye'nin Biyoaktif Bitkileri Bibliyografyasının veritabanına aktarımı

Bitki İsimlerinin Girişi

Girişi yapılacak literatürde yer alan bilimsel bitki isimleri, Türkiye ve Doğu Ege Adaları Florası'nda yer alan bitkilerden oluşan ve önceki veritabanı çalışmalarımızda entegre edilmiş olan listeden seçilmiştir (Şekil 3). Listede bulunamayan bitkilerin, sinonim olma ihtimalleri göz önünde bulundurularak "Türkiye Bitkileri Listesi (Damarlı Bitkiler)" adlı eserden kontrolleri sağlanmıştır [17]. Bu çalışma kapsamında, içeriğinde sadece Latince bilimsel bitki ismi bulunan literatürlerin veritabanına aktarımı gerçekleştirilmiştir. Cins bazında net olarak belirtilmeyen veya sadece İngilizce isimleriyle tanımlanan bitkilerin yer aldığı literatür veritabanına kaydedilmemiştir. Literatürde birden fazla takson söz konusu ise her biri için ayrı giriş yapılmıştır.



Şekil 3. Bitki isimlerinin kaydedilmesi

Lokalitelerin Girişi

Bitki adlarının girilmesinin ardından her bitkinin toplandığı lokalite, Türkiye'nin il ve ilçelerinin kayıtlı olduğu listeden seçilerek girilmiştir. Literatürde lokalite bilgisinin yer almadığı durumlarda, şehir kısmına "belirtilmemiş" olarak giriş yapılmıştır. Literatürde bir bitkinin birden çok lokaliteden toplandığı durumlarda alt satırlar eklenerek tüm lokalitelerin girişleri yapılmıştır (Şekil 4). İl sınırlarında meydana gelen değişimler sebebiyle, bazı literatürlerde belirtilen il-ilçe eşleşmeleri farklılık gösterebilmektedir. Bu durumda lokalite bilgileri girilirken ilçe bilgisi dikkate alınmış ve güncel il bilgilerinin girişi T.C. İçişleri Bakanlığı İller İdaresi Genel Müdürlüğü tarafından hazırlanmış olan "İlçeler Genel Listesi"nden kontrol edilerek yapılmıştır. Bazı literatürlerde bitkilerin Türkiye Cumhuriyeti sınırlarına ek olarak farklı ülkelere ait lokasyonlardan da temin edildiği görülmüştür. Bu durumda sadece ülkemiz sınırlarına ait lokaliteler belirtilmiştir. Araştırma için kullanılan bitki, aktar veya market kanallarından elde edildiyse mevki bölümünde "aktar" veya "market" olarak belirtilmiştir.

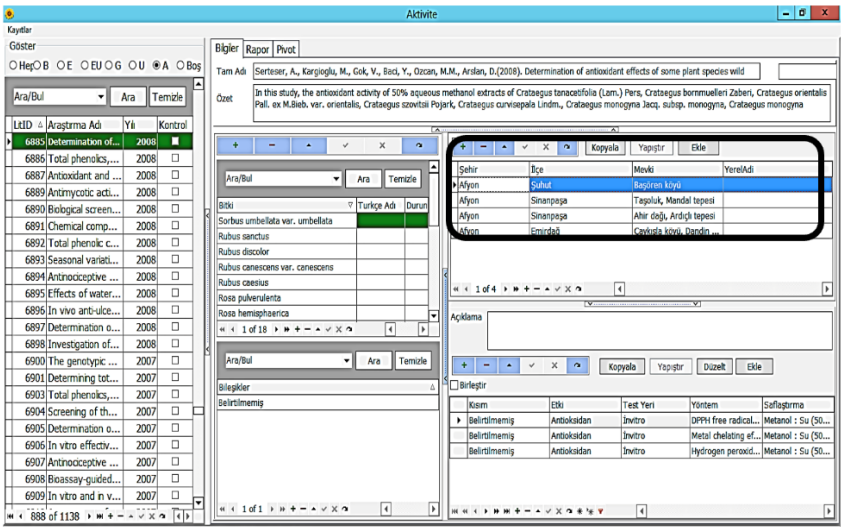
Kimyasal İçerik Girişi

Verileri girilecek literatürde, biyoaktivite çalışmalarının yanı sıra kimyasal içerik analizleri de mevcutsa elde edilen veriler bu bilgi paketine, her takson için ayrı ayrı aktarılmıştır (Şekil 5).

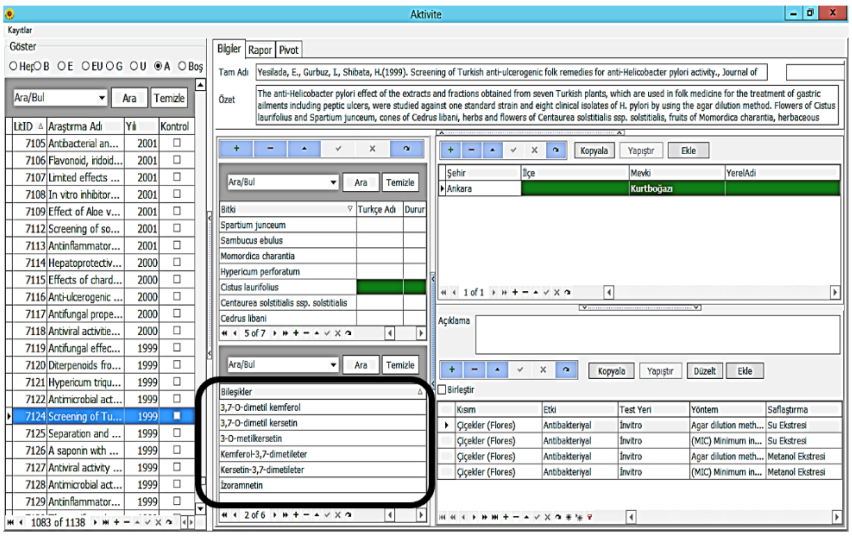
Majör bileşikler belirtilmiş ise ilk 3 tanesi, belirtilmemişse içerik bölümünde yer alan ilk 3 bileşik kaydedilmiştir. Ancak bu kayıtlar bileşik-biyoaktivite ilişkisine dayanmamaktadır.

Literatürde geçen kimyasal içerik ile ilgili veriler, bir başka literatüre dayandırılıyor veya literatürde bu konu hakkında hiçbir veri bulunmuyorsa bu durumda bileşik kısmına "belirtilmemiş" olarak giriş yapılmıştır. Kimyasal içerik ile ilgili veriler aktarılırken bileşikler, hem İngilizce hem de

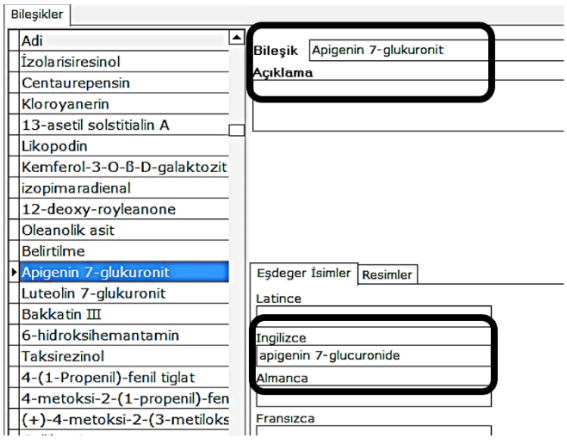
Türkçe olarak kaydedilmiştir (Şekil 6).



Şekil 4. Lokalite bilgilerinin kaydedilmesi



Şekil 5. Kimyasal içeriğin kaydedilmesi



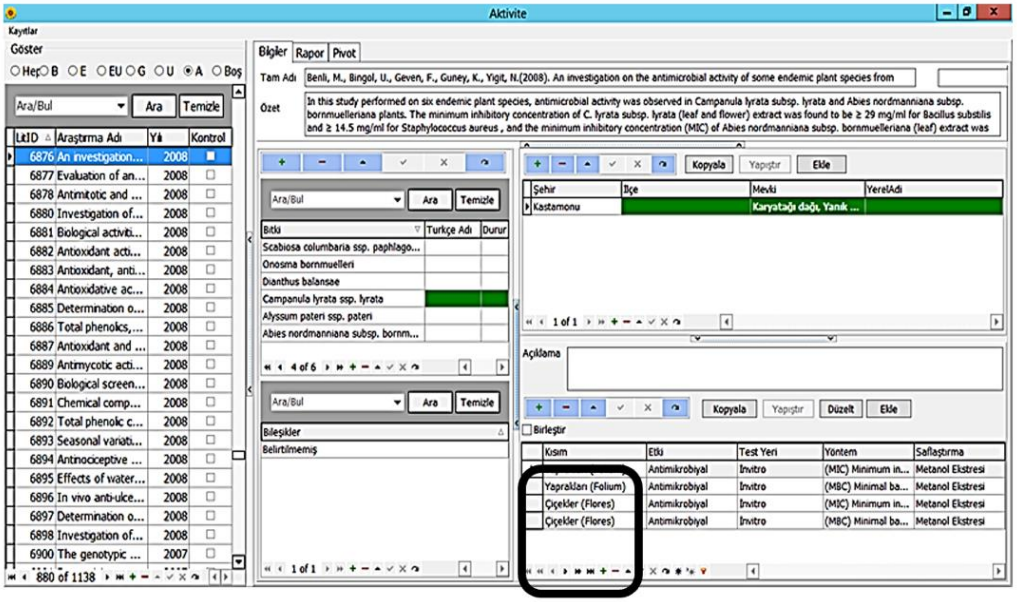
Şekil 6. Kimyasal içeriğin İngilizce ve Türkçe olarak kaydedilmesi

Kullanılan Kısmın Girişi

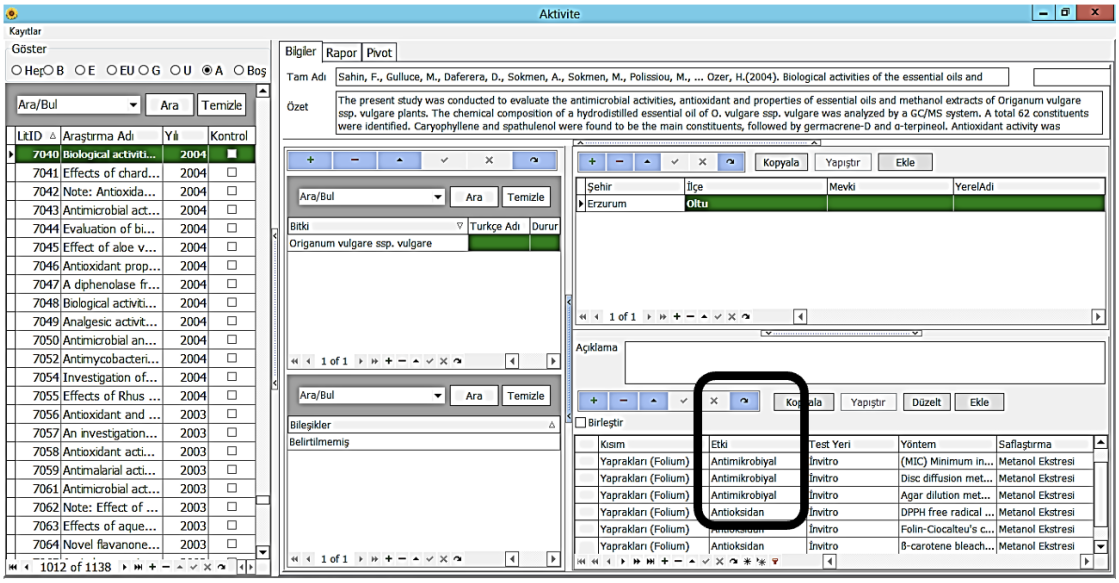
Bitkilerin kullanılan kısımlarının kaydı, bilimsel drog isimlendirme kurallarına göre hazırlanan listeden seçilerek gerçekleştirilmiştir. Ayrıca bu listeye drogların Türkçe karşılıkları da eklenmiştir. Bir bitki için birden fazla kısım kullanılması durumunda, alt satırlar eklenerek tüm kısımların girişi yapılmış, kullanılan kısmın belirtilmemesi durumunda ise kayıt, "belirtilmemiş" olarak gerçekleştirilmiştir (Şekil 7).

Biyoaktivitenin Girişi

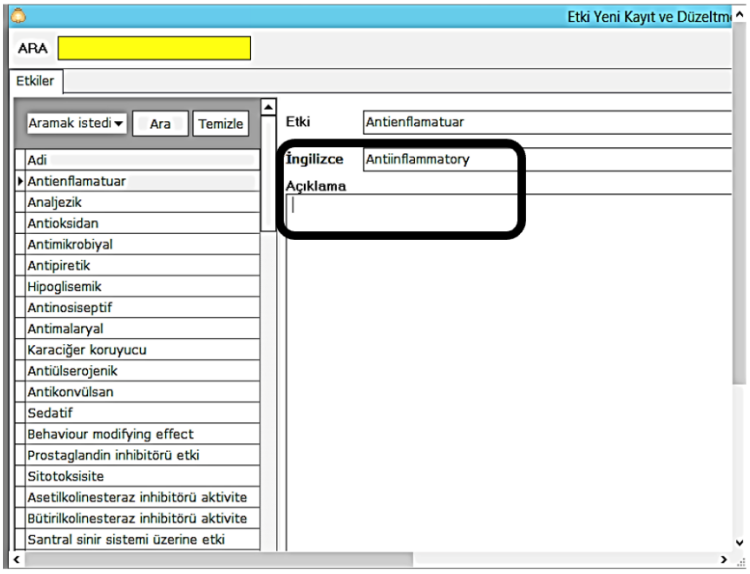
Biyoaktivite verileri, anlamlı bir sonuç elde edilip edilmemesine bakılmaksızın literatürde yer alan her bitkinin, her lokalitesi ve her kısmı için ayrı ayrı girilmiştir (Şekil 8). Biyoaktivite, hem İngilizce hem de Türkçe olarak kaydedilmiştir (Şekil 9). Bu aşamada terimlerin girişi literatürde belirtildiği şekliyle yapılmıştır.



Şekil 7. Kullanılan kısmın kaydedilmesi



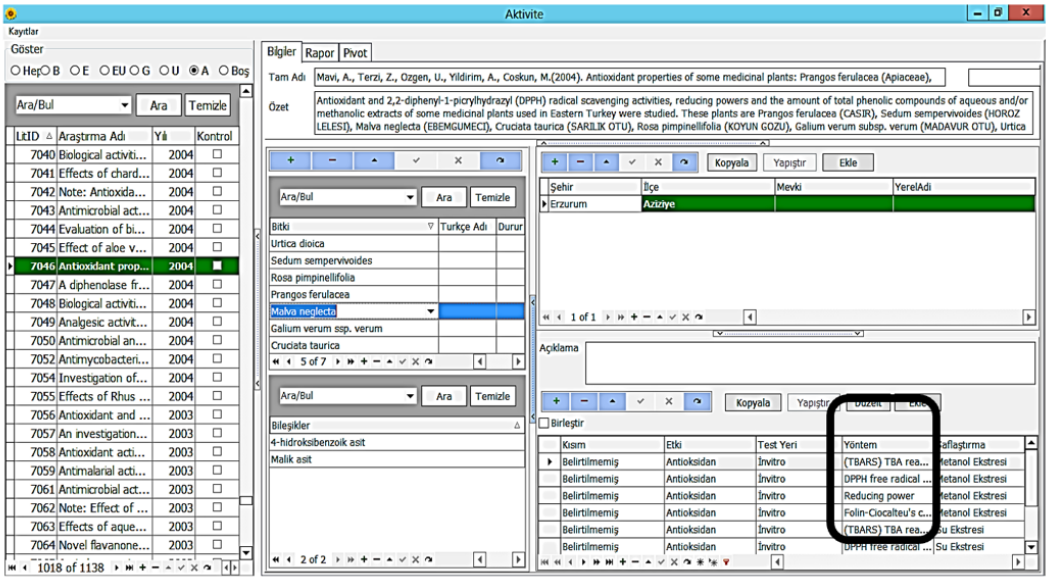
Şekil 8. Biyoaktivitenin kaydedilmesi



Şekil 9. Biyoaktivitenin İngilizce ve Türkçe olarak kaydedilmesi

Biyoaktivite Tarama Yöntemi Girişi

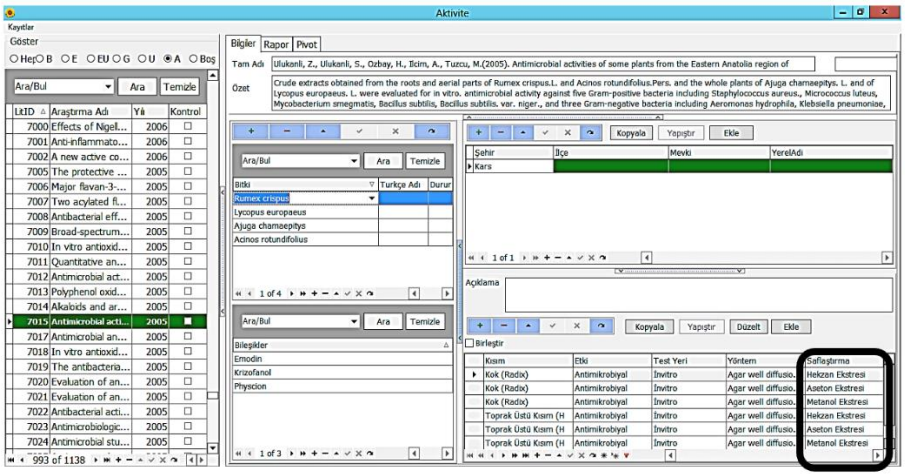
Araştırılan aktivitenin taranması amacıyla kullanılan yöntemler, literatürde yer alan her bitkinin, her lokasyonu ve her kısmı için ayrı ayrı girilmiştir. Bu veriler, orijinalliği ve anlaşılabilirliği bozmamak adına İngilizce olarak kaydedilmiştir (Şekil 10).



Şekil 10. Biyoaktivite tarama yönteminin kaydedilmesi

Ekstraksiyon Solvanının Girişi

Biyoaktivitenin taranması amacıyla bitkilerden elde edilen farklı ekstreler, her bitkinin her lokalitesi ve her kısmı için ayrı ayrı kaydedilmiştir (Şekil 11). Fraksiyonlar ise bu alana dahil edilmemiştir.



Şekil 11. Ekstraksiyon solvanının kaydedilmesi

Test Yeri Girişi

Araştırılan biyoaktivitenin taranması amacıyla kullanılan yöntemler için, test yeri "*in vitro*" veya "*in vivo*" olarak kaydedilmiştir.

SONUÇ VE TARTIŞMA

Çalışmamız kapsamında yapılan literatür taramaları ve daha önce yayınlanmış olan bibliyografyaların gözden geçirilmesi sonucunda 3452 literatüre ulaşılmıştır. Bunlardan 1307'sinin tezimizin kapsamına uygun olması ve dahil edilme kriterlerini sağlaması nedeniyle "Türkiye'nin Biyoaktif Bitkileri Bibliyografyası"na dahil edilmesine karar verilmiştir. Bibliyografyaya dahil edilen 1307 literatürden 1088'inin, veritabanına aktarılması uygun bulunmuştur. Veritabanına aktarılması uygun bulunmasına rağmen ulaşılamadığı için detaylı incelemesi yapılamayan 44 literatür ise ayrı bir liste olarak kayıt altına alınmıştır. Veritabanına aktarılan 1088 çalışmada toplam 49.486 satır veri girişi yapılmıştır.

Veritabanında sorgulama sonucu, bir yayında geçen bir bitkinin hangi kısmında, hangi aktivitenin, hangi yöntemler kullanılarak araştırıldığı bilgisinin elde edilmesi, çalışmanın temel hedefleri arasında yer almaktadır. Bu nedenle, bir yayında bir bitkinin birden çok kullanılan kısmının olması, birden çok aktivitesinin taranmış olması, herhangi bir aktivitenin birden çok tarama yöntemi ile taranmış olması durumunda, veriler ayrı satırlar halinde girilmiştir. Böylece verilerin net bir şekilde ve karışıklığa yol açmadan sunulabilmesi sağlanmıştır.

Kaydedilen verilerin analizi yapıldığında; 107 familyaya ait 430 cins ve 1594 taksonun biyoaktivite çalışmalarının yapıldığı belirlenmiştir. Ülkemizin 7 coğrafi bölgesi için biyoaktivitesi taranan taksonların sayıları incelendiğinde; en fazla taksonun İç Anadolu Bölgesi'nden (386 takson) temin edildiği, bunu Karadeniz Bölgesi (335 takson), Akdeniz Bölgesi (293 takson), Doğu Anadolu Bölgesi (287 takson), Ege Bölgesi (274 takson) ve Marmara Bölgesi'nin (210 takson) takip ettiği, en az taksonun ise Güneydoğu Anadolu Bölgesi'nden (73 takson) temin edildiği saptanmıştır (Şekil 12).

İllere göre dağılım incelendiğinde ise en fazla taksonun sırasıyla Ankara, Antalya, Erzurum, Bolu ve Konya'dan temin edildiği tespit edilmiştir (Şekil 13).

Yine lokalite verilerine bakıldığında 28 literatürde yer alan 41 taksonun kültür alanından, 43 literatürde yer alan 116 taksonun ise aktar veya marketlerden temin edildiği görülmüştür.

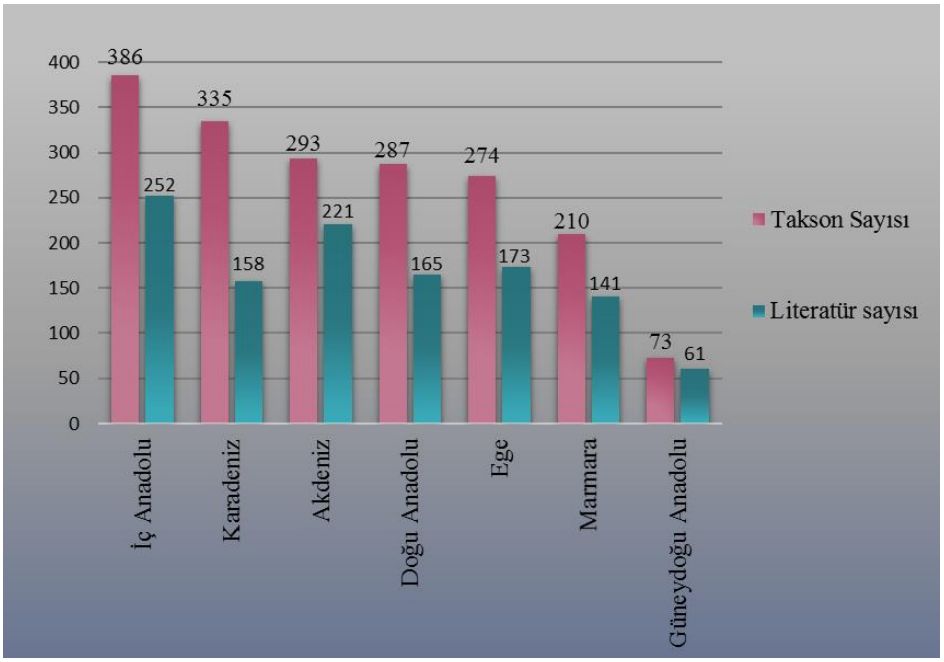
Bitkilerin en sık toprak üstü kısım (905 takson), yaprak (391 takson), toprak altı kısım (278 takson), çiçek (232 takson) ve meyvelerinin (174 takson) kullanıldığı belirlenmiştir.

Kimyasal molekül kayıtları incelendiğinde ise 563 farklı molekülün kaydedildiği görülmüştür.

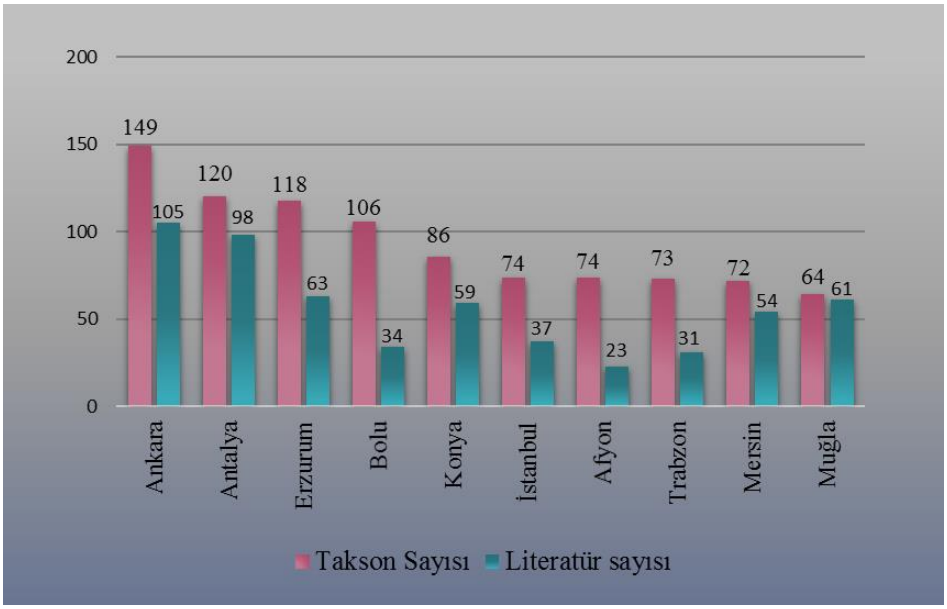
Yöntemlerle ilgili kayıtlara bakıldığında; biyoaktivite çalışmalarında *in vitro* yöntemlerin (903 literatür), *in vivo* yöntemlere göre (230 literatür) daha fazla uygulandığı, klinik çalışmalara ise sınırlı yer verilebildiği görülmüştür.

Kayıtların familyalara göre dağılımlarına bakılacak olursa, en çok çalışılan taksonların yer aldığı familyalar Lamiaceae (307 takson), Asteraceae (271 takson) ve Fabaceae (125 takson) olarak belirlenmiştir. 28 familyada ise sadece birer taksonun çalışıldığı saptanmıştır. Şekil 14'te en sık çalışılan familyalar ve takson sayıları sunulmaktadır (Şekil 14).

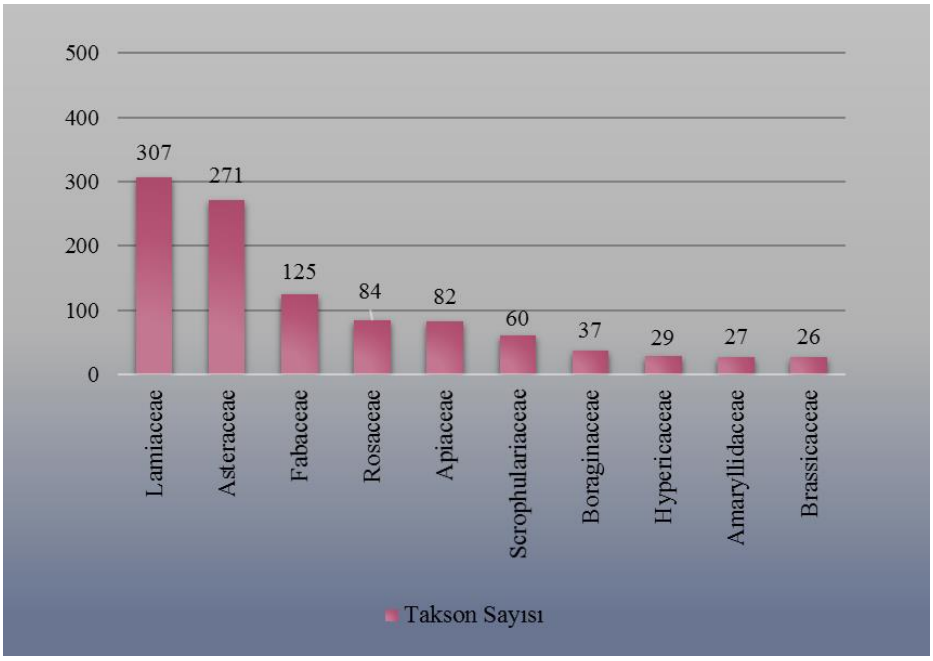
Ekstraksiyon kayıtları incelendiğinde en fazla çalışmanın metanol ekstreleri üzerinde yapıldığı (537 literatür), bunu etanol (267 literatür) ve su (263 literatür) ekstrelerinin izlediği görülmüştür. Araştırmalarda en sık kullanılan ilk 10 solvanın literatür sayısına göre dağılımı Şekil 15'te sunulmaktadır (Şekil 15).



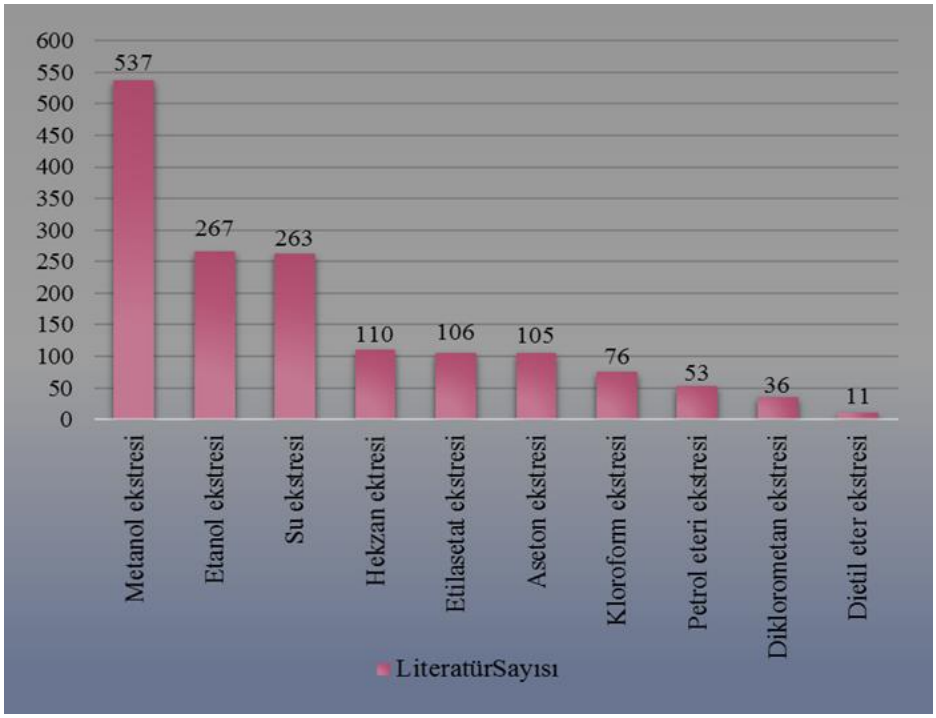
Şekil 12. Bitkilerin toplandıkları coğrafi bölgelere göre takson ve literatür sayısı



Şekil 13. Bitkilerin toplandığı illere göre dağılımı



Şekil 14. En sık çalışılan familyalar ve takson sayıları



Şekil 15. En sık kullanılan ilk 10 solvanın literatür sayısına göre dağılımı

Veritabanına, 115 farklı aktivitenin kaydı yapılmıştır (Tablo 2). Takson sayılarının biyoaktivitelere göre dağılımı incelendiğinde ise en fazla sayıda taksonun antimikrobiyal aktivite (962 takson), antioksidan aktivite (950 takson), sitotoksik aktivite (220 takson), antienflamatuar aktivite (160 takson) ve analjezik-antinosiseptif aktivite (113 takson) açısından araştırıldığı görülmektedir.

Tablo 2. Veri tabanına kaydı yapılan aktiviteler

Aktivite	**Aktivite**
(ACE) Anjiyotensin dönüştürücü enzim inhibisyonu	Apoptotik etki
(hCA) Karbonik anhidraz enzim aktivite	Asetilkolinesteraz inhibitörü aktivite
(LOX) Lipoksijenaz inhibisyonu	Bakterisidal
(MPO) Miyeloperoksidaz aktivite	Behaviour modifying effect
(NO) Nitrik oksit inhibisyonu	Bütirilkolinesteraz inhibitörü aktivite
(PPC) Protein çökeltme kapasitesi	Büyümeyi inhibe edici etki
(PPO) Polifenol oksidaz aktivite	DNA bölünmesi
(TrxR) Tiyoredoksin redüktaz aktivite	DNA hasarına neden olucu etki
α-amilaz inhibitör etki	DNA hasarınından koruyucu etki
Adaptojenik	DNA topoizomeraz I inhibisyonu
α-glukozidaz inhibitör etki	Elastaz inhibisyonu
Aldoz redüktaz (AR) intibitör aktivite	Epileptiform aktivite
Amebisidal aktivite	Fibroblast gelişimini stimüle edici etki
Analjezik	Fungisidal
Anti-(XO) ksantin oksidaz	Gastroprotektif
Antiamnezik	Genotoksisite
Antiartritik etki	Hemaglütinasyon etki
Antibakteriyal	Hemolitik aktivite
Antibiyofilm aktivite	Hipoglisemik
Anti-blood coagulation activity	Hücre agregasyonu inhibisyonu
Antidiyabetik	Hyaluronidaz inhibisyonu
Antienflamatuar	İlaç metabolize edici enzimler üzerine etkiler
Antifeedant	İmmünmodülatör
Antifertilite	İmmünostimulan etki
Antifungal	İnsektisidal
Anti-*Helicobacter pylori* aktivite	Kapiler geçirgenlik
Antihelmentik	Karaciğer koruyucu
Antihemolitik	Kardiyoaktif
Antihepatotoksik	Kemotaktik aktivite
Antihiperglisemik	Koleretik aktivite
Antihiperkolesterolemik	Kolojenaz inhibisyonu
Antihipertansif	Ksantin oksidaz aktivite
Antikandidal	Larvisidal
Antikanser	Mitojenik aktivite
Antikolinerjik aktivite	Mitotik aktivite
Antikolinesteraz	Motor koordinasyon
Antikonvülsan	Nefrolitiyatik etki
Antileishmanial aktivite	Nefrotoksik etki
Antimalaryal	Opioid reseptör bağlama
Antimikobakteriyel	Pankreatik lipaz inhibitör etki
Antimikrobiyal	Repellent etki
Antimitotik	Santral sinir sistemi üzerine etki
Antimutajenik aktivite	Sedatif
Antineoplastik etki	Serotonerjik aktivite
Antinosiseptif	Sitokin biyosentesi üzerine etki
Antioksidan	Sitostatik aktivite
Antipiretik	Sitotoksisite
Antiplatelet	Spazmojenik
Antiplatelet agregasyonu	Tiroid hormonu geliştirici aktivite
Antiproliferatif aktivite	Tirozinaz inhibitör etki
Antiprotozoal	Uterus kasıcı etki
Antispazmodik	Üreaz inhibitör aktivite
Antitripanozomal	Vazodepresör etki
Antitrombotik	Vazodilatasyon
Antitümör	Vazorelaksan
Antiülserojenik	Vücut iyon konsantrayonu üzerine etki
Antiürolitiyatik etki	Yara iyileştirici etki
Antiviral	

Antimikrobiyal aktivite ile ilgili kayıtlar incelendiğinde 394 literatürden veri girişi yapıldığı ve 962 takson üzerinde çalışıldığı görülmektedir. Antimikrobiyal aktivite için en sık kullanılan tarama yöntemleri ise MIC, disk difüzyon, mikrobroth dilüsyon, mikropleyt Alamar mavisi metodu ve agar difüzyon olarak saptanmıştır. Tablo-3'te antimikrobiyal aktivite tarama yötemlerinin takson ve literatür sayısına göre dağılımı gösterilmektedir. Antimikrobiyal aktivitesi çalışılan taksonlar ve familyaları ise bir liste halinde kayıt altına alınmıştır.

Tablo 3. En sık kullanılan antimikrobiyal aktivite tarama yöntemlerinin dağılımı

Yöntem	Takson sayısı	Literatür sayısı
MIC	626	194
Disk difüzyon	451	178
Mikrobroth dilüsyon	253	108
Mikropleyt Alamar mavisi	146	6
Agar difüzyon	143	40

Antioksidan aktivite ile ilgili kayıtlar incelendiğinde 544 literatürden veri girişi yapıldığı ve 950 takson üzerinde çalışıldığı görülmektedir. Antioksidan aktivite için en sık kullanılan tarama yöntemleri ise DPPH yöntemi, metal şelatlayıcı etki, β-karoten ağartma yöntemi, FRAP ve TEAC yöntemi olarak saptanmıştır. Tablo 4'te antioksidan aktivite tarama yötemlerinin takson ve literatür sayısına göre dağılımı gösterilmektedir. Antioksidan aktivitesi çalışılan taksonlar ve familyaları ise bir liste halinde kayıt altına alınmıştır.

Tablo 4. En sık kullanılan antioksidan aktivite tarama yöntemlerinin takson ve literatür sayısına göre dağılımı

Yöntem	Takson sayısı	Literatür sayısı
DPPH	846	402
Metal şelatlayıcı etki	344	115
***β*-karoten ağartma yöntemi**	297	141
FRAP	280	124
TEAC	249	131

Sitotoksik aktivite ile ilgili kayıtlar incelendiğinde 120 literatürden veri girişi yapıldığı ve 220 takson üzerinde çalışıldığı görülmektedir. Sitotoksik aktivite için en sık kullanılan tarama yöntemleri ise MTT metodu, Brine shrimp yöntemi, Alamar mavisi yöntemi, Tripan mavisi yöntemi ve Canlılık testi (Viability) olarak saptanmıştır. Tablo 5'te sitotoksik aktivite tarama yötemlerinin takson ve literatür sayısına göre dağılımı gösterilmektedir. Sitotoksik aktivitesi çalışılan taksonlar ve familyaları ise bir liste halinde kayıt altına alınmıştır.

Tablo 5. En sık kullanılan sitotoksik aktivite tarama yöntemlerinin takson ve literatür sayısına göre dağılımı

Yöntem	Takson sayısı	Literatür sayısı
MTT	79	37
Brine shrimp	43	20
Alamar mavisi	21	4
Tripan mavisi	20	10
Viability	19	2

Antienflamatuar aktivite ile ilgili kayıtlar incelendiğinde 90 literatürden veri girişi yapıldığı ve 160 takson üzerinde çalışıldığı görülmektedir. Antienflamatuar aktivite için en sık kullanılan tarama yöntemleri ise karragen ile oluşturulmuş pençe ödemi, TPA ile oluşturulan kulak ödemi, PGE2 ile oluşturulan pençe ödemi, asetik asitle indüklenen artmış kapiler geçirgenlik ve serotonin ile oluşturulan pençe ödemi olarak saptanmıştır. Tablo 6'da antienflamatuar aktivite tarama yöntemlerinin takson ve literatür sayısına göre dağılımı gösterilmektedir. Antienflamatuar aktivitesi çalışılan taksonlar ve familyaları ise bir liste halinde kayıt altına alınmıştır.

Tablo 6. En sık kullanılan antienflamatuar aktivite tarama yöntemlerinin takson ve literatür sayısına göre dağılımı

Yöntem	Takson sayısı	Literatür sayısı
Karragen ile oluşturulmuş pençe ödemi	114	61
TPA ile oluşturulan kulak ödemi	47	19
PGE_2 ile oluşturulan pençe ödemi	37	13
Asetik asitle indüklenen artmış kapiler geçirgenlik	35	22
Serotonin ile oluşturulan pençe ödemi	20	12

Analjezik ve antinosiseptif aktiviteler ile ilgili kayıtlar incelendiğinde 64 literatürden veri girişi yapıldığı ve 113 takson üzerinde çalışıldığı görülmektedir. Analjezik ve antinosiseptif aktiviteler için en sık kullanılan tarama yöntemleri ise p-benzokinon ile sağlanan karın kasılması, p-benzokinon kaynaklı kıvranma testi, kuyruk hareketliliği yöntemi (tail-flick), asetik asit kaynaklı kıvranma testi ve sıcak plaka (hot plate) yöntemi olarak saptanmıştır. Tablo 7'de analjezik ve antinosiseptif aktivite tarama yöntemlerinin takson ve literatür sayısına göre dağılımı gösterilmektedir. Analjezik ve antinosiseptif aktiviteleri çalışılan taksonlar ve familyaları ise bir liste halinde kayıt altına alınmıştır.

Tablo 7. En sık kullanılan analjezik ve antinosiseptif aktivite tarama yöntemlerinin takson ve literatür sayısına göre dağılımı

Yöntem	Takson sayısı	Literatür sayısı
***p*-benzokinon ile sağlanan karın kasılması**	60	27
***p*-benzokinon kaynaklı kıvranma testi**	32	13
Tail-flick	14	12
Asetik asit kaynaklı kıvranma testi	13	13
Hot plate	9	6

Taksonlar, taranan aktiviteler yönünden incelendiğinde en fazla aktivite çalışmasının *Urtica dioica* (18 aktivite), *Hypericum perforatum* (17 aktivite), *Cistus laurifolius* (16 aktivite), *Plantago lanceolate*a (13 aktivite), *Salvia tomentosa* (13 aktivite), *Taxus baccata* (13 aktivite), *Rumex patientia* (12 aktivite), *Buxus sempervirens* (11 aktivite), *Centaurea depressa* (11 aktivite) ve *Viscum album* subsp. *album* (11 aktivite) üzerinde yapıldığı görülmüştür. İlk 3 tür ile gerçekleştirilen aktivite çalışmaları Tablo 8'de belirtilmiştir.

Veritabanına kaydı yapılan literatürlerin dağılımları incelendiğinde; 2002 yılının öncesinde 179, 2002-2007 yılları arasında 252, 2008-2012 yılları arasında 391 ve 2013-2017 yılları arasında 316 literatür olduğu görülmüştür.

Veritabanının hazırlanması sürecinde pek çok biyoaktivite çalışması detaylı olarak incenlenmiş ve çalışmalara ilişkin bazı sınırlılıklar tespit edilmiştir. Bu sınırlılıklar veritabanına yapılan kayıtları da etkilemiştir.

Tablo 8. En fazla aktivite çalışmasının yapıldığı tespit edilen ilk 3 tür ve gerçekleştirilen çalışmalar

Bitki	**Biyoaktivite**
Urtica dioica	Tiyoredoksin redüktaz (TrxR) aktivite *a*-amilaz inhibitör aktivite *a*-glukozidaz inhibitör aktivite Antibakteriyal aktivite Antifungal aktivite Antihiperkolesterolemik aktivite Antikandidal aktivite Antimikobakteriyel aktivite Antioksidan aktivite Antiviral aktivite Asetilkolinesteraz inhibitör aktivite Bütirilkolinesteraz inhibitor aktivite Genotoksik aktivite İmmünmodülatör aktivite Hepatoprotektif aktivite Kemotaktik aktivite Mitojenik aktivite Sitotoksik aktivite
Hypericum perforatum	Miyeloperoksidaz (MPO) aktivite Antibakteriyal aktivite Antidiyabetik aktivite Antienflamatuar aktivite Antifeedant aktivite Antifungal aktivite Anti-*Helicobacter pylori* aktivite Antimutajenik aktivite Antioksidan aktivite Antiülserojenik aktivite Asetilkolinesteraz inhibitör aktivite Bütirilkolinesteraz inhibitör aktivite Hepatoprotektif aktivite Santral sinir sistemi üzerine etki Sitotoksik aktivite Tirozinaz inhibitor aktivite Yara iyileştirici etki
Cistus laurifolius	Aldoz redüktaz (AR) inhibitör aktivite Analjezik aktivite Antibakteriyal aktivite Kan pıhtılaşmasını engelleyici aktivite Antienflamatuar aktivite Anti-*Helicobacter pylori* aktivite Antimikobakteriyel aktivite Antifungal aktivite Antinosiseptif aktivite Antioksidan aktivite Antiplatelet agregasyonu aktivite Antiülserojenik aktivite Antiviral aktivite Asetilkolinesteraz inhibitör aktivite Bütirilkolinesteraz inhibitör aktivite Hepatoprotektif aktivite

Bilginin doğru aktarılabilmesi ve sonraki araştırmalarda etkili bir kaynak olabilmesi için çalışmalarda bitki teşhisinin tam olarak gerçekleştirilmesi, isimlendirmenin en az tür düzeyinde yapılması, eğer varsa tür altı kategorilerin de mutlaka belirtilmesi gerekmektedir. Ayrıca sadece yerel ismin ya da İngilizce ismin verilmesi de bilgi eksikliğine ve bilgi karışıklığına neden olacağı için bu yaklaşımdan kaçınılmalıdır. Araştırmada botanik bilimi hakkında eğitimli araştırmacıların bulunması da teşhis ve isimlendirme konusunda hata payını en aza indirecektir.

Bitkiler üzerine yapılan araştırmalarda lokalite de oldukça önemli bir bilgi olduğundan bitkinin toplandığı lokalite il, ilçe ve mevki olarak net şekilde belirtilmelidir.

Yine bitkilerin, araştırmalarda kullanılan kısımları da çalışmalarda mutlaka eksiksiz olarak verilmelidir. Biyoaktivite terimlerine bakıldığında aynı ya da benzer aktivitelerin farklı şekillerde ifade edildiği, bazı aktivitelerin de yanlış ifade edildiği görülmüştür. Bu konuda da bir standart oluşturulması araştırmacılar açısından büyük önem taşımaktadır.

Çalışmalarda *in vitro* yöntemler sıkça kullanılırken, *in vivo* yöntemlerin ya da klinik çalışmaların daha az uygulandığı görülmektedir. Elbette *in vitro* çalışmalar yapılmadan, bir üst basamak olan *in vivo* çalışmalara ve sonrasında da klinik çalışmalara geçilemeyecektir. Ancak bu çalışma da açıkça göstermiştir ki bitki biyoaktivite araştırmaları üzerinde gerçekleştirilecek olan *in vivo* ve klinik çalışmaların arttırılması gerekmektedir.

Türkiye'de yürütülmüş bitki biyoaktivite çalışmalarının bulgularının bir veritabanına kaydedilmesinin ve sorgulanabilir hale getirilmesinin, veriye ulaşmak ve verileri yönetmek için kapsamlı, hızlı ve fonksiyonel bir bilgi kaynağı oluşturacak olmasının yanı sıra kayda alınacak verilerin yönetilmesi sonrasında hangi bitki ya da kimyasal bileşiklerin araştırıldığı, hangilerinin ise araştırılmayı beklediği netleşecektir. Tıbbi bitki zenginliğimize ait veriler daha iyi yönetilebilir olacaktır.

Türkiye'nin Biyoaktif Bitkileri Veritabanı, bu konuda şimdiye kadar Türkiye'de hazırlanmış ilk detaylı veritabanı olup araştırılacak bitkiye, lokaliteye, kullanılan kısma, aktiviteye, aktivite tarama ve ekstraksiyon yöntemine göre literatür destekli ve Türkçe-İngilizce olarak sorgulama yapılabilecek şekilde tasarlanmıştır. Hazırlanan veritabanı, ülkemiz bitkileri üzerinde yürütülecek ulusal ve uluslararası biyoaktivite araştırmalarının kapsam ve niteliğine önemli katkılar sağlayacaktır.

Örneğin; literatürde *Lavandula stoechas* L. ekstrelerinin H_2O_2 toksisite modeline karşı koruyucu etkinliğinin değerlendirilmesi üzerine herhangi bir çalışmanın bulunmadığı saptanmış ve bu yönde bir aktivite çalışması ekibimizin bir kısmı tarafından gerçekleştirilmiştir [23]. Bu, veritabanının kullanım potansiyeline ilişkin iyi bir örnek oluşturmaktadır. Konu, bağlantılı yeni projelerin geliştirilmesine açıktır.

Veritabanının, içerdiği bilgilerin basılı olarak yayınlanmasının ve son düzenlemelerin yapılmasının ardından çevrimiçi ortamda araştırmacıların kullanımına sunulması planlanmaktadır.

YAZAR KATKILARI

Kavram: S.Ö.K., M.A.E., B.Ö., G.E.C.; Tasarım: S.Ö.K., M.A.E., B.Ö., G.E.C.; Denetim: M.A.E., B.Ö., G.E.C.; Kaynaklar: S.Ö.K., M.A.E., B.Ö., G.E.C.; Malzemeler: S.Ö.K., M.A.E.; Veri Toplama ve/veya İşleme: S.Ö.K., M.A.E.; Analiz ve/veya Yorumlama: S.Ö.K., M.A.E., B.Ö., G.E.C.; Literatür Taraması: S.Ö.K.; Makalenin Yazılması: S.Ö.K., M.A.E., B.Ö., G.E.C.; Kritik İnceleme: S.Ö.K., M.A.E., B.Ö., G.E.C.; 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, 47(3): 883-893, 2023
Doi: 10.33483/jfpau.1266421

ÖZGÜN MAKALE / ORIGINAL ARTICLE

SALVIA VERTICILLATA L., *ACHILLEA BIEBERSTEINII* AFAN., *TRAGOPOGON AUREUS* BOISS. VE *CEPHALARIA PROCERA* FISCH. & AVÉ-LALL.'NIN HEMOSTATİK PERFORMANSLARININ *İN VİTRO* DEĞERLENDİRİLMESİ

IN VITRO ASSESSMENT OF HEMOSTATIC PERFORMANCES OF SALVIA VERTICILLATA L, ACHILLEA BIEBERSTEINII AFAN., TRAGOPOGON AUREUS BOISS., AND CEPHALARIA PROCERA FISCH. & AVÉ-LALL.

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ÖZ

Amaç: *Hemostaz, kanamayı önlemek veya durdurmak için doğal bir işlev ve doğal bir süreçtir. Günümüzde kanama kontrolü için yeni, ekonomik ve yüksek performanslı ürünler geliştirmek için büyük çaba sarf edilmektedir. Bu çalışmada, halk hekimliğinde farklı amaçlarla kullanılan Salvia verticillata, Achillea biebersteinii, Tragopogon aureus ve Cephalaria procera gibi dört farklı bitki türünün in vitro hemostatik etkilerini değerlendirmeyi amaçladık.*

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Gönderilme / Submitted : 16.03.2023
Kabul / Accepted : 03.08.2023
Yayınlanma / Published : 20.09.2023

Gereç ve Yöntem: *Farklı polaritelerde ekstreler hazırlandı ve hemostatik etkinlikleri optik agregometri kullanılarak belirlendi.*
Sonuç ve Tartışma: *Mevcut sonuçlar, diğer bitki özleri ile karşılaştırıldığında S. verticillata ekstrelerinin adenozin-difosfat (ADP) (%80.77), kollajen (%80.78) ve araşidonik asit (AA) (%73.71) varlığında trombosit agregasyonu üzerinde en yüksek etkinliği gösterdiğini açıkça ortaya koymuştur. Yine epinefrin (EPI) varlığında en etkili trombosit agregasyonu (%47.27) C. procera uygulamasından sonra belirlendi. Ayrıca, öncelikle etil asetat ekstrelerinin APD, kollajen, AA ve EPI olgusunda en yüksek trombosit agregasyonu yüzdelerini göstermiştir. Sonuç olarak, bulgularımız, özellikle S. verticillata ve C. procera'nın etkili hemostatik ajanların yeni ve doğal kaynakları olabileceğini gösterdi.*
Anahtar Kelimeler: *Achillea biebersteinii, Cephalaria procera, hemostaz, Salvia verticillata, Tragopogon aureus*

ABSTRACT

Objective: *Hemostasis is an inherent function and natural process to prevent or stop bleeding. Nowadays great efforts are being made to develop novel, economical and high-performance products to control bleeding. In this study, we aimed to assess the in vitro hemostatic effects of four several plant species used in folk medicine for different purposes including Salvia verticillata, Achillea biebersteinii, Tragopogon aureus, and Cephalaria procera.*
Material and Method: *The extracts with different solvent nature were prepared and their hemostatic efficacy were determined using optical aggregometry.*
Result and Discussion: *The present results clearly revealed that the extracts of S. verticillata showed the highest efficacy on platelet aggregation in presence of adenosine-diphosphate (ADP) (80.77%), collagen (80.78%), and arachidonic acid (AA) (73.71%) when compared to other plant extracts. Again, the most effective platelet aggregation (47.27%) was determined after the application C. procera within the presence of epinephrine (EPI). Moreover, we first executed that ethyl acetate extracts led to the highest percentages of platelet aggregation in the fact of APD, collagen, AA and EPI. In a conclusion, our findings suggested that the tested medicinal plants in particular S. verticillata and C. procera could be novel and natural sources of effective hemostatic agents.*
Keywords: *Achillea biebersteinii, Cephalaria procera, hemostasis, Salvia verticillata, Tragopogon aureus*

GİRİŞ

Kanama, travma tedavisi ve cerrahi müdahalelerde hastaların ve yaralı kişilerin güvenliğini tehdit eder. Kanama, tüm travma kazalarının %80'ini oluşturan travmadan sonra 48 saat içindeki ölümün başlıca nedenidir. Hemostaz, kan akışının durdurulması anlamına gelen bir Yunanca kelime kökenlidir. Birkaç sistemin hassas bir dengeye dayandığı karmaşık bir düzenlenmiş sistemden oluşur. Hemostatik süreçte yer alan en önemli sistemler arasında vasküler sistem, trombositler, pıhtılaşma sistemi ve fibrinolitik sistem bulunur [1].

Kanama, yaralanmalar veya diğer bazı sebeplerle damardan dışarı çıkan kandır. Bu durum, tüm insanlar ve hayvanlar için hayati bir öneme sahiptir ve ciddi risklere, hatta ölüme yol açabilir. Bu nedenle, kanamayı önlemek veya durdurmak için gerçek teknikler ve yeni ilaçlar/bileşikler kullanılarak kapsamlı çabalar gösterilmektedir. Bu alandaki ilk uygulamalar, eski Mısır ve Yunan halkının kullandığı kazıma tekniğidir. Bu teknik uzun bir süredir kullanılmakta olup, 16. yüzyılda ligasyon tekniğiyle değişime uğramıştır. 20. yüzyılın başlarında bypass tekniği uygulanmaya ve yaygınlaşmaya başlamıştır. Birinci ve İkinci Dünya Savaşı gibi dünyanın farklı bölgelerindeki savaşlar ve çatışmalar, bu alandaki ihtiyacın ve tecrübenin artmasına neden olmuştur. Vasküler yaralanmaları iyileştirmenin temel amacı kanamayı durdurmak ve normal dolaşımı sürdürmektir [2]. İnsan trombositleri, normal hemostaz, patolojik kanama ve trombozda farklı işlevlerle bu süreçte önemli bir rol oynar. Temel hemostaz, damar duvarları ve trombositler arasındaki etkileşime dayanır. Bir damar duvarının hasar gördüğü bölgede, trombositler hızlı bir fonksiyonel yanıtta adezyon, aktivasyon ve agregasyon yoluyla trombosit aktivasyonuna katılırlar [3]. Trombositlerin çeşitli işlevleri, kapsamlı bir araştırma yelpazesiyle güvenilir bir şekilde tanımlanabilir [4,5]. Erken kanama kontrolü hayati önem taşır, çünkü kan yaşamın

değerli özüdür ve kontrolsüz kanama hayati tehlike oluşturabilir. Vücudun doğal hemostatik sistemi hafif ila orta düzeydeki kanamaları yönetme kabiliyetine sahiptir [6].

1960'larda, trombosit açısından zengin plazmada (PRP) devrim niteliğindeki trombosit agregasyon deneyi olan ışık geçirgenliği agregometrisi, trombosit fonksiyonunu teşhis etmek için anahtar teknikti [7]. Bu yaklaşım, adenozin-difosfat (ADP), araşidonik asit (AA), kollajen ve epinefrin (EPI) gibi harici agregasyon ajanlarına veya agonistlerine yanıt olarak trombositlerin *in vitro* olarak birbirlerini aglomere etme yeteneklerinin belirlenmesini sağlar. Günümüzde kan pıhtılaşmasını değiştiren yeni ajanlar geliştirmek için büyük çaba sarf edilmektedir. Pıhtılaşmayı değiştiren ilaçların endikasyonları, trombosit inhibitörleri ile yakından ilişkilidir. Çoğu trombosit inhibitörü, trombositler üzerindeki reseptörleri bloke ederek yapışmayı önlemeyi amaçlar. Kanama (diş fırçalama gibi basit kanama durumlarından, yaralanma sonrası aşırı kanamaya kadar) en yaygın yan etkidir [8]. Bu soruna yönelik birçok ürün piyasada bulunmaktadır. Bununla birlikte, hala keşfedilmemiş ürünler mevcuttur ve daha iyi performans ve daha ekonomik özellikler sunan yeni adaylar elde etmek için artan sayıda araştırma yapılmaktadır. Ankaferd Blood Stopper (ABS) adı verilen bir ilaç, çiçek aşısı sonrasında Türk uzmanların en büyük icadı olarak kabul edilen bu alandaki en yeni üründür. Bu ilacın hiçbir yan etkisi olmadığı ve kanamayı diğerlerine göre çok daha kısa sürede durdurduğu bilinmektedir. Gerçekten de ABS, dış kanama ve diş operasyonu kanamalarının tedavisinde etkinliği kanıtlanmış bitkisel ektre karışımıdır. Geleneksel olarak yıllar boyunca topikal bir hemostatik ajan olarak kullanılmıştır. İçeriğinde *Glycyrrhiza glabra* L., *Thymus vulgaris* L., *Urtica dioica* L., *Alpinia officinarum* Hance ve *Vitis vinifera* L olmak üzere beş farklı bitki bulunduğu bildirilmiştir L [9,10]. Bu ilaç tamamen bitkisel karışımla hazırlanmıştır ve halk tıbbına dayanmaktadır [11]. Lamiaceae familyasının en yaygın üyelerinden biri *Salvia* L. cinsidir ve yaklaşık 700 türü vardır. *Salvia* türünden elde edilen terpenoid bileşikler, fenolik türevler ve uçucu yağlar gibi çeşitli kimyasal gruplardan faydalı ikincil metabolitler elde edilmiştir ve bu içerikler, birçok ülkenin farmakopelerinde belirgin bir şekilde yer almaktadır [12]. *S. verticillata* L., leylak adaçayı olarak da bilinen bir Avrasya türüdür ve geleneksel olarak halk tıbbında ve bahçe düzenlemesinde kullanılır. *S. verticillata*'nın iyi antioksidan ve antikolinesteraz aktiviteleri olduğu bildirilmiştir [13]. *Achillea*, Truva Savaşı'nda askerlerin yaralarını iyileştirmek için kullanılan ayva yağından dolayı Aşil'in adını almıştır. *A. biebersteinii* Hub.-Mor. (Asteraceae) ise odunsu olmayan, aromatik ve otsu bir bitkidir. *A. biebersteinii*; antiplatelet, antikanser, antioksidan, antiülser, yara iyileştirme, antigut, hipoglisemik, antibakteriyel, antiinflamatuar, anti epimastigot, nöroprotektif gibi çok çeşitli biyolojik ve farmakolojik etkilere sahiptir. Ayrıca antikolinesteraz özelliği de bulunmaktadır. Bu tür aynı zamanda endometriozis tedavisinde de etkili olmuştur [14]. Bitki, peynirin korunması için peynir yapım sürecinde kullanılmıştır. *S. verticillata* toprak üstü kısımlarından hazırlanan çay balgam söktürücü olarak, ağız dezenfeksiyonunda ve yaraların iyileşmesinde kullanılmaktadır [15].

A. biebersteinii Hub.-Mor. 2012'den beri *A. arabica* Kotschy'nin sinonimi olarak kabul edilmektedir [16]. *Tragopogon aureus* Boiss. (Asteraceae) adlı bitki, Avrasya'dan Atlantik ve Pasifik Okyanuslarına kadar yayılan, endemik ve çok yıllık bir türdür. Olgun *T. aureus* genellikle 10-30 cm boyunda olup, gövdeleri dalsız ve yaprakları mızrak şeklindedir. Bu tür, 1700-2300 m yükseklikteki kayalık yamaçlar, bozkırlar ve tarla kenarlarında yetişir ve Türkiye'de "sarı yemlik, spıng, spink, yemlik" gibi isimlerle tanınır. Bu bitkiden elde edilen lateks, halk hekimliğinde hemostatik (kanama durdurucu) olarak kullanılmaktadır. Ayrıca bitkinin yaprakları taze olarak sebze olarak da tüketilebilir [17-20]. *Cephalaria procera* Fisch & Lall. (Caprifoliaceae) adlı bitki, kuzey Ermenistan, İran ve Türkiye'de yetişir. Bu bitki çok yıllıktır ve gövdeleri 2 metreye kadar uzayabilir. Çiçekler genellikle krem veya soluk sarı renktedir. *C. procera*, 1120-2450 metre yükseklikteki çayırlar, bozkır alanları ve kayalık yamaçlarda yaygın olarak bulunur. Halk arasında "cipreş, gevrek, ganteper" gibi isimlerle bilinir ve bu bitkinin taze toprak üstü kısımları ezilerek geleneksel olarak yara iyileşmesinde hemostatik (kanama durdurucu) olarak kullanılır [18,19,21,22].

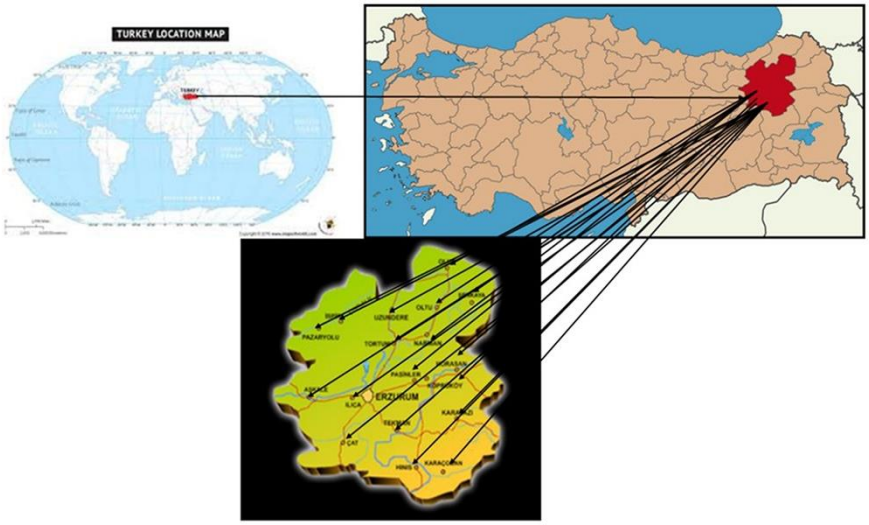
Bu araştırmada, *S. verticillata, A. biebersteinii, T. aureus ve C. procera in vitro* deneylerle hemostatik potansiyelleri değerlendirilmiştir. Bu bitkiler, Orman ve Su İşleri Bakanlığı Doğa Koruma ve Milli Parklar Genel Müdürlüğü'nün "Erzurum İlinde Biyoçeşitliliğe Dayalı Geleneksel Bilgilerin Belirlenmesi" projesi kapsamında halk tarafından kan durdurucu amaçla yaygın olarak kullanılan bitkiler tespit edilmiştir. Bu amaçla, bitkilerin trombositlerin maksimum agregasyonunu ölçmek için

optik agregometri yöntemiyle incelenmesi, bu projede ilk kez önerilen bir yaklaşımdır. Bu şekilde, bitkilerin hemostatik özellikleri araştırılmış ve değerlendirilmiştir.

GEREÇ VE YÖNTEM

Bitki Materyalleri

Salvia verticillata, Achillea biebersteinii, Tragopogon aureus ve *Cephalaria procera*, "Erzurum İlinde Biyoçeşitliliğe Dayalı Geleneksel Bilgilerin Belirlenmesi" adlı mevcut projenin kapsamında toplanmıştır. Araştırma bölgesinin coğrafi konumu Şekil 1'de sunulmuştur. Bitkiler, Prof. Dr. Özkan Aksakal tarafından teşhis edilmiştir. Bitki örnekleri, Atatürk Üniversitesi Biyoçeşitlilik Uygulama ve Araştırma Merkezi Herbaryumu'na kaydedilmiştir. Toplanan bitki örneklerinin lokaliteleri ve herbaryum numaraları Tablo 1'de gösterilmiştir.



Şekil 1. Araştırma bölgesinin coğrafi konumu

Tablo 1. Toplanan bitki örneklerinin lokaliteleri ve herbaryum örnekleri

Türler	Lokaliteler	Herbaryum kodu
***Salvia verticillata* L.**	B8 Erzurum: Narman, Göllü köyü, 03.06.2018, 1900 m	AUEF 1264
***Achillea biebersteinii* Hub.-Mor.**	B8 Erzurum: Atatürk Üniversitesi kampüsü, Eczacılık Fakültesi bahçesi, 05.06.2018, 1890 m	AUEF 1359
***Tragopogon aureus* Boiss.**	B8 Erzurum: Erzurum Kent ormanı, 07.06.2018, 1910 m	AUEF 1360
***Cephalaria procera* Fisch. & Avé-Lall.**	B8 Erzurum: Erzurum Kent ormanı, 07.06.2018, 1910 m	AUEF 1361

Ekstraksiyon ve Fraksiyonlama

S. verticillata, A. biebersteinii ve *C. procera*'nın toprak üstü kısımları (50 g) ile *T. aureus*'un kökleri (50 g) öğütüldü ve oda sıcaklığında üç gün boyunca metanolle hareketli maserasyona tabi tutuldu (3 x 200 ml). Ekstreler kurutuldu ve metanol: su (1: 9) karışımında disperse edildikten sonra sırasıyla *n*-hekzan (3 x 150 ml), diklorometan (3 x 150 ml), etil asetat (3 x 150 ml) ve *n*-butanol (3 x 150 ml) ile fraksiyonlandı. Her bir fraksiyon kurutuldu, tartıldı. Çalışmada tüm ekstre ve alt-ekstreler test edilmiştir. *S. verticillata, A. biebersteinii, T. aureus* ve *C. procera*'dan elde edilen ekstrelerin ve alt-ekstrelerin miktarları Tablo 2'de sunulmuştur. Ayrıca, bitkilerin halk arasındaki kullanımı dikkate alındığında, bitkiler taze iken toprak üstü kısımları havanda dövülerek elde edildi. Sadece *T. aureus* kökünün lateksi kesim yoluyla elde edildi.

Tablo 2. *Salvia verticillata, Achillea biebersteinii, Tragopogon aureus* ve *Cephalaria procera*'dan elde edilen ekstre ve alt ekstrelerin miktarları

Estre ve alt ekstreler	*Salvia verticillata*	*Achillea biebersteinii*	*Tragopogon aureus*	*Cephalaria procera*
MeOH (g)	10.09	10.25	11.24	9.87
Hekzan (g)	1.20	1.22	1.51	1.26
CH_2Cl_2 (g)	2.89	2.92	3.02	2.04
EtOAc (g)	0.76	0.69	1.03	0.93
BuOH (g)	2.77	2.91	3.01	2.28
Sulu kısım (g)	2.23	2.04	2.41	2.22

Kimyasallar

ADP, Kollajen, AA ve EPI için trombosit agregasyonu, Hart Biologicals (Hartlepool, İngiltere) tarafından satın alınmıştır.

Kan Toplama ve PRP'nin Hazırlanması

Bu çalışma, Atatürk Üniversitesi Tıp Fakültesi Klinik Araştırmalar Etik Kurulu tarafından onaylanmıştır (B.30.2.ATA.0.01.00/). Kan, 20-25 yaş aralığında (22.5 ± 1.3) üç sağlıklı ve sigara kullanmayan kadın gönüllüden 0.10^5 M tamponlu sodyum sitrat içeren polipropilen tüpe toplanmıştır. Vericilerden hiçbiri, kan toplamadan en az 10 gün önce trombosit fonksiyonunu etkileyen herhangi bir ilaç almamıştır. PRP, tam kanın 150 g'de 15 dakika boyunca RT'de santrifüj edilmesiyle toplanmıştır. Daha sonra, trombosit yoksun plazma (PPP) 2000 g'de 20 dakika boyunca RT'de santrifüj edilerek toplanmıştır. Trombosit sayısı PPP ile 2 x 10^8 hücre/ml'ye ayarlanmıştır.

PRP'nin Ekstrelerle İnkübasyonu

PRP, ekstrelerle (1/5 ve 1/10) 37 °C'de 15 dakika boyunca inkübe edilmiştir. Herhangi bir bileşik içermeyen PRP kontrol grubu olarak kullanılmıştır.

Optik Agregometri ile Maksimum Agregasyon Ölçümü

PRP'deki trombosit agregasyonu, dört kanallı agregometre (APACT 4004; LABiTec, Ahrensburg, Almanya) kullanılarak ölçülmüştür. PRP'nin ekstraktlarla inkübasyonundan sonra, trombosit agregasyonu ADP (5µM), Kollajen (10µg/ml), AA (5mM) ve EPI (10µM) ile uyarılmış ve agregasyon yüzdesi 10 dakika boyunca izlenmiştir. Test, kan toplandıktan hemen sonra yapılıp 2 saat içinde tamamlanmıştır. Trombosit agregasyon deneyleri üç kez tekrarlanmıştır.

Toplam Fenolik İçerik

250 µl Folin-Ciocalteu, 50 µl materyal ve %500 Na_2CO_3 su çözeltisinin göstergesi karıştırılmış ve 5 ml suya tamamlanmıştır. Absorbans 765 nm'de ölçülmüştür. 30 dakika RT inkübasyonu sonrasında referans eğrisi galik asit ile yapılmıştır. Toplam polifenoller galik asit eşdeğerleri (GAE) olarak sunulmuştur ve mg GAE/g materyal (dw) ± standart sapma (SD) olarak belirtilmiştir [23-25].

Kantitatif DPPH

Materyallerin 0.1 ml'si, 2.9 ml DPPH çözeltisine serbest radikal kaynağı olarak konuldu. Karışımlar, karıştırıldı, 30 °C'deki bir su banyosunda 30 dakika inkübe edildi ve her materyalin absorpsiyonu 517 nm'de ölçüldü. Referans olarak klorojenik asit, rutin ve propil gallat kullanıldı. Veriler, DPPH'nin radikal süpürme aktivitesinin yüzdesi (%) olarak gösterildi ve [(Ao-As) / Ao] × 100 formülüyle hesaplandı, burada Ao materyallerin varlığında absorpsiyonu ifade etmektedir [26].

İstatistiksel Analiz

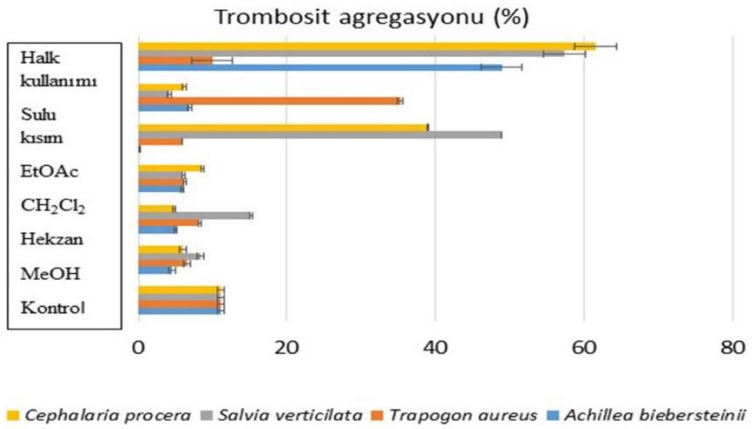
Bulguların istatistiksel analizinde SPSS 20.0 paket programı (SPSS Inc., Chicago, USA) kullanıldı. Varyans analizi, kontrol ve farklı örneklerin işlem görmüş grupları arasında trombosit

agregasyonunun farklı olup olmadığını belirlemek için kullanıldı. Oneway Anova ve Fisher Least Significant Difference (LSD) testleri varyans analizi için kullanıldı. 0.05'ten küçük bir p değeri istatistiksel olarak anlamlı olarak kabul edildi.

SONUÇ VE TARTIŞMA

S. verticillata, A. biebersteinii, T. aureus ve *C. procera* bitkilerinin toprak üstü kısımlarının metanolik ekstreleri ve hekzan, CH_2Cl_2, EtOAc, BuOH, sulu kısım alt ekstrelerinin ADP, kolajen, AA ve EPI varlığında kanama durdurma performansları test edilmiştir. Ayrıca, *S. verticillata, A. biebersteinii* ve *C. procera* toprak üstü kısımlarının usareleri ile *T. aureus* kök lateksi de test edilmiştir. Numunelerin ADP, kolajen, AA ve EPI varlığında kanama durdurma performansı sonuçları Şekil 2-5'te sunulmuştur.

A. biebersteinii numuneleri arasında ADP varlığında trombosit agregasyonu üzerine en etkili numunenin halk arasındaki kullanımının (yaprakların tazeyken ezilerek kanayan bölge üzerinde bekletilerek) olduğu görülmüştür (%45.62). *S. verticillata,* örnekler arasında, ADP varlığında en etkili örnek olarak bulundu (%80.77). *T. aureus* numuneleri arasında ADP varlığında trombosit agregasyonu üzerine en etkili numunenin halk arasındaki kullanımının (köklerden elde edilen latekslerin toplanıp sakız kıvamına getirilip kanayan bölge üzerinde bekletilerek) olduğu görülmüştür (%37.94). *C. procera* numuneleri arasında ADP varlığında trombosit agregasyonu üzerine en etkili numunenin halk arasındaki kullanımının (pedunkullar tazeyken ezilerek kanayan bölge üzerinde bekletilerek) olduğu görülmüştür (%61.61). Tüm numuneler arasında ADP varlığında trombosit agregasyonu üzerine en etkili numunenin *S. verticillata* bitkisi olduğu görülmüştür (Şekil 2).

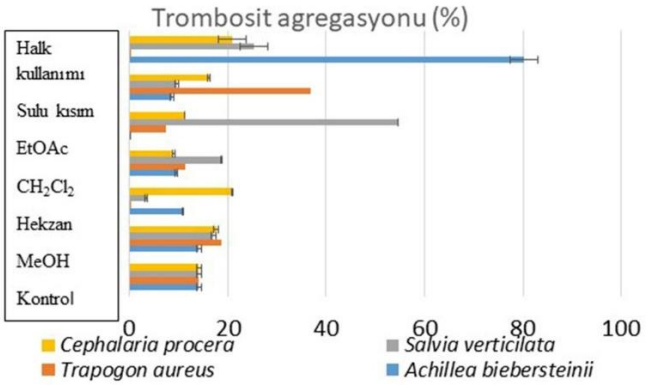


Şekil 2. Farklı çözücülerle elde edilen bitki ekstrelerinin ADP varlığında trombosit agregasyonuna etkisi. Değerler ortalama+SD olarak gösterilmiştir

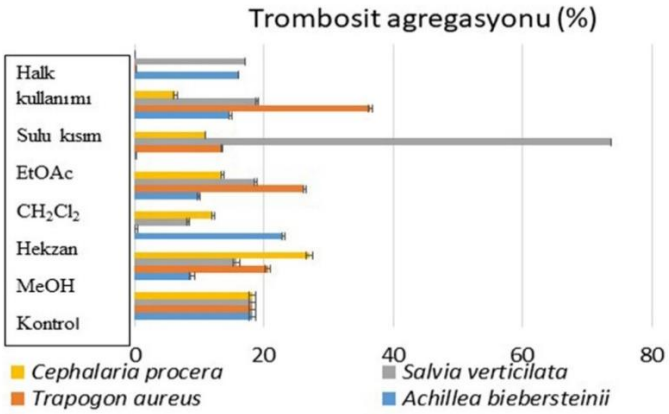
Sonuçlarımıza göre, *A. biebersteinii* numunelerinin halk arasında kullanımıyla (yaprakların tazeyken ezilerek kanayan bölge üzerinde bekletilmesi) kolajen varlığında trombosit agregasyonunu en etkili şekilde inhibe ettiği (%80.27) görüldü. Benzer şekilde, *S. verticillata* numuneleri de halk arasında kullanıldığında (yaprakların tazeyken ezilerek kanayan bölge üzerinde bekletilmesi) kolajen varlığında trombosit agregasyonunu etkili bir şekilde inhibe ettiği belirlendi (%80.78). *T. aureus* numuneleri arasında sulu fraksiyonunun kolajen varlığında trombosit agregasyonunu en etkili şekilde inhibe ettiği (%36.74) tespit edildi. Benzer şekilde, *C. procera* numuneleri arasında da sulu fraksiyonunun kolajen varlığında trombosit agregasyonunu etkili bir şekilde inhibe ettiği (%38.55) gözlendi. Tüm numuneler arasında kolajen varlığında trombosit agregasyonunu en etkili şekilde inhibe eden bitkinin *S. verticillata* olduğu belirlendi (Şekil 3).

Sonuçlarımıza göre, *A. biebersteinii* numunelerinin araşidonik asit varlığında trombosit agregasyonunu en etkili şekilde inhibe eden fraksiyonun hekzan fraksiyonu olduğu belirlendi (%22.92). Benzer şekilde, *S. verticillata* numunelerinde ise araşidonik asit varlığında trombosit agregasyonunu en

etkili şekilde inhibe eden fraksiyonun etilasetat fraksiyonu olduğu tespit edildi (%73.71). *T. aureus* numuneleri arasında ise araşidonik asit varlığında trombosit agregasyonunu en etkili şekilde inhibe eden fraksiyonun sulu fraksiyon olduğu görüldü (%36.39). *C. procera* numunelerinde ise araşidonik asit varlığında trombosit agregasyonunu en etkili şekilde inhibe eden numunenin metanol ekstresi olduğu belirlendi (%26.99). Tüm numuneler arasında araşidonik asit varlığında trombosit agregasyonunu en etkili şekilde inhibe eden bitkinin *S. verticillata* olduğu sonucuna ulaşıldı (Şekil 4).



Şekil 3. Farklı çözücülerle elde edilen bitki ekstrelerinin kollajen varlığında trombosit agregasyonuna etkisi. Değerler ortalama+SD olarak gösterilmiştir

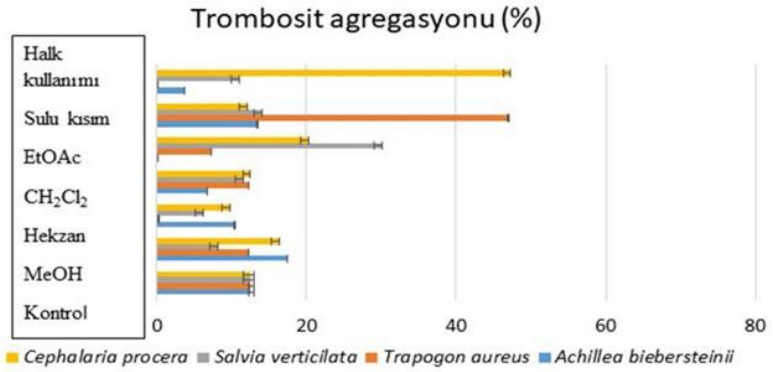


Şekil 4. Farklı çözücülerle elde edilen bitki ekstrelerinin araşidonik asit varlığında trombosit agregasyonuna etkisi. Değerler ortalama + SD olarak gösterilmiştir

Elde ettiğimiz sonuçlara göre, *A. biebersteinii* numunelerinde epinefrin varlığında trombosit agregasyonunu en etkili şekilde inhibe eden numunenin metanol ekstresi olduğu saptandı (%13.45). Benzer şekilde, *S. verticillata* numunelerinde epinefrin varlığında trombosit agregasyonunu en etkili şekilde inhibe eden numunenin etilasetat fraksiyonu olduğu tespit edildi (%30.14). *T. aureus* numuneleri arasında epinefrin varlığında trombosit agregasyonunu en etkili şekilde inhibe eden numunenin sulu fraksiyon olduğu gözlendi (%47.02). *C. procera* numunelerinde ise epinefrin varlığında trombosit agregasyonunu en etkili şekilde inhibe eden numunenin halk arasında kullanılan yöntemle (pedunkulların tazeyken ezilip kanayan bölge üzerinde bekletilmesi) elde edilen numune olduğu görüldü (%47.27). Tüm numuneler arasında epinefrin varlığında trombosit agregasyonunu en etkili şekilde inhibe eden bitkinin *C. procera* olduğu sonucuna ulaşıldı (Şekil 5).

Tüm numuneler arasında ADP, kolajen ve araşidonik asit varlığında trombosit agregasyonu üzerine en etkili bitkinin *S. verticillata* olduğu görülmüştür. Epinefrin varlığında trombosit agregasyonu üzerine en etkili bitkinin ise *C. procera* olduğu görülmüştür. Her bir bitkinin halk arasında kullandığı

şekli ve çeşitli kısımlarından hazırlanan ekstreler üzerine uygulanan "Platelet Agregasyon Testi" sonucunda, yüksek performans gösterenler tespit edilmiş, yeni bir ürün geliştirme potansiyelinin olup-olmadığı araştırılmış ve bazı örneklerin çok yüksek aktivite gösterdiği tespit edilmiştir. Çalışma sonucunda, bazı yüksek performanslara dikkat çekilirken, *in vivo* çalışmaların da yapılması gerektiği kanısına varılmıştır. Ancak bu sonuçlar, bu konudaki uzun soluklu çalışmaların ilk basamağı olarak değerlendirilmelidir.



Şekil 5. Farklı çözücülerle elde edilen bitki ekstrelerinin epinefrin varlığında trombosit agregasyonuna etkisi. Değerler ortalama + SD olarak gösterilmiştir

Oksidatif stres ROS tarafından arttırıldığında dokularda hasara, DNA, protein ve lipid hasarına neden olduğu bilinmektedir [27]. En yüksek toplam fenolik içerik geleneksel kullanımda *S. verticillata*'da (1287.83 ± 1.77 mg GAE/g kuru ağırlık) gösterilmiştir (Tablo 3). Ayrıca, *S. verticillata*'nın geleneksel kullanımı (8.65 µg/ml), en yüksek radikal süpürme aktivitesini göstermiştir (Tablo 3).

Tablo 3. Yüksek aktivite gösteren örneklerin toplam fenolik içerikleri ve DPPH radikal süpürme aktivitesi (µg/ml)

Test edilen örnekler	**Toplam fenolik içerik (mg GAE/g) ± SD***
S. verticillata'nın geleneksel kullanımı	1287.83 ± 1.77
S. verticillata'nın EtOAc alt-ekstraktı	988.25 ± 2.56
C. procera'nın geleneksel kullanımı	1092.66 ± 1.92
Test edilen örnekler	**IC_{50} değerleri (µg/ml) ± SD***
S. verticillata'nın geleneksel kullanımı	8.65 ± 2.87
S. verticillata'nın EtOAc alt-ekstraktı	13.77 ± 1.60
C. procera'nın geleneksel kullanımı	10.77 ± 0.91
Klorojenik asit	2.66 ± 0.72
Propil galat	0.015 ± 0.82
Rutin	2.99 ± 0.90

*Standard deviation

Kanama, yaralanma, kan hastalıkları veya ilaç etkisi gibi nedenlerle oluşabilir. Kanamayı durdurmak sıkça talep edilen tıbbi bir müdahaledir. Desmopressin, aprotinin ve antifibrinolitik amino asitler (traneksamik asit ve aminokaproik asit gibi) gibi etkili birçok hemostatik ilaç mevcuttur. Bunlar doğuştan gelen kanamalar, hastalıklar, iç kanamalar veya kalp cerrahisi gibi durumlarda kullanılırlar, ancak bu ilaçlar lokal kullanım için tasarlanmamıştır. Fibrin doku yapıştırıcıları, protrombin, kollajen ve trombin gibi topikal hemostatik ajanlar pahalı ve kolayca erişilebilir değildir. Ayrıca, bazıları sınırlı aktivite sergilemiştir. Bu nedenle, kritik kanama durumları için değil, aynı zamanda yaygın yaralar veya mukozal kanamalar için acil olarak etkili topikal ajanlara ihtiyaç vardır. Bazı doğal bileşikler geleneksel olarak kanamayı durdurmak için etkili bir şekilde kullanılmıştır [28]. Cerrahi işlemlerin neden olduğu

kanama, yanıkları içeren plastik cerrahi uygulamaları dışında ölümün nadir bir nedenidir. Konvansiyonel tıbbi bitki özleri, dermal, harici travmatik ve postoperatif, ve diş kanamalarının yönetimi için kullanılan bir alternatif tedavi modalitesidir [29]. Fitokimyasal bulgular, incelenen tüm numunelerin polifenolik bileşenler içerdiğini göstermiştir. Aslında, *Arbutus unedo, Equisetum arvense, Petroselinum crispum, Cistus ladaniferus* ve *Urtica dioica*'da flavonoidler ve tanenlerin mevcut olduğu bildirilmiştir. Test edilen bitki türlerinin gözlemlenen hemostatik özellikleri, biyolojik olarak aktif bileşenlerine atfedilebilir. Ve önceki araştırmalar, flavonoidlerin trombosit agregasyonunu, sekresyonunu ve yapışmasını önemli ölçüde inhibe ettiğini göstermiştir. Aslında, kırmızı şarapta bulunan bir polifenolik bileşik (3,5,4′-trihidroksi-trans-stilben veya t-resveratrol), hiperkolesterolemik tavşanlarda trombosit agregasyonunu inhibe ettiği gösterilmiştir [30]. Oksidatif stres, kan damarlarının hasarına neden olarak kanamaya yol açabilir. Serbest radikaller, endotel hücrelerinde hasara neden olarak damarların bütünlüğünü bozabilir ve kanamaya neden olabilir. Serbest radikaller, pıhtılaşma faktörlerini etkileyebilir ve kanın normal pıhtılaşmasını engelleyebilir. Bu durumda kanama eğilimi artabilir. Trombositler, kanamanın durdurulmasında önemli bir rol oynar ve oksidatif stres trombosit aktivasyonunu artırabilir veya inhibe edebilir. Bbu da kanama üzerinde etkili olabilir ve oksidatif stres kan damarlarında hasara yol açarak kanama riskini artırabilir.

Kanamayı durdurmak veya azaltmak için geleneksel tıbbi bitkiler uzun süredir kullanılmaktadır. Bu bitkiler, içerdikleri biyoaktif bileşikler sayesinde kanama kontrolüne yardımcı olabilir ve yara iyileşmesini destekleyebilir. İşte kanamada kullanılan geleneksel tıbbi bitkilerin önemini vurgulayan bazı noktalar:

Hemostatik etki: Kanamayı durdurmak veya azaltmak için kullanılan bitkiler, içerdikleri aktif bileşikler sayesinde kan pıhtılaşmasını artırabilir veya kan damarlarının daralmasını sağlayabilir. Bu şekilde, kanama kontrol edilir ve kan pıhtılaşması hızlanır.

Yara iyileşmesi: Kanama, yaralanmaların doğal bir sonucu olarak ortaya çıkar. Geleneksel tıbbi bitkiler, yara iyileşmesini hızlandırmaya yardımcı olabilir. Antioksidan ve anti-inflamatuar özelliklere sahip bileşikler içerdikleri için yara bölgesinde iyileşme sürecini desteklerler.

Doğal ve yan etkisiz: Geleneksel tıbbi bitkiler genellikle doğal kaynaklardan elde edilir ve kimyasal bileşenler içermezler. Bu nedenle, sentetik ilaçlara kıyasla daha az yan etki riski taşırlar. Bununla birlikte, herhangi bir bitki veya bitkisel ürün kullanırken, kişinin özel sağlık durumu ve alerjik reaksiyonlar gibi faktörleri göz önünde bulundurması önemlidir.

Kolay erişilebilirlik: Geleneksel tıbbi bitkiler, birçok coğrafi bölgede yetişir ve kolayca erişilebilir olabilir. Bu, insanların kırsal bölgelerde veya sınırlı tıbbi kaynaklara sahip olan toplumlarda dahi kanamayı kontrol etmek için bitkileri kullanabilmesini sağlar.

Ancak, geleneksel tıbbi bitkilerin kullanımı konusunda dikkatli olunması önemlidir. Uzman olmayan kişilerin bitki kullanımı konusunda bilgili olmaması veya yanlış dozaj ve yöntemlerin uygulanması istenmeyen etkilere neden olabilir. Bu nedenle, bitkisel tedavilerden yararlanırken uzman bir sağlık uzmanından veya bitki uzmanından destek almak önemlidir.

Bu tür bitki türlerinin kanamayı durdurucu olarak kullanımının altında yatan mekanizmaları ve pıhtılaşmayı basitleştiren veya fibrinolizi inhibe eden ilişkili aktif bileşik veya bileşiklerin ortaya çıkarılması için daha fazla araştırmalar gereklidir. Öncelikle, bitkilerin klinik kullanımları için denenmesi gereklidir ve güvenlik profilleri belirlenmelidir, çünkü infüzyonlar ve topikal uygulamalar gibi başarılı kullanımlarına rağmen. Yüksek etkinliğe sahip yeni ve doğal hemostatik ajanların diş ve kardiyovasküler cerrahide, gastrointestinal kanamada ve antikoagülan veya trombolitik ilaç tedavisi sonrası kanamada kritik gereksinimlerin varlığından dolayı, çalışmamız etkili hemostatiklerin geliştirilmesi için yeni bakış açıları sağlayacaktır. Aslında, bu bulgular öncelikle *S. verticillata* gibi test edilen dört bitki türünün hem deri hem de mukoza hasarları için ucuz, doğal, güvenli ve kolay kullanılabilir bir topikal hemostatik ajan kaynağı olabileceğini önermektedir.

Sonuçlar, bu dört bitki türünün farklı ekstreleri ve bileşenlerinin kanama durdurma yeteneklerini ortaya koymaktadır. Hemostatik performansları, ADP, kolajen, AA ve EPI gibi kanama tetikleyicilerinin varlığında test edilmiştir. *S. verticillata*, diğer bitkiler arasında en etkili hemostatik performansa sahip olduğu bulunmuştur. Bu sonuçlar, geleneksel tıpta kullanılan bitkilerin hemostatik özelliklerini desteklemektedir ve bitkinin potansiyel olarak kanama kontrolünde kullanılabileceğini göstermektedir. Ancak, bu çalışma sadece *in vitro* koşullarda yapılmış olup, klinik uygulamalar için daha fazla araştırma

gerekmektedir. İleri çalışmalar, bu bitkilerin etkin bileşenlerini ve mekanizmalarını daha ayrıntılı olarak değerlendirmeli ve potansiyel yan etkileri veya etkileşimleri de göz önünde bulundurmalıdır.

Sonuç olarak, bu çalışma, S. *verticillata, A. biebersteinii, T. aureus ve C. procera* gibi bitkilerin hemostatik performanslarının *in vitro* olarak değerlendirildiğini göstermektedir. Bu bitkilerin kanama kontrolünde potansiyel kullanımını destekleyen bulgular elde edilmiştir. Ancak, klinik uygulamalar için daha fazla araştırma yapılması gerekmektedir.

TEŞEKKÜR

Bu çalışma Atatürk Üniversitesi Araştırma Fonu (FBA-2018-6832) tarafından desteklenmiştir.

YAZAR KATKILARI

Kavram: S.K., U.İ., H.T., O.A.; Tasarım: S.K., O.O., U.İ., H.T., O.S., O.A.; Denetim: S.K., U.İ., H.T., O.S.; Kaynaklar: S.K., O.O., U.İ., H.T., O.S., O.A.; Malzemeler: S.K., U.İ., H.T., O.A.; Veri Toplama ve/veya İşleme: S.K., O.O., U.İ., H.T., O.S., O.A.; Analiz ve/veya Yorumlama: S.K., O.O., U.İ., H.T., O.S., O.A.; Literatür Taraması: S.K., U.İ., H.T., O.S.; Makalenin Yazılması: S.K., U.İ., H.T., O.S.; Kritik İnceleme: S.K., O.O., U.İ., H.T., O.S., O.A.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Bu çalışma, Atatürk Üniversitesi Tıp Fakültesi Klinik Araştırmalar Etik kurulu tarafından onaylanmıştır (B.30.2.ATA.0.01.00/).

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 894-906, 2023
Doi: 10.33483/jfpau.1319958

ORIGINAL ARTICLE / ÖZGÜN MAKALE

GREEN PROCEDURE INDEX ASSESSMENT OF THE NOVEL STABILITY-INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF CAPTOPRIL FROM PHARMACEUTICAL DOSAGE FORM

FARMASÖTİK DOZAJ FORMUNDAN KAPTOPRİL TAYİNİ İÇİN YENİ STABİLİTE-GÖSTERGELİ RP-HPLC YÖNTEMİNİN YEŞİL PROSEDÜR İNDEKSİ DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *In this study, it was aimed to develop a novel reverse-phase liquid chromatography method for the ultra-sensitive determination of the antihypertensive drug captopril, using paracetamol, which is the common pain killer, as the internal standard. Optimization of all experimental conditions including composition of mobile phase, flow rate, and column temperature was carried out step by step, and the method validity of the developed method was examined according to international validation guidelines. Calibration range, linearity, the limit of determination, the limit of quantification, robustness, accuracy from commercial tablet samples, and method stability were examined in detail. In addition, the greenness profile for the developed method was assessed with the Green Analytical Procedure Index and Analytical Greenness Calculator techniques, which are frequently used in the literature.*

Material and Method: *The chromatographic method was conducted with an XBridge C18 column (25 cm x 4.6 mm ID; 5 µm) packed with fully porous silica materials. All analyses were performed isocratically with a mobile phase containing acetonitrile:5 mM, pH 7.0 ammonium acetate solution (50:50, v/v) at a flow rate of 1.5 ml min^{-1}. The injection volume was 5 µl, and the column was kept at 25°C in a column oven. The column eluate was monitored at 220 nm. Under optimized conditions, retention times of captopril, and paracetamol were approximately 1.59, and 2.0 min, respectively.*

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Submitted / Gönderilme : 25.06.2023
Accepted / Kabul : 04.08.2023
Published / Yayınlanma : 20.09.2023

Result and Discussion: *This study described a fully validated, simple, sensitive, accurate, linear, precise, and reproducible reversed-phase liquid chromatography method for the determination of captopril in tablet samples. Under optimal experimental conditions, the linear range was found in the range of 0.5-200 µg ml^{-1} and the correlation coefficient was greater than 0.99. Method precision was acceptable, with coefficients of variation between 0.05% and 0.61%. In addition, as a result of the recovery studies carried out on the tablet samples, the accuracy was found to be within satisfactory limits between 99.45% and 102.55%. Moreover, the greenness profile of the developed method also showed that the method is environmentally friendly.*
Keywords: *Analytical greenness assessment, captopril, HPLC, optimization, validation*

ÖZ

Amaç: *Bu çalışmada, yaygın bir ağrı kesici olan parasetamol iç standart olarak kullanarak antihipertansif ilaç kaptopril'in ultra-hassas tayini için yeni bir ters fazlı sıvı kromatografi yönteminin geliştirilmesi amaçlanmıştır. Mobil faz bileşimi, akış hızı, kolon sıcaklığı gibi tüm deneysel koşulların optimizasyonu adım adım gerçekleştirilmiş ve geliştirilen yöntemin yöntem geçerliliği uluslararası validasyon kılavuzlarına göre incelenmiştir. Kalibrasyon aralığı, doğrusallık, tespit limiti, tayin limiti, sağlamlık, ticari tablet numunelerinden doğruluk ve metot stabilitesi detaylı olarak incelenmiştir. Ayrıca geliştirilen yöntemin yeşillik profili, literatürde sıklıkla kullanılan Yeşil Analitik Prosedür İndeksi ve Analitik Yeşillik Hesaplayıcı teknikleri ile değerlendirilmiştir.*
Gereç ve Yöntem: *Kromatografik yöntem, tamamen gözenekli silika materyaller ile doldurulmuş bir XBridge C18 kolonu (25 cm x 4.6 mm ID; 5 µm) ile gerçekleştirildi. Tüm analizler, 1.5 ml dk^{-1} akış hızında asetonitril:5 mM, pH 7.0 amonyum asetat çözeltisi (50:50, v/v) içeren bir mobil faz ile izokratik olarak yapıldı. Enjeksiyon hacmi 5 µl idi ve kolon, bir kolon fırınında 25°C'de sabit tutuldu. Kolon eluatı 220 nm'de izlendi. Optimize edilmiş koşullar altında, kaptopril ve parasetamolün alıkonma süreleri sırasıyla yaklaşık 1.59 ve 2.0 dakika olmuştur.*
Sonuç ve Tartışma: *Bu çalışma, tablet numunelerinde kaptopril tayini için tam olarak doğrulanmış, basit, hassas, doğru, doğrusal, kesin ve tekrar üretilebilir bir ters fazlı sıvı kromatografi yöntemini tanımlamıştır. Optimal deneysel koşullar altında lineer aralık 0.5-200 µg ml^{-1} aralığında bulundu ve korelasyon katsayısı 0,99'dan büyüktü. Yöntem kesinliği, %0.05 ile %0.61 arasındaki varyasyon katsayıları ile kabul edilebilir düzeydeydi. Ayrıca tablet numuneleri üzerinde yapılan geri kazanım çalışmaları sonucunda doğruluğun %99.45 ile %102.55 arasında tatmin edici sınırlar içinde olduğu görülmüştür. Ayrıca geliştirilen yöntemin yeşillik profili de yöntemin çevre dostu olduğunu göstermiştir.*
Anahtar Kelimeler: *Analitik yeşillik değerlendirmesi, HPLC, kaptopril, optimizasyon, validasyon*

INTRODUCTION

A serious medical disease called hypertension raises a person's risk of developing a number of problems that could impair the operation of their heart, brain, kidneys, and other vital organs. World Health Organization (WHO) data show that approximately 1.28 billion people between the ages of 30-79 have hypertension. Reports also show that 972 million people will be added to this prevalence in 2025. Hypertension treatment is challenging, particularly for people over 60. Because the pharmacological effects of initial conventional doses of antihypertensive drugs are sensitive to these patients [1,2]. Chemically known as ((2S)-1[(2S)-S-2-methyl-3-sulfanylpropanoyl]pyrrolidine-2-carboxylic acid), captopril (CAP) is a frequently used anti-hypertensive drug that is also used to treat different diseases such as rheumatoid arthritis, diabetic nephropathy, and vascular diseases. To keep the body's blood pressure stable, CAP is utilized as an angiotensin-converting enzyme inhibitor. However, agranulocytosis, teratogenicity, acute renal failure, proteinuria, angioedema, hyperkalemia, taste changes, proteinuria, postural hypotension, diarrhea, nephrotic syndrome, and skin rashes are some of the negative side effects of excessive CAP use in humans [3-6]. For this reason, CAP as an anti-hypertension drug should be analyzed carefully because it is frequently used in the treatment of diseases and because of its negative effects on the human body. Looking at the research in the literature, it is clear that several traditional approaches have been successfully used to identify the presence of CAP in pharmaceutical products and human fluid samples including spectrophotometric [7], Raman

spectroscopy [8], capillary electrophoresis [9], electrochemical [10-13], high-performance liquid chromatography (HPLC) [14-18]. HPLC is the most widely utilized method in the pharmaceutical industry for the manufacturing, development, and analysis of pharmaceuticals, particularly in quality control laboratories, as can be shown from literature studies. However, HPLC still uses a lot of organic solvents that can harm the environment and produce a lot of waste that needs to be disposed of, which poses a threat to worker safety and has an adverse effect on the environment. The purpose of this study is to present a novel chromatographic technique for CAP determination that can be viewed as a commonly employed, environmentally acceptable replacement using less organic solvent. This study focuses on how easily less harmful and environmentally friendly solvents can replace traditional mobile phases by enhancing technique performance.

MATERIAL AND METHOD

Reagents and Materials

The CAP and paracetamol (PAR), which was used as an internal standard (IS), were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium acetate and potassium bromide were purchased from Sigma-Aldrich (St. Louis, MO). Other reagents, including sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) and were provided from Sigma-Aldrich (St. Louis, MO). All chemicals were analytical reagent grade. Chromatography grade acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid and hydrochloric acid (HCl) were purchased from Merck (Merck KGaA, Darmstadt, Germany). A Milli-Q® system (Millipore, Milford, MA, USA) was used to obtain chromatographic grade water to prepare the required solutions. Pharmaceutical dosage form known as Kaptopril® was provided local pharmacy.

Instruments

The reversed-phase (RP)-HPLC method was developed using the Agilent 1100 series LC system, which is furnished with a degasser, quaternary pump, autosampler, and diode array detector (DAD). As the stationary phase, an XBridge C18 (25 cm x 4.6 mm ID; 5 µm) column from Waters in Milford, Massachusetts, USA, was used for the analysis. pH measurements were taken using an Orion 3 Star Plus benchtop pH meter from Thermo Scientific (USA).

Chromatographic Conditions

For the RP-HPLC study, a stationary phase of XBridge C18 (25 cm x 4.6 mm ID; 5 µm) with a detection wavelength of 220 nm was used in conjunction with a mobile phase in isocratic mode (1.5 ml min^{-1} of flow rate) that contained a mixture of acetonitrile: 5 mM Ammonium acetate (50:50; v/v). Using 1 M NaOH, the mobile phase's pH was adjusted up to 7.0. The prepared mobile phase was first degassed using an ultrasonic bath, and then these solutions were filtered through a 0.45 µm filter in a vacuum. About 25 minutes prior to the injection, the column was prepared, and the injection volume was set at 5 µl. By injecting potassium bromide, which was predetermined for each mobile phase composition, the dead time (t_0) was calculated.

Preparation of Solutions

To prepare a 1000 µg ml^{-1} stock solution of CAP and PAR, 5 mg of CAP or PAR was first weighed and dissolved in 5 ml of acetonitrile by the same procedure. For around 10 minutes, both solutions were allowed to dissolve in an ultrasonic bath. At 5°C ± 3°C, all solutions were stored in the refrigerator. Working solutions were arranged by diluting the stock solution with mobile phase in a range of 0.5 to 200.0 µg ml^{-1} while maintaining a constant PAR content of 100 µg ml^{-1}. To obtain the calibration plot, the linearity was established as the ratio of the peak area of the CAP against the peak area of the PAR. Related parameters and calculations were reported for this graph in the results and discussion section.

Pharmaceutical Dosage Form Analysis and Accuracy Study

Five Kaptopril® film-coated tablets (one tablet contains 25 mg of CAP) were precisely weighed and ground into a fine, homogenous powder in a mortar for the RP-HPLC analysis. This powder was

accurately weighted to represent one tablet's worth of substance, then deposited into a flask calibrated for 100 ml, volume completed using acetonitrile as a solvent, mixed for approximately 10 minutes, and then diluted to the desired volume using the acetonitrile. The clear solution was collected separately by filtration of this solution. A reasonable aliquot of the clear filtrate was taken, and the correct amount of PAR (100 μg ml^{-1}) was added before dilution with the mobile phase to provide the appropriate solutions. The associated regression equations were used to determine the CAP's content amount.

To demonstrate the applicability of the RP-HPLC method, by adding a specified amount of pure CAP in a pre-analyzed tablet, recovery studies were carried out. For this purpose, the dosage form of the tablet was supplemented with specified amounts of pure CAP (and at a 100 μg ml^{-1} PAR), and the mixtures were analyzed at optimal conditions. By comparing the concentration obtained from spiked samples with the additional concentration, the percent recovery was computed. In this way, to better understand how common excipients in tablet form affect chromatograms (such as tailing and broadening), the developed method was tested.

Preparation of Stressed Samples

According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) recommendations, a forced degradation study on the bulk form of CAP was assessed in this study [19,20]. For this purpose, degradation experiments were applied for up to 6 hours to examine the effects of acidic hydrolysis (0.01 N and 0.1 N HCl), alkaline hydrolysis (0.01 N and 0.1 N NaOH), and oxidative degradation (3% and 30% H_2O_2). An air oven was set at 80°C for 6 hours to allow for the thermal degradation of a solid form of CAP. Additionally, for 6 hours at room temperature, the CAP solid form was exposed to 254 nm UV radiation. Prior to the examination of the forced degradation samples, a suitable blank was injected and 50 μg ml^{-1} of the constant concentration of solutions was used throughout.

RESULT AND DISCUSSION

Optimization of Chromatographic Conditions

In this study, it was aimed to determine CAP with lower analysis time and sharp peak shapes compared to other studies in the literature. To determine the optimal experimental conditions, the effects of wavelength, organic solvent type, mobile phase composition, pH of the buffer solution, temperature, and flow rate parameters were investigated. Moreover, the precision and accuracy of the developed method can be enhanced by using an appropriate IS. The use of IS also supports correcting fluctuations in the response of the detector. For this purpose, PAR was chosen as the most suitable IS. Initially, five different wavelengths were tested to obtain optimum high selectivity. When the peak heights and areas for CAP and PAR were evaluated, it was found that the most suitable wavelength for the analysis was 220 nm to obtain highly selective and sensitive results (Figure 1A). Organic solvents used as mobile phase components are directly related to the retention of substances in the column. For this purpose, experiments were carried out using methanol and acetonitrile. When methanol was utilized as the organic solvent, CAP was not retained because of its polar nature (logP: 0.34) (Figure 1B). By preparing various amounts of ammonium acetate concentration (5-40 mM), the concentration of the buffer solution was examined as a further parameter. Based on the shape and selectivity of the CAP peak, 5 mM was chosen for further investigation (Figure 1C). The ratio of organic solvent has a significant impact on the peak shape and retention in chromatographic investigations. Therefore, varying proportions of mobile phase compositions by buffer solution and acetonitrile in the range of 50-85% have been evaluated in studies. Finally, it was discovered that the ideal mobile phase composition consisted of 50% buffer solution and 50% acetonitrile (Figure 1D). According to the literature, CAP has pKa values of 3.7 (carboxyl group) and 9.8 (thiol group) [9], so different pH of the mobile phase was evaluated to achieve maximum retention. The buffer solution's pH of 7.0 was determined to be the optimal pH based on the resistance of the utilized stationary phase to pH (Figure 1E). As one of the important parameters, the column temperature was varied between 25°C and 45°C. Low temperatures resulted in better peak shape and shorter retention time. This is why 25°C was selected as the ideal column temperature (Figure 1F). Lastly, to obtain the best performance, the flow rate of the method was varied from 1.0 to 1.5 ml min^{-1}.

When the results are evaluated, the flow rate was set constant at 1.5 ml min^{-1} (Figure 1G).

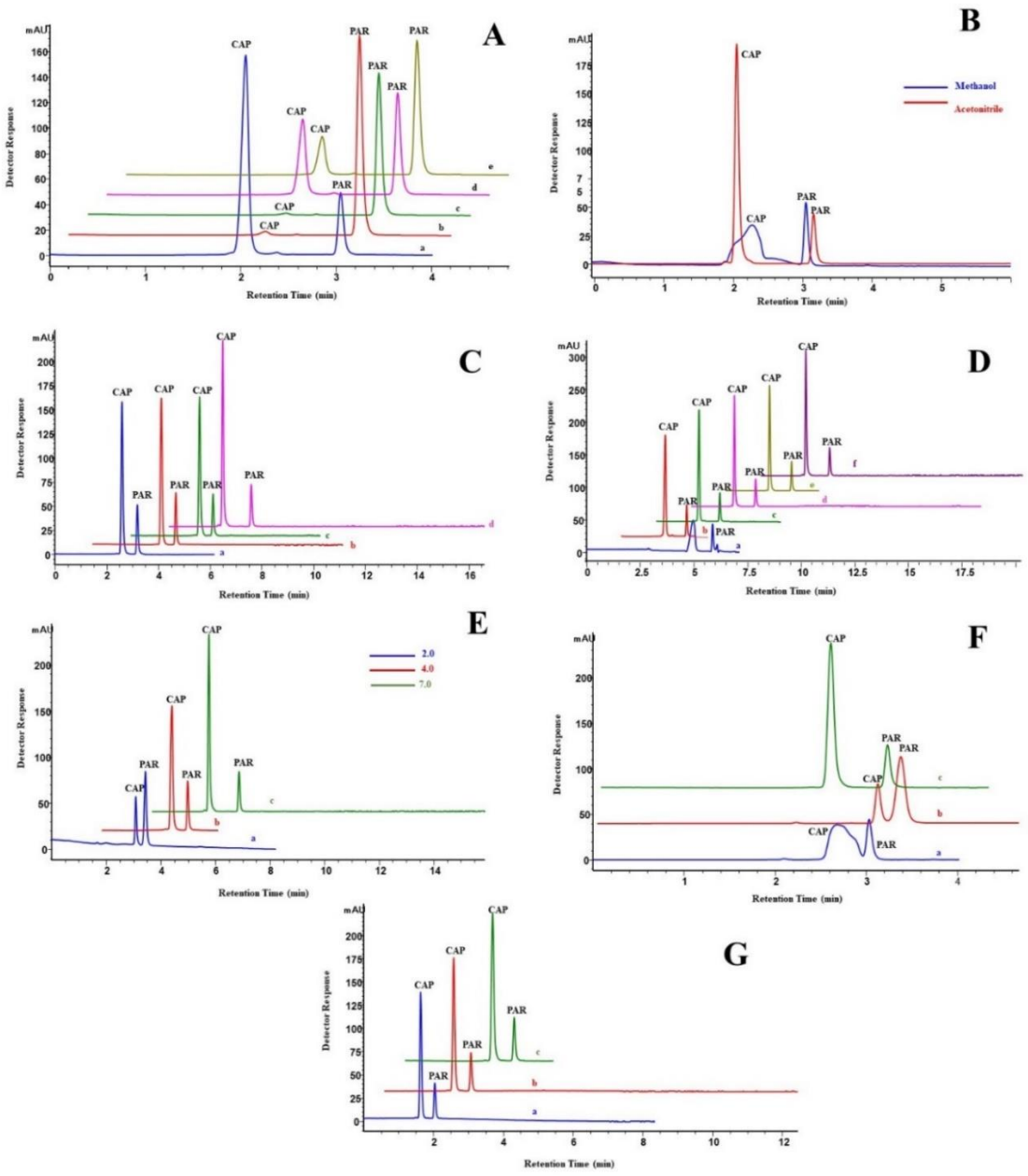

Figure 1. A) Effect of wavelength; 220 nm(a), 250 nm (b), 260 nm (c), 230 nm (d), 210 nm (e), **B)** Effect of organic solvent, **C)** Effect of buffer solution concentration; 10 mM Ammonium acetate (a), 20 mM Ammonium acetate (b), 40 mM Ammonium acetate (c), 5 mM Ammonium acetate (d), **D)** Effect of mobile phase composition; Acetonitrile: Ammonium acetate; (85/15; v/v) (a), (80/20; v/v) (b), (75/25; v/v) (c), (70/30; v/v) (d), (60/40; v/v) (e), (50/50; v/v) (f), **E)** Effect of pH of buffer solution, **F)** Effect of column temperature; 45°C (a), 35°C (b), 25°C (c); **G)** Effect of flow rate; 1.5 ml min^{-1} (a), 1.25 ml min^{-1} (b), 1.0 ml min^{-1} (c)

After the optimum conditions were determined, the system suitability test (SUT) parameters were evaluated based on the ICH guidelines for the determination and quantification of CAP. Under optimized conditions, the retention time of CAP was found as 1.59 min, and other parameters were presented in Table 1.

Table 1. SUT parameters in optimized conditions

Parameters	CAP	PAR
Retention time (min)	1.59	2.00
Capacity factor (k')	0.13	0.43
Resolution (R_s)	4.40	-
Theoretical plates (N)	3476	6004
Selectivity factor (α)	3.31	-
Tailing factor	0.81	0.82

Analytical Performance and Validation

In this study, the developed RP-HPLC method was validated in accordance with standard validation guidelines [19-21]. A series of standard solutions (eleven different concentrations) containing CAP (0.5-200 µg ml^{-1}) and PAR (100 µg ml^{-1}) were analyzed with the optimized method (Figure 2). The linearity of the developed RP-HPLC method was evaluated by a correlation coefficient ($R^2 > 0.999$) of linear in the stated ranges. The limit of detection (LOD) and limit of quantification (LOQ) were evaluated to demonstrate the sensitivity of the developed RP-HPLC method based on the ICH guideline.

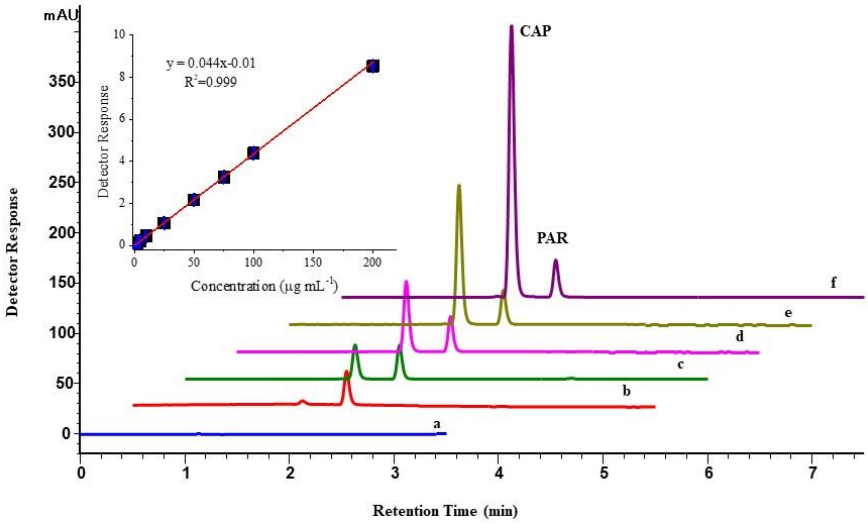

Figure 2. Calibration chromatograms of CAP mobile phase as a blank (a), 2.5 µg ml^{-1} (b), 10 µg ml^{-1} (c), 50 µg ml^{-1} (d), 100 µg ml^{-1} (e), 200 µg ml^{-1} (f) in optimized conditions (Inset: Calibration curve for CAP analysis)

The 3 s/m and 10 s/m criteria, where 's' was taken as the standard deviation of the peak area of the lowest concentration and, 'm' was taken as the slope of the associated calibration curve, were used to determine LOD and LOQ, respectively. Based on the calculations, LOD was determined to be 0.51 ng ml^{-1} and LOQ was determined to be 1.56 ng ml^{-1}. Furthermore, the accuracy of the developed method was also tested intraday and between days by injecting three different calibration solution levels on the same day and three days in a row, respectively. The relative standard deviation (RSD%) was determined as a percentage obtained to be acceptable and less than 2%. Table 2 provides a summary of the calibration results' characteristics and the associated validation parameters. Moreover, the analysis of CAP from tablet samples was also achieved and reported in terms of labeled and found amount in table 2.

Table 2. Validation parameters for the determination of CAP by the developed RP-HPLC method

Parameters	CAP
Linear concentration range (µg ml^{-1})	0.5-200
Slope of calibration curve	0.044
Intercept of calibration curve	-0.01
Correlation Coefficient	0.999
Standard error of slope	5.41×10^{-4}
Standard error of intercept	2.72×10^{-4}
LOD (µg ml^{-1})	5.10×10^{-4}
LOQ (µg ml^{-1})	1.56×10^{-3}
Within-day Precision (RSD %)*	0.05
Between-day Precision (RSD %)*	0.61
Labeled amount (µg ml^{-1})	20.00
Amount found (µg ml^{-1})	19.68
RSD (%)*	0.63
Bias (%)	1.59

* Each result was obtained by means of five experiments

The method accuracy was examined according to recovery experiments from the tablet dosage form of CAP. After analyzing the known amount of CAP in tablet form, these samples were spiked with the bulk CAP solution by rates ranging from 25–150% (Figure 3). The recovery data were shown together with the RSD% and Bias% after each measurement was carried out 5 times. The recoveries were found in the range between 99.45%–102.55% for the commercial dosage form, and all results were tabulated in Table 3.

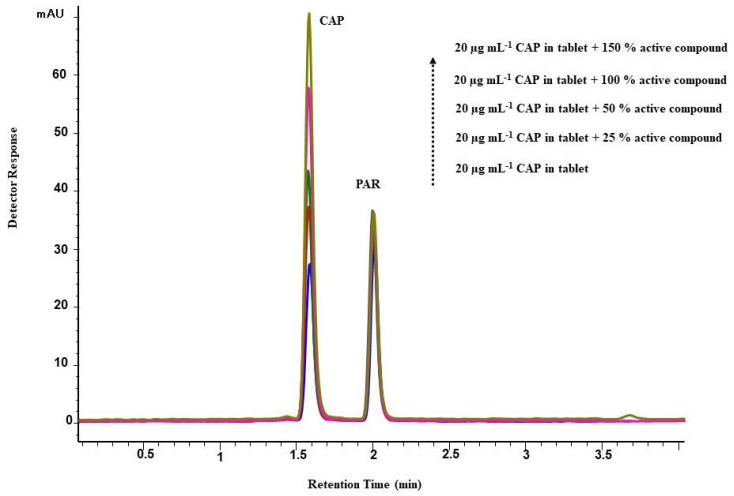

Figure 3. Recovery chromatograms in optimized conditions

Table 3. The accuracy results of the CAP determination in tablet dosage form by the developed RP-HPLC method

Parameters	Accuracy Levels			
Added (µg ml^{-1})	5 *(for 25%)*	10 *(for 50%)*	20 *(for 100%)*	30 *(for 150%)*
Found (µg ml^{-1})	5.64	10.14	20.32	28.77
Recovery (%)	102.55	100.45	100.80	99.45
RSD% of recovery*	1.16	0.86	0.64	0.38
Bias (%)	-2.55	-0.45	-0.80	0.55

* Each result was obtained by means of five experiments

The robustness of the developed method was examined as part of validation studies by assessing the bulk form of CAP under the deliberate modifications of the optimal conditions. For this assay, 50 µg ml^{-1} of standard CAP was used. The resulting RSD% values (<2) were used to evaluate the deliberate changes in the wavelength (±2 nm), the flow rate of the developed method (±0.1 ml min^{-1}), and the temperature of the column (±2°C). The robustness study's findings show that minor adjustments to the optimal conditions had little impact on the developed chromatographic method that had been adjusted (Table 4).

Table 4. Robustness results for CAP determination

Changed Parameters	R_t (min)	Response (concentration µg ml^{-1})
Wavelength: 220 nm	1.590	49.79
SD	0.002	0.21
RSD%	0.13	0.42
Wavelength: 218 nm	1.590	52.54
SD	0.003	0.36
RSD%	0.21	0.69
Wavelength: 222 nm	1.589	48.17
SD	0.001	0.31
RSD%	0.08	0.64
Flow rate: 1.50 ml min^{-1}	1.590	49.79
SD	0.002	0.21
RSD%	0.13	0.42
Flow rate: 1.40 ml min^{-1}	1.690	52.74
SD	0.008	0.53
RSD%	0.48	1.00
Flow rate: 1.60 ml min^{-1}	1.485	53.20
SD	0.004	0.45
RSD%	0.27	0.85
Temperature: 25°C	1.590	49.79
SD	0.002	0.21
RSD%	0.13	0.42
Temperature: 23°C	1.594	51.80
SD	0.004	0.54
RSD%	0.24	1.03
Temperature: 27°C	1.589	50.43
SD	0.006	0.25
RSD%	0.39	0.49

Finally, forced degradation studies were carried out to produce possible degradation products, and the specificity of the developed RP-HPLC method was investigated among the possible interferences. Table 5 and Figure 4 include the findings of the degradation studies. Under drastic conditions including 0.1 N HCl, 0.1 N NaOH, and 0.3% H_2O_2 within 5 min. CAP completely degraded. When the CAP was exposed to 0.01 N HCl for 6 h at room temperature, the acidic hydrolysis was carried out, and degradation of CAP was found 28% at the end of this period. In basic hydrolysis with 0.01 N NaOH, only 7% of CAP was degraded. Under oxidative degradation using 0.03% H_2O_2, almost total CAP degraded gradually over the end of 6 hours. Moreover, when photodegradation and thermal degradation were investigated, it can be said that the CAP did not degrade for 6 hours.

Moreover, there are many research studies for the determination of CAP using HPLC. From the reported methods, CAP was detected in human serum, human plasma, and tablets. This novel RP-HPLC method is compared with the reported methods, as shown in Table 6. Consequently, it can be said that the more sensitive RP-HPLC method, which was less time-consuming and has good recovery values, was developed.

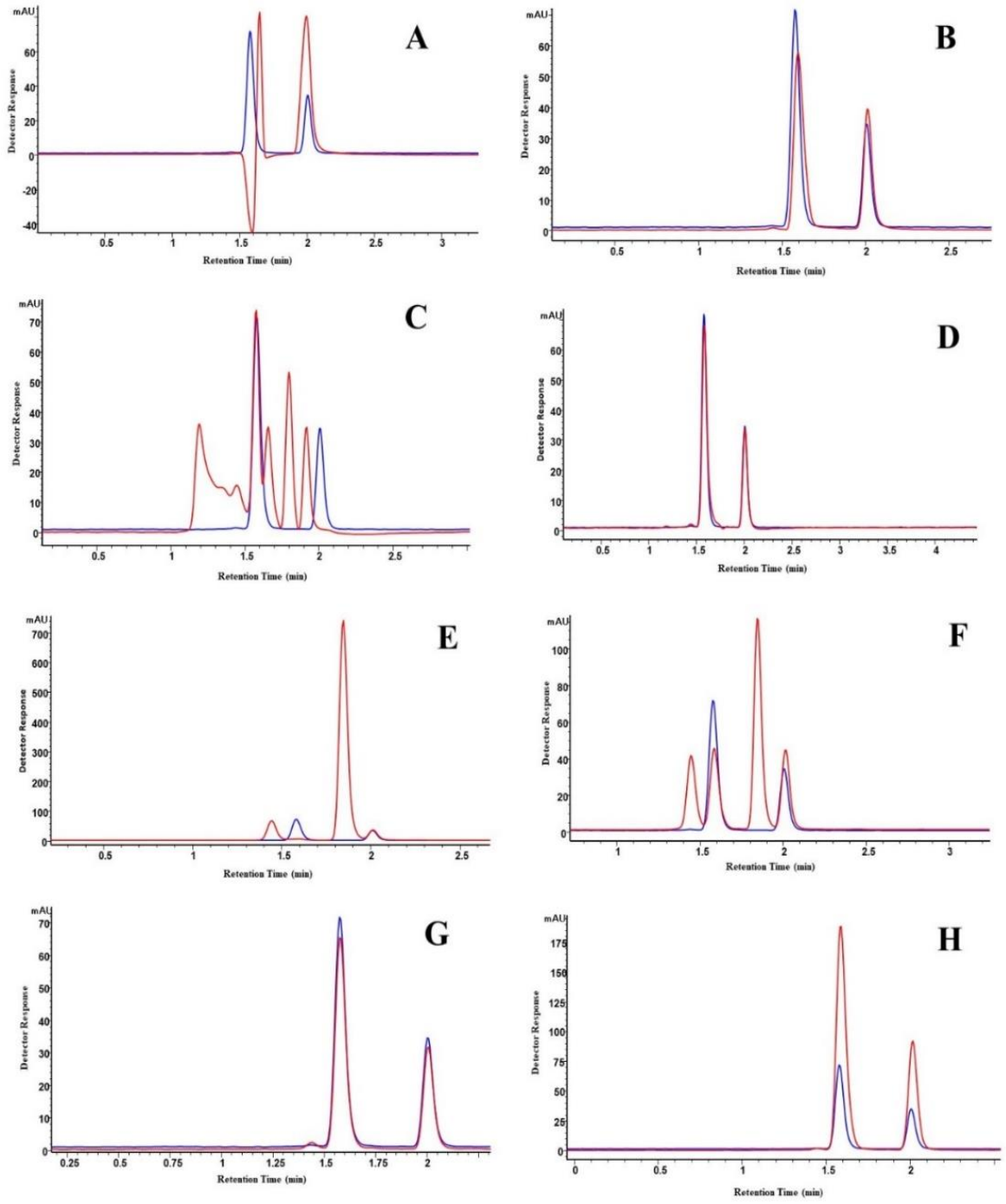

Figure 4. Chromatograms for acid degradation after 12 h; A: 0.1 N HCl, B: 0.01 N HCl, alkaline degradation; C: 0.1 N NaOH, D: 0.01 N NaOH, oxidative degradation; E: 0.3% H_2O_2, F: 0.03% H_2O_2, G: photodegradation, H: thermal degradation (blue line: original chromatograms, red line degradation chromatograms)

Table 5. % Degradation results for CAP

Degradation type	Degradation condition	% Degradation
Acid degradation	HCl (*0.1 N*), after 5 min	100 %
	HCl (*0.01 N*), after 5 min	22 %
	HCl (*0.01 N*), after 1 h	22 %
	HCl (*0.01 N*), after 3 h	26 %
	HCl (*0.01 N*), after 6 h	28 %
Alkaline degradation	NaOH (*0.1 N*), after 5 min	100 %
	NaOH (*0.01 N*), after 5 min	4 %
	NaOH (*0.01 N*), after 1 h	4 %
	NaOH (*0.01 N*), after 3 h	7 %
	NaOH (*0.01 N*), after 6 h	7 %
Oxidative degradation	H_2O_2 (*0.3%*), after 5 min	100 %
	H_2O_2 (*0.03%*), after 5 min	8 %
	H_2O_2 (*0.03%*), after 1 h	33 %
	H_2O_2 (*0.03%*), after 3 h	76 %
	H_2O_2 (*0.03%*), after 6 h	95 %
Photodegradation	After 5 min	1 %
	After 1 h	6 %
	After 3 h	7 %
	After 6 h	7 %
Thermal degradation	After 5 min	2 %
	After 1 h	3 %
	After 3 h	4 %
	After 6 h	4 %

Table 6. Comparison of studies for determination of CAP

Method	IS	Linear range (µg ml^{-1})	LOD/LOQ (µg ml^{-1})	Applications	R_t	References
HPLC-DAD	-	500-1200 100-240	-	Tablet	~ 2 min	[14]
HPLC-DAD	Thiol	30-130	LOD: 0.018 LOQ: 0.05	Tablet	7.21 min	[15]
HPLC–UV	2-propene-1-thiol	0.003-2	LOQ: 0.003	Human plasma	8.4 min	[16]
HPLC-PDA	-	5-35	LOD: 0.4763 LOQ: 1.4434	Bulk form	1.589 min	[17]
HPLC–UV	-	5.05-50.5	LOD: 1.13 LOQ: 3.394	Bulk form	~ 12 min	[18]
HPLC–UV	-	2.5-250	LOD:0.145 LOQ: 0.441	Tablet and human serum	4.78 min	[22]
HPLC–UV	-	30-300	LOD:0.204 LOQ: 0.620	Tablet and human serum	1.09 min	[23]
LC–UV	-	0.25-25	LOD:0.0013 LOQ: 0.0042	Human serum	2.80 min	[24]
HPLC–UV	-	0.25-200	LOD:0.08 LOQ: 0.25	Tablet	~ 2 min	[25]
RP-HPLC-DAD	PAR	0.5-200	LOD: 0.00051 LOQ: 0.00156	Tablet	1.60 min	This study

Green Assessment Using Analytical Greenness Calculator (AGREE) and Green Analytical Procedure Index (GAPI)

In the literature, the greenness of the developed analytical methods is evaluated using a variety of tools. In this work, the greenness of the RP-HPLC method for CAP was assessed using two different online software. For this purpose, covering the entire procedure from sample preparation to the end of the analysis, the Green Analytical Procedure Index (GAPI) based on the 12 principles of green analytical chemistry (SIGNIFICANCE) and the newly suggested Analytical Greenness Calculator (AGREE) techniques were used. These evaluations have the advantages of being quick, easy, efficient, and producing clear results. The AGREE and GAPI approaches display the evaluation as a graph that resembles a clock, with the final score and color representation in the centre, which are the outcomes of the evaluation of the different criteria. While the fifteen pentagrams that make up GAPI each represent a different stage of the analysis process and are color-coded according to their influence on the environment, AGREE gives a numeric number from 0 to 1 for evaluation within the core pictogram [26-28]. As shown in Figure 5, AGREE has an overall score of 0.7 but has 8 yellow and 6 green pentagrams and a single red zone in all criteria in the GAPI assessment. These results showed the green effect of the developed new method on both GAPI and AGREE metrics for CAP analysis. In addition, when the developed RP-HPLC method was compared with the reported methods in the literature, it stands out with its shorter analysis time and is environmentally friendly [17,29,30].

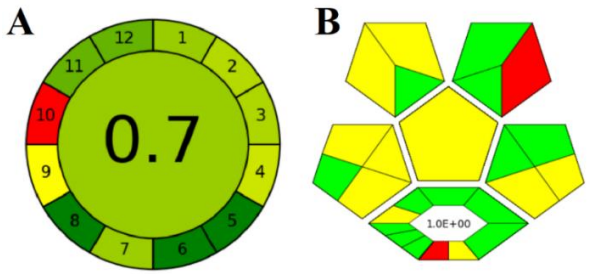

Figure 5. Greenness assessment of the developed HPLC method by AGREE (A) and GAPI (B) approaches

In conclusion, the correct use of drugs in recommended doses has important effects on the quality of the treatment of diseases and on human health. Therefore, it is necessary to monitor the active ingredient content in commercial dosage forms. In this research, a novel RP-HPLC method is introduced for the determination of the antihypertensive drug CAP. The method, which was validated by optimizing the parameters required for the analysis, was successfully applied to the tablet dosage form of CAP. Analyzing CAP in a short time like 1.59 minutes without any interference effect and with higher sensitivity has outperformed the methods in the literature. Also, looking at the greenness profile of the method, it can be said that the developed HPLC method was cheaper and environmentally friendly.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

The authors declare that there is no real, potential 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, 47(3): 907-914, 2023
Doi: 10.33483/jfpau.1291975

ÖZGÜN MAKALE / ORIGINAL ARTICLE

AKCİĞER KANSERİ VE MEME KANSERİ HÜCRELERİNDE SAFRANAL BİLEŞİĞİNİN SİTOTOKSİK AKTİVİTESİNİN GERÇEK ZAMANLI İZLENMESİ

REAL-TIME MONITORING OF CYTOTOXIC ACTIVITY OF SAFRANAL COMPOUND ON LUNG CANCER AND BREAST CANCER

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ÖZ

Amaç: *Akciğer adenokarsinomu ve meme kanseri en sık görülen kanser türleridir ve dünya çapında kansere bağlı ölümlerin önde gelen nedenlerindendir. Safranal bileşiğinin çeşitli farmakolojik etkileri ve sitotoksik özellikleri vardır. Bu çalışmanın amacı, safranal bileşiğinin insan akciğer karsinomu hücre hattı (A549), insan meme kanseri hücre hattı (MCF-7) ve insan bronşiyal epitel sağlıklı hücre (Beas-2b) hatları üzerindeki sitotoksisitesini sürekli izleme yoluyla tahlil etmektir.*
Gereç ve Yöntem: *Hücreler, safranal ile 1, 10, 100 µM konsantrasyonda muamele edildi ve bu bileşiğin hücre canlılığı üzerindeki etkisini belirlemek için xCELLigence gerçek zamanlı hücre analizörü kullanılmıştır. E-plaka kuyularının empedansı aracılığıyla hücre indeksi görüntülenerek her 15 dakikada bir izlenmiştir.*
Sonuç ve Tartışma: *Yapılan çalışmalar sonucunda safranal bileşiğinin MCF-7 hücre hattı üzerinde sitotoksik etkiye sahip olduğu, A549 ve Beas-2b hücreleri üzerinde toksik etkisinin olmadığı belirlenmiştir. MCF-7 hücre hattında, hücre indeksi değişiklikleri, kontrol grubu ile karşılaştırıldığında tüm konsantrasyonlarda azaldığı tespit edilmiştir. Kanser türüne göre bu ilaçların etkinliğini artırmak için geleneksel antikanser ilaçlar ve safranal bileşiği kombinasyonu kullanılabilir.*
Anahtar Kelimeler: *A549 hücre hattı, MCF-7 hücre hattı, safranal, sitotoksisite, xCELLigence*

ABSTRACT

Objective: *Lung adenocarcinoma and breast cancer are the most commonly occurring cancer types and they are the leading cause of cancer-related deaths worldwide. Safranal has various pharmacological effects and have cytotoxic properties. Aim of this study was by continuous monitoring to assay the cytotoxicity of safranal on human lung carcinoma cell line (A549), human*

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Gönderilme / Submitted : 03.05.2023
Kabul / Accepted : 08.08.2023
Yayınlanma / Published : 20.09.2023

breast cancer cell line (MCF-7) and human bronchial epithelial cell lines (Beas-2b).
Material and Method: *Cells were treated 1, 10, 100 μM concentrations with safranal and xCELLigence real-time cell analyzer were used to determine this compound effect on cell viability. The cell index was monitored every 15 minutes by visualizing the impedance of the E-plate wells.*
Result and Discussion: *Consequently the studies, it was determined that the safranal has cytotoxic effect on MCF-7 cell line and did not has a toxic effect on A549 and Beas-2b cells. In the MCF-7 cell line, cell index alterations were found to be decreased at all concentrations compared with the untreated control. The combination of conventional anticancer drugs and safranal can be used to increase the effectiveness of these drugs considering cancer type.*
Keywords: *A549 cell line, cytotoxicity, MCF-7 cell line, safranal, xCELLigence*

GİRİŞ

Meme kanseri ve akciğer adenokarsinomu, dünyada kansere bağlı ölümlere yol açan en yaygın kanser türleridir [1]. Hedefe yönelik ilaç tedavileri, immünoterapi ve cerrahi yaklaşımlar dahil olmak üzere çeşitli tedavi yaklaşımları halen devam etmektedir. Diğer tüm kanserler gibi, anormal hücre proliferasyonu bu kanser türlerinin temel özelliğidir [2]. Mevcut terapötiklerin, özellikle gelişmiş ülkelerde, meme kanseri ölümlerini başarılı bir şekilde kontrol etmesine rağmen, meme kanseri dünya çapında kadınlar arasında en yaygın kanser ve önde gelen ölüm nedeni haline gelmiştir [3]. Kanser hücrelerinin çoğalmasına karşı takviyeler, meme kanserinin önlenmesine ve tedavisine katkıda bulunabilir. Ek olarak, östrojen biyosentezini katalize eden aromataz enziminin inhibe edilmesi, meme kanseri için etkili bir tedavi seçeneği olarak kabul edilmektedir [2]. Kanserde ilaç tedavileri için güçlü bileşiklere olan gereklilik nedeniyle, ilerleme gösteren araştırmalar değerli bir yaklaşımdır. Meme kanseri ve akciğer kanserinin en yaygın olarak görülen kanser tipleri olması, tedaviye katkı sunabilecek her türlü araştırmayı daha da önemli kılmaktadır. Kanser tedavisinde kullanılan ilaçların ağır yan etkileri vardır. Bu nedenle etkinliği artırabilecek veya daha az yan etkileri olabilecek alternatif moleküllerin antikanserojen etkinliklerinin araştırılmasının yeni farmakolojik etkilerinin açığa çıkarılmasına katkıda bulunabileceği düşünülmektedir.

Safranal, dehidrojenasyon yoluyla beta-siklositralden türetilen bir monoterpenoid ve *Crocus sativus* L.'de bulunan doğal bir üründür [4]. Antidiyabetik, antienflamatuar, antioksidan, antitüssif, antitümör ve antikonvülsanları içeren geniş bir terapötik özellik yelpazesi, safrandan türetilen özel nutrasötik türlerine atfedilmiştir. Safranal, antikanser özellik sergileyen bir bileşiktir; safran ve temel bileşenlerin normal hücreler üzerinde hiçbir sitotoksik etkisinin olmadığı, ancak kanser hücreleri için öldürücü olduğu tespit edilmiştir. Çalışmalar, safranın ve bileşenlerinin kanser gelişimine karşı uygun şekilde hareket ettiğini ve tümörlere karşı seçici toksisite gösterdiğini kanıtlamıştır [5-7].

Canlı hücrelerin metabolik aktivitesini ölçen MTT testi gibi yaygın sitotoksisite testleri, kanser hücre dizilerinin, hücre canlılığının belirlenmesinde hala yaygın olarak kullanılmaktadır [8]. Tüm durumlar için evrensel olarak uygun tek bir yöntem yoktur ve her birinin belirli koşullar altında potansiyel olarak sınırlamaları vardır [9]. Ancak bu testler, yalnızca test edilen bileşiğin seçilen kesin zaman noktası üzerindeki etkisini yansıtan son nokta testleridir. Bazen ilaç adayı, bu yaygın sitotoksisite testleri tarafından test edilen zaman noktasından/noktalarından daha erken zaman noktalarında akut bir etkiye neden olabilir. Bu nedenle, gerçek zamanlı bir değerlendirme için gerçek zamanlı sitotoksisite testlerini kaçınılmaz kılan bu tür uç nokta testleri ile bu akut etkiler gözden kaçırılabilir. xCELLigence Sistemi, hücrelerin kantitatif ve gerçek zamanlı izlenmesiyle ve yapışık hücrelerin durumunu *in vitro* olarak analiz etmek için empedans ölçümlerine dayalı olarak hücre proliferasyonu ve sitotoksisitenin değerlendirilmesine olanak tanımaktadır. Elektrik empedansının ölçülmesi, hücrelerin yapışması, çoğalması ve yaşayabilirliği hakkında fikir vermektedir. Hücre bağlanması ve yayılması nedeniyle empedansta gözlenen değişiklikler Hücre İndeksi (CI) olarak adlandırılan parametre olarak ifade edilmektedir. CI, hücre canlılığını, dolayısıyla hücre sayısını, bağlanma kalitesini ve hücre tipini yansıtmaktadır [10-12]. Hücre canlılığının izlenmesi kritiktir ve xCELLigence sistemi, hücrelerin sürekli olarak ölçülmesini ve kantifikasyonunu sağlamaktadır [13,14]. Ayrıca, xCELLigence sistemi, zamana bağlı IC_{50} değerlerinin gerçek zamanlı olarak hesaplanmasına izin vermektedir [11]. Bu analizin özellikle büyük ölçekli ilaç taramasında yararlı olduğu kanıtlanmıştır [10-12].

Mevcut çalışmada, safranal bileşiğinin A549, MCF-7 hücre hatlarına karşı antiproliferatif etkileri araştırılmıştır ve antikanser aktivitesinin, sağlıklı hücre olan Beas-2b hücre hattı ile kıyaslanarak gerçek zamanlı hücre analizörü ile değerlendirilmesi amaçlanmıştır. Bu amaçla safranal bileşiğinin A549, MCF-7 ve Beas-2b hücre hatlarındaki etkileri gerçek zamanlı hücre analizi ile ilk kez gösterilmiştir.

GEREÇ VE YÖNTEM

A549 (ATCC, CCL-185) insan akciğer karsinomu hücre hattı, MCF-7 (ATCC, HTB-22) insan meme kanseri hücre hattı ve Beas-2b (ATCC, CRL-9609) insan bronşiyal epitel sağlıklı hücre hattı American Type Culture Collection'dan (ATCC) satın alınmıştır. Dulbecco's Modifiye Eagle Ortamı/Besin Karışımı F-12 (DMEM/F12), DMEM, RPMI besiyeri, 100 U/ml penisilin-100 ug/ml streptomisin, fetal sığır serumu (FBS), tripsin-EDTA, Capricorn Scientific'ten (Ebsdorfergrund, Almanya) satın alınmıştır. 96 kuyucuklu E-plaka, ACEA Biosciences'tan (San Diego, ABD) satın alınmıştır. Safranal, Tamoksifen ve Gemsitabin MedChemExpress'ten (NJ, ABD) satın alınmıştır.

Hücre Kültürü

%10 FBS, %1 L-glutamin, 100 U/ml Penisilin ve 100 µg/ml Streptomisin içeren sırasıyla DMEM-F12, DMEM ve RPMI besiyeri ile A549, MCF-7 ve Beas-2b hücreleri çoğaltılmıştır. Hücreler, %5 $C0_2$ içeren nemli bir atmosferde 37°C'de inkübe edilmiştir. Safranal dimetilsülfoksit (DMSO) içinde çözülmüştür ve nihai DMSO konsantrasyonunun %0.1'den az olduğu belirlenmiştir.

Gerçek Zamanlı Hücre Analizi (RTCA)

A549 hücrelerinin optimum ekim konsantrasyonu belirlenmiştir ve ardından hücreler (12500 hücre/kuyu), MCF-7 hücreleri (10000 hücre/kuyu) ve Beas-2b hücreleri (10000 hücre/kuyu) 96 kuyucuklu E-plakaya ekilmiştir. Hücreler yaklaşık %80 doluluğa ulaştığında, %0.25 tripsin-EDTA uygulanmıştır. Daha sonra 25°C'de 5 dakika 1000 rpm'de santrifüjlenip 96 kuyucuklu xCELLigence E-plakasına ekilmiştir. Hücreler, E-plakaya ekildikten yaklaşık 24 saat sonra logaritmik olarak büyüme aşamasına ulaştığında, 1, 10, 100 µM konsantrasyonlarında safranal uygulanmıştır. Tamoksifen ve Gemsitabin pozitif kontrol olarak kullanılmıştır. E-plaka kuyularının empedansı aracılığıyla her 15 dakikada bir izlenmiştir. Deneyler yaklaşık 72 saat süreyle yürütülmüştür. Bu bileşiklerin etkisi, küçük değişikliklerle üreticinin talimatlarında (Roche Applied Science ve ACEA Biosciences) açıklandığı gibi RTCA sistemi ile izlenmiştir. RTCA elektriksel empedansı ölçmekte ve hücre canlılığını, hücre sayısını, bağlanma kalitesini ve hücre tipini ifade eden bir parametre olan hücre indeksini görüntülemektedir. IC_{50} (yarı maksimum inhibisyon konsantrasyonu) değerleri, 24 saatte RTCA entegre yazılımı aracılığıyla hesaplanmıştır.

İstatistiksel Analiz

Tüm hesaplamalar, xCELLigence sisteminin RTCA entegre yazılımı kullanılarak elde edilmiştir. Veriler ortalama ± standart sapma olarak ifade edilmiştir. CI, xCELLigence sistemi ile tekrarlanan deneylerden (n = 4) hesaplanmıştır. Kontrol ve deney grubu arasındaki değerlerdeki farklılıkları karşılaştırmak için GraphPad Prism Version 9.5.1 programı kullanılarak istatistiksel analiz yapılmıştır. Gruplar arasındaki farklılıklar tek yönlü ANOVA varyans analizi ardından Dunnett's post-hoc analizi yapılarak belirlenmiştir. $p < 0.05$ değerleri istatistiksel olarak anlamlı kabul edilmiştir.

SONUÇ VE TARTIŞMA

Safranalın Etkilerinin RTCA Kullanılarak Gerçek Zamanlı Olarak İzlenmesi

Gerçek zamanlı hücre analizi; hücre proliferasyonunu ve morfoloji değişimini, işaretçi kullanmadan saptama, fizyolojik temas gerektirmeksizin gerçek zamanlı izleme ile otomatik ölçümler yapabilme, yüksek duyarlılık ve doğrulukta görüntüleme için kullanılan bir sistemdir. Elektronik plakalara entegre edilmiş mikroelektronik hücre sensörü dizisi, elektrodların elektronik empedansının ölçülmesi, elektrodlar üzerindeki değişikliklerin saptanması ve izlenmesini sağlamaktadır. Bu çalışmada safranal, iki kanser hücre hattına (A549 ve MCF-7) ve sağlıklı hücre olan Beas-2b hücre hattına karşı

sitotoksisitesi açısından araştırılmıştır. Sitotoksisite, potansiyel terapötik bir ajanın antikanser aktivitesini tespit etmek açısından önemli bir ölçüt olduğu için bu çalışmada safranal bileşiğinin etkisini belirlemek amaçlanmıştır. Safranal doz-yanıt eğrilerinin tekrarlanan xCELLigence ölçümleri ile elde edilmiştir. Hücrelerin indeksi, hücre sayısı ile doğru orantılı olarak artarken, hücreler bileşik ile muamele edilmiştir. Biyolojik aktivite çalışmalarında safranal bileşiğinin sitotoksik incelemeleri olumlu sonuçlar vermiştir ve konsantrasyona bağlı bir şekilde MCF-7 hücre hattında hücre proliferasyonunu inhibe etmiştir. Safranalın A549 ve Beas-2b hücre hattında sitotoksik etkisinin olmadığı belirlenmiştir (Şekil 1). Etkisi olan potansiyel bir antikanser bileşiğin, kabul edilebilir olması için sağlıklı hücreler üzerinde herhangi bir toksik etkisinin olmaması veya çok düşük düzeyde olması beklenmektedir. Safranal bileşiğinin Beas-2b sağlıklı hücre hattında toksik etkisi bulunmadığı gözlemlenmiştir (Şekil 1c).

Uygulama sonrası 24 saat ve 48 saatlik maruziyetten sonra hücre tipleri için CI değişiklikleri aşağıda verilmiştir (Tablo 1). Safranalın MCF-7 hücre hattında uygulamadan sonra 24. saatteki IC_{50} değeri 12.94 µM (Şekil 2a); 48. saatteki IC_{50} değeri 13.83 µM olarak hesaplanmıştır (Şekil 2b); ve sigmoidal konsantrasyon yanıt eğrisi verilmiştir. Bu eğri, deneysel veri noktalarına en uygun eğriyi matematiksel fonksiyonlara göre oluşturan sigmoidal konsantrasyon-yanıt denklemini kullanarak IC_{50} değerlerinin hesaplanmasını sağlamaktadır.

MCF-7 hücrelerinde gerçek zamanlı hücre analiz sonuçları değerlendirildiği zaman uygulama sonrası 24. saatte, 10 µM Tamoksifen'in ($p<0.0001$) ve 1 µM, 10 µM ve 100 µM safranalın ($p<0.001$) hücre canlılığını anlamlı olarak azalttığı tespit edilmiştir (Şekil 3a). Uygulama sonrası 48. saatte ise, 10 µM Tamoksifen ($p<0.0001$), 1 µM safranal ($p<0.05$), 10 µM safranal ($p<0.05$) ve 100 µM safranalın ($p<0.01$) hücre canlılığını anlamlı olarak azalttığı tespit edilmiştir (Şekil 3b).

Aktif biyomoleküllerin sağlığı geliştirdiği ve çeşitli hastalıkları önlemek veya tedavi etmek için kullanılabileceği bilinmektedir. Potansiyel antikanser bileşiklerinin saptanması için biyomoleküller ile yapılan antikanser aktivite tarama çalışmaları önem kazanmıştır. Bu nedenle, yeni antikanser ilaçların geliştirilmesi ve kanser için daha etkili tedavi stratejilerinin bulunmasında aktif biyomoleküllerin tespiti değerli bir yaklaşım olarak görülmektedir [15]. Safranalın kanser hücrelerinde etkinliği ile ilgili literatürde çeşitli çalışmalar bulunmaktadır. Bir çalışmada, HeLa hücrelerinde safrandan türetilen farklı bileşiklerin potansiyel sitotoksik aktiviteleri değerlendirilmiştir. Safran özlerinin, insan tümör hücrelerinin hücre büyümesini engellediği bildirilmiştir. Safranalın HeLa hücrelerinin büyümesini *in vitro* olarak inhibe edebildiği, yüksek LD_{50} değeri ile birlikte baharattaki düşük yüzdesinin büyümeyi inhibe edici aktiviteye işaret ettiği belirlenmiştir [16].

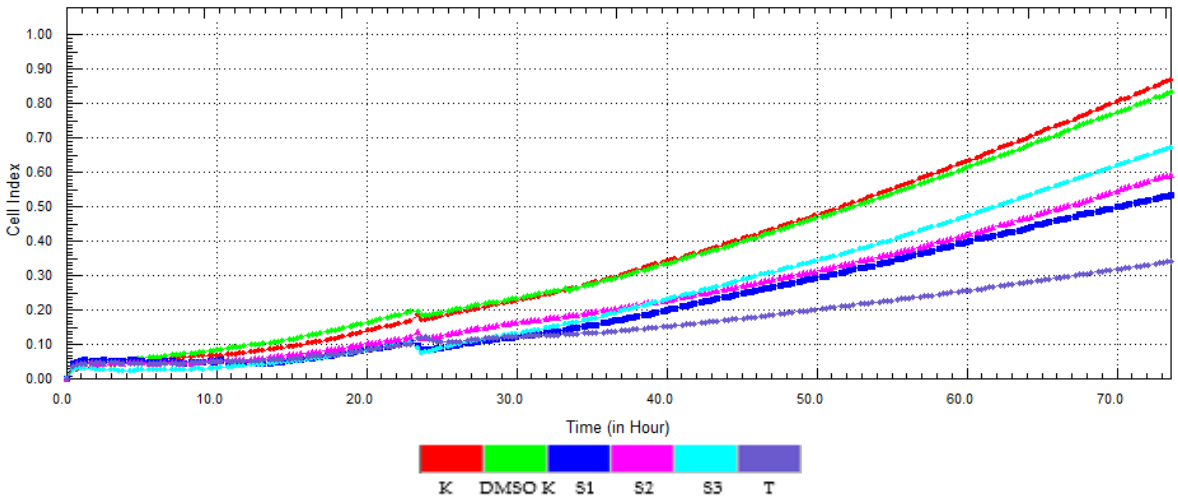
Safranın kanser önleyici özelliklerinin kesin mekanizması belirsizliğini korumaktadır, ancak bazı çalışmalarda birkaç hipotez belirtilmiştir. Bir çalışmada, safranalın antikanser potansiyeli, antianjiyojenik kapasitesiyle ilgilenerek araştırılmıştır. Safranalın, VEGF'nin indüklediği anjiyogenezi *in vitro* ve *ex vivo* olarak çizik yara testi, tüp oluşumu testi, transmembran testi ve aortik halka testi yoluyla inhibe ettiği tespit edilmiştir [5].

Safranalın potansiyel bir kanser önleyici madde olarak kabul edilebileceği ve tübüline bağlanmasının mikrotübül polimerizasyonunun inhibisyonuna yol açabileceği belirtilmiştir [17].

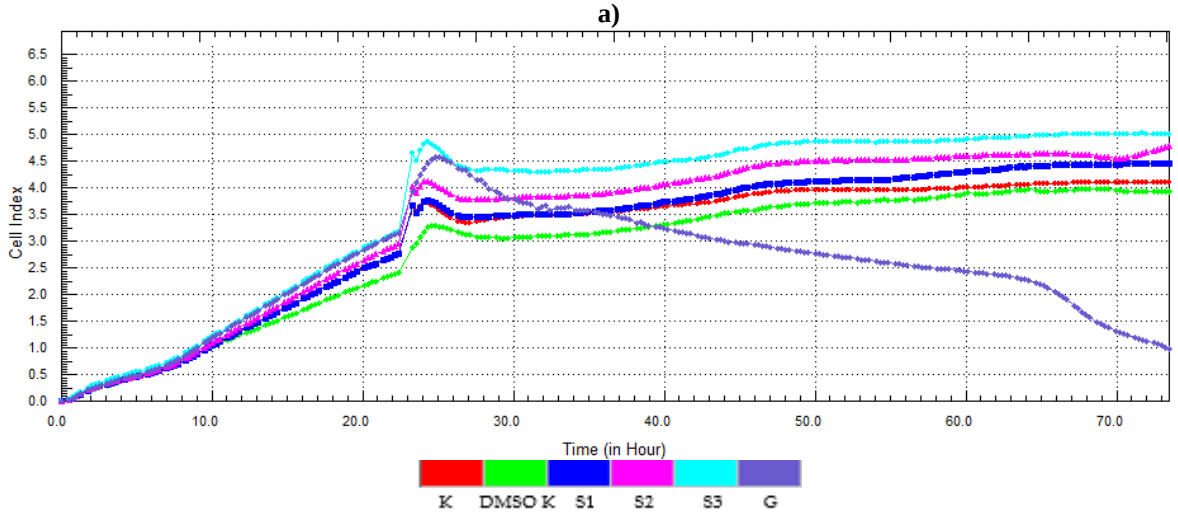
Tablo 1. xCELLigence sistemi kullanılarak safranal uygulandıktan 24 saat ve 48 saat sonra hücre tipleri için CI değişiklikleri

Hücre tipi	**A549**	**A549**	**MCF-7**	**MCF-7**	**Beas-2b**	**Beas-2b**
Zaman	**48. saat**	**72. saat**	**48. saat**	**72. saat**	**48. saat**	**72. saat**
Hücre sayıları (hücre/kuyu)	**12500**	**12500**	**10000**	**10000**	**10000**	**10000**
S1	4.02±0.06	4.36±0.34	0.23±0.01	0.54±0.01	4.44±0.41	3.99±0.48
S2	4.39±0.06	4.61±0.19	0.33±0.09	0.62±0.17	4.66±0.33	4.54±0.12
S3	4.74±0.99	4.91±0.94	0.32±0.01	0.64±0.01	4.69±0.24	4.61±0.13

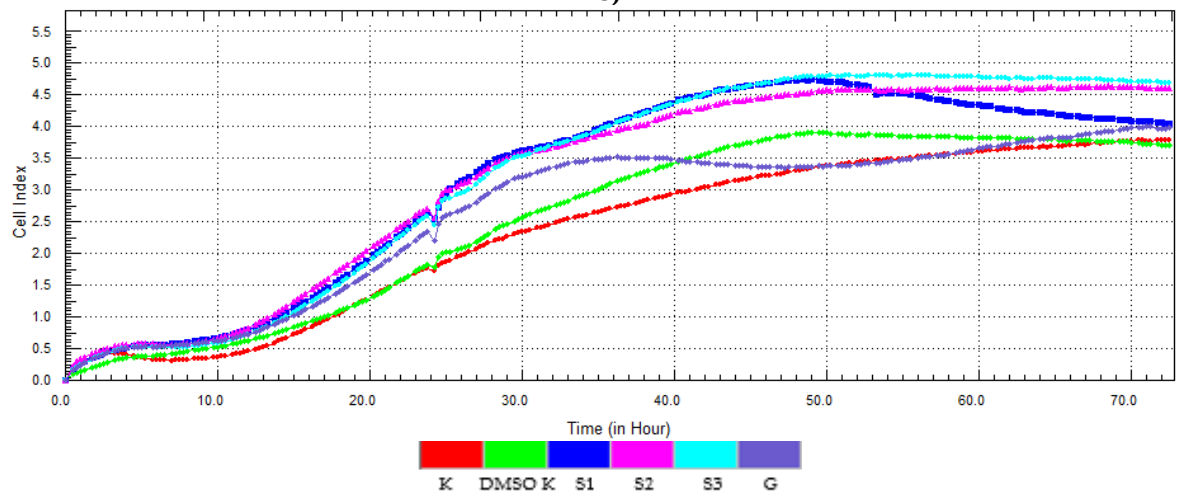
xCELLigence sistemi ile tekrarlanan deneylerden elde edilen hücre indeksi verileri kullanılarak hesaplanmıştır (n = 4). Hücrelere 24. saatte safranal uygulanmıştır. Veriler ortalama ± SD olarak ifade edilmiştir. S1: 100 µM safranal; S2: 10 µM safranal; S3: 1 µM safranal



a)



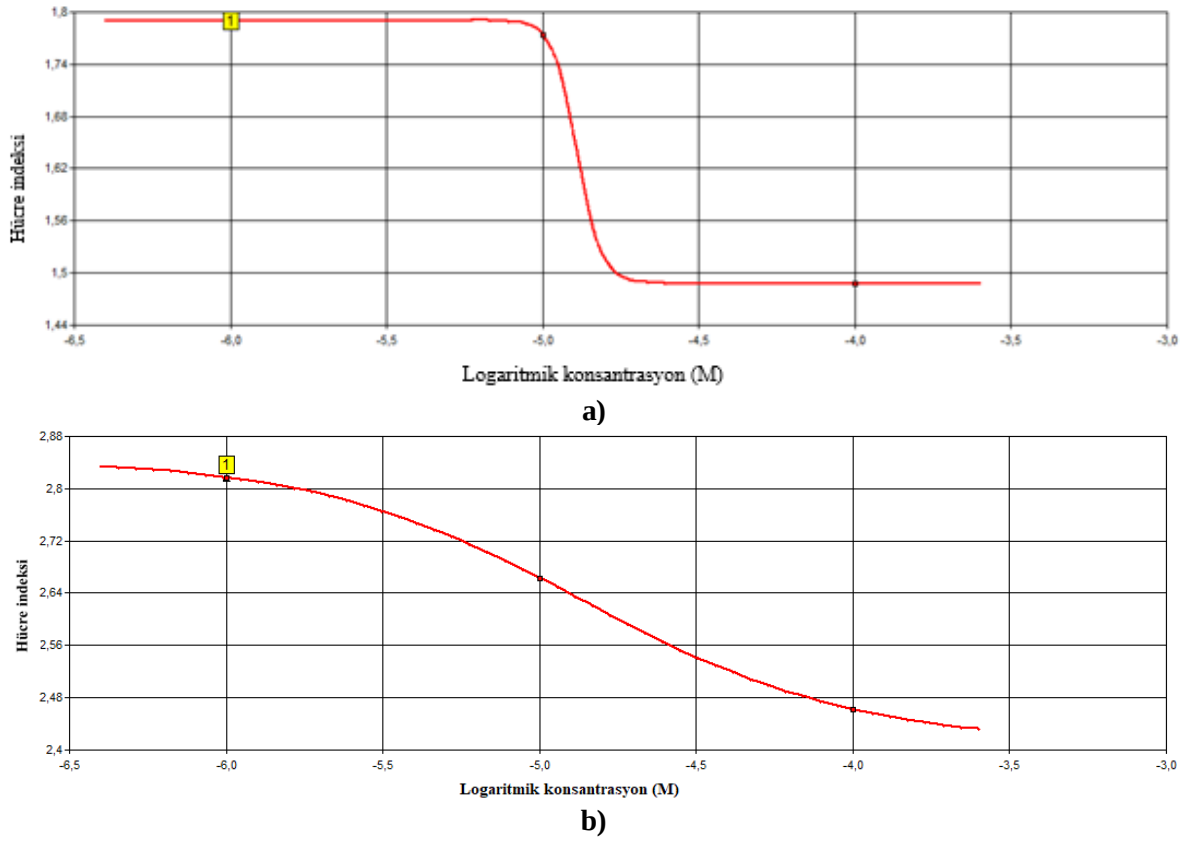
b)



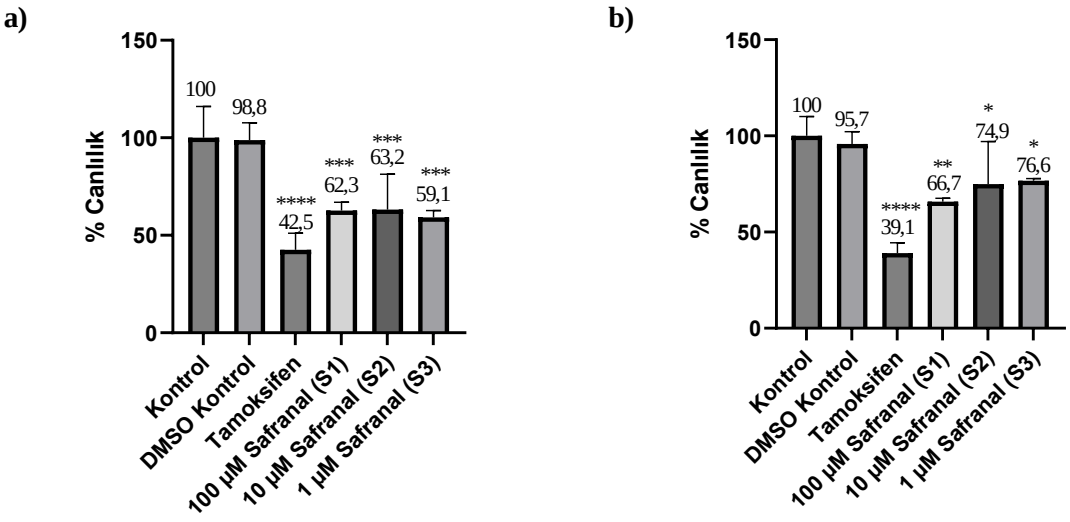
c)

Şekil 1. Hücre çoğalmasına karşı safranal bileşiğinin gerçek zamanlı etkileri
a) MCF-7 b) A549 c) Beas-2b

MCF-7, A549 ve Beas-2b hücreleri, çeşitli konsantrasyonlarda safranal ile muamele edilmiştir. Tüm safranal konsantrasyonları, tedaviden 24 saat sonra belirgin bir şekilde MCF-7 hücrelerinin hücre çoğalmasını azaltmıştır.
K: Kontrol; DMSO K: DMSO Kontrol;
S1: 100 µM safranal; S2: 10 µM safranal; S3: 1 µM safranal; T: 10 µM Tamoksifen; G: 10 µM Gemsitabin



Şekil 2. MCF-7 hücre hattında safranal uygulamasında sigmoidal konsantrasyon yanıt eğrisi
a) 24 saatlik b) 48 saatlik maruziyet



Şekil 3. MCF-7 hücre hattında gerçek zamanlı hücre analiz sonuçları
a) 24 saatlik b) 48 saatlik maruziyet
Değerler GraphPad Prism 9.5.1 programında One-way ANOVA ve post-hoc Dunnett testi ile analiz edildi. *p<0.05 kontrol grubuna kıyasla. Anlamlılık aralığı; *<0.05, **<0.01, ***<0.001 ve ****<0.0001 (n=4). Sonuçlar, ortalama ± ortalamanın standart hatası olarak sunuldu

Safranalın HeLa ve MCF-7 hücrelerinde safranın neden olduğu hücre ölümünde rol oynayabileceği sonucuna varılabileceği belirtilmiştir. Lipozom kapsüllemenin safranalın anti-tümör etkisini geliştirdiği ve özellikle lipozomal formunun kanserde ümit verici kemoterapötik ajanlar olarak araştırılabileceği belirlenmiştir [18].

Literatürdeki çalışmalar, birkaç insan karsinom hücre hattının hem canlılık hem de hücre döngüsü inhibisyonu açısından safranaldan etkilendiğini göstermektedir [19,20].

Yapılan bir çalışma safranal bileşiğinin oral skuamöz hücreli karsinoma karşı (KB hücre hattı) kısmen seçici sitotoksik ve apoptojenik etkilere sahip olduğunu ve normal fibroblast (NIH 3T3) hücreleri üzerinde çok düşük düzeyde etkilere sahip olduğunu göstermektedir [21].

Yıllar boyunca yapılan kapsamlı araştırmalar, safranalın kanser de dahil olmak üzere birçok hastalığın tedavisine yönelik umut verici farmakolojik özelliklere sahip olduğunu göstermiş olsa da, hücresel ve moleküler mekanizmalar aydınlatılmayı beklemektedir. Sonuç olarak safranal, antikanser potansiyeli açısından daha fazla araştırılmaya değecek nitelikte bir bileşiktir. Safranalın etkilerinde yer alan olası mekanizmaların daha fazla incelenmesi, klinik deneyler de dahil olmak üzere sonraki çalışmalar için güçlü bir doğrulamaya yol açacaktır.

YAZAR KATKILARI

Kavram: E.U.; Tasarım: E.U.; Denetim: E.U.; Kaynaklar: E.U.; Malzemeler: E.U.; Veri Toplama ve/veya İşleme: E.U.; Analiz ve/veya Yorumlama E.U.; Literatür Taraması: E.U.; Makalenin Yazılması E.U.; Kritik İnceleme: E.U.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan eder.

ETİK KURUL ONAYI

Yazar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 915-926, 2023
Doi: 10.33483/jfpau.1311157

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IMIDAZOLINONE-BASED SULFONAMIDE DERIVATIVES: SYNTHESIS, CHARACTERIZATION, AND INHIBITORY PROPERTY AGAINST SOME METABOLIC ENZYMES

İMİDAZOLİNON BAZLI SÜLFONAMİD TÜREVLERİ: SENTEZ, KARAKTERİZASYON VE BAZI METABOLİK ENZİMLERE KARŞI İNHİBİTÖR ÖZELLİKLERİ

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ABSTRACT

Objective: *The purpose of the work was to investigate new synthetic compounds of imidazolinone-based sulfonamide derivatives as potent and selective enyzme inhibitors. A number of compounds synthesized and their inhibitory action against acetylcholine esterase (AChE), and human (h) carbonic anhydrase (CA) isoforms I and II were investigated.*
Material and Method: *The identity of the compounds has been confirmed by HRMS, ^{1}H NMR, and ^{13}C NMR. The pharmacological potential of the compounds has been determined by in vitro enzyme-based assays.*

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Submitted / Gönderilme : 08.06.2023
Accepted / Kabul : 08.08.2023
Published / Yayınlanma : 20.09.2023

Result and Discussion: *In this study, a series of imidazolinone-based sulfonamide derivatives were synthesized from 4-(2,4-dimethoxybenzylidene)-2-phenyloxazol-5(4H)-one, sodium acetate, glacial acetic acid, and suitable sulfonamide derivatives such as sulfaguanidine (3), sulfanilamide (4), sulfadiazine (5). These compounds showed potent inhibitory action against acetylcholine esterase (AChE), and human (h) carbonic anhydrase (CA) isoforms I and II. Compound 4 (Ki=19.53±1.23 nM) was a potent and selective inhibitor against hCA I while compound 3 (Ki=16.49±2.20 nM) was found to be potent inhibitor against hCA II. Compound 5 with Ki of 11.68±1.45 nM showed a potent inhibitory effect against the AChE enzyme. Imidazolinone-based sulfonamides can be used in the design of selective CAs inhibitors and anti-Alzheimer's compounds for further studies.*
Keywords: *Imidazolinone, synthesis, acetylcholinesterase, carbonic anhydrase, Alzheimer's disease*

ÖZ

Amaç: *Çalışmanın amacı, güçlü ve seçici enzim inhibitörleri olarak imidazolinon bazlı sülfonamid türevlerinin yeni sentetik bileşiklerini araştırmaktır. Sentezlenen bir dizi bileşik ve bunların asetilkolin esteraz (AChE) ve insan (h) karbonik anhidraz (CA) izoformları I ve II'ye karşı inhibe edici etkileri araştırılmıştır.*
Gereç ve Yöntem: *Bileşiklerin yapısı HRMS, ^{1}H ve ^{13}C NMR ile doğrulanmıştır. Bileşiklerin farmakolojik potansiyeli, in vitro enzim bazlı analizler ile belirlenmiştir.*
Sonuç ve Tartışma: *Bu çalışmada, imidazolinon bazlı sülfonamit bileşikleri serisi 4-(2,4-dimetoksibenziliden)-2-feniloksazol-5(4H)-on, sodyum asetat, buzlu asetik asit ve sulfaguanidin (3), sulfanilamid (4), sulfadiazin (5) gibi uygun sülfonamid türevlerinden hareketle sentezlendi. Bu bileşikler, asetilkolin esteraz (AChE) ve insan (h) karbonik anhidraz (CA) izoformları I ve II'ye karşı güçlü inhibe edici etki gösterdi. Bileşik 4 (Ki= 19.53±1.23 nM), hCA I'e karşı güçlü ve seçici bir inhibitör iken, bileşik 3'ün (Ki=16.49±2.20 nM) hCA II'ye karşı güçlü inhibitör olduğu bulundu. 11.68±1.45 nM Ki'ye sahip bileşik 5, AChE enzimine karşı güçlü bir inhibitör etki gösterdi. İmidazolinon bazlı sülfonamidler, seçici CA inhibitörleri ve anti-Alzheimer bileşiklerinin tasarımında ileriki çalışmalarda kullanılabilirler.*
Anahtar Kelimeler: *İmidazolin, sentez, asetilkolinesteraz, karbonik anhidraz, Alzheimer hastalığı*

INTRODUCTION

Heteroatoms comprise a very prevalent part of a number of active pharmaceutical materials [1]. Statistics indicate that greater than 85 % of all biologically active chemical components have a heterocyclic structure [1]. Using heterocycles to modify the ADME-Tox (absorption, distribution, metabolism, and excretion – toxicity) properties of the compounds is advantageous to obtain drug candidates having favorable pharmacokinetics [2]. An essential and original class of medicinal chemistry comprises nitrogen-based compounds [3-5]. These types of molecules continue to be the focus of increasing attention in recent research in the pharmaceutical sciences [6-8]. Vitamins, nucleic acids, pharmaceuticals, antibiotics, and agrochemicals which has *N*-heterocyclic structure are mainly distributed in nature and exhibit physiological and pharmacological properties [9-11].

Aromatic heterocyclic ring system with five members $C_3N_2H_4$ is an imidazole ring structure with three carbon and two nitrogen atoms. Because of the presence of non-adjacent nitrogen in the ring structure, a diazole is an aromatic heterocyclic ring. The scientific community refers to this group of keto dihydroimidazoles as oxoimidazolines or imidazolinones. They are made up of an imidazoline derivative with carbon-nitrogen double bonds at positions 1 and 3 and carbon-oxygen double bonds at positions 2, 4 or 5 (Figure 1) [12].

Imidazolinones offer a variety of therapeutic properties, including anticonvulsant, antidepressant, antibacterial, anti-inflammatory, MAO inhibitory, anti-Parkinson, antihypertensive, anti carbonic anhydrase, and [13] acetylcholine esterase inhibitory, among others [14]. Kagthara et al. synthesized new imidazolidinone derivatives (Figure 2, compound 3 derivatives) incorporating benzimidazole and tested them *in vitro* for anti-microbial activity against bacteria like *S. citrus*, *E. coli*, and *S. typhi*, and these compounds were compared to standard drugs like ampicillin, norfloxacin, and chloramphenicol. They discovered that the majority of active molecules included imidazolinone ring [15].

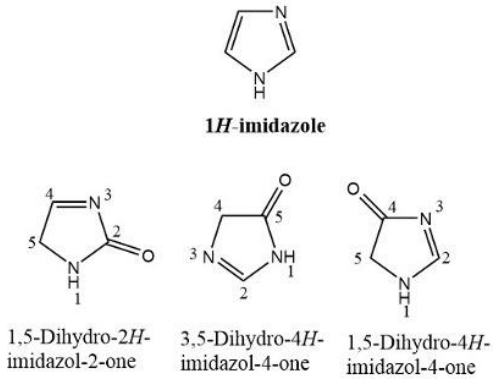


Figure 1. Different imidazolinones' chemical structures

A series of imidazolinone bearing compounds (Figure 2, compound 25 derivatives) were tested for their ability to reduce inflammation and bacterial development. Compound 25's derivatives, 25a, 25b, and 25c, demonstrated significant activity. The existence imidazolinone, styryl, and dipeptidyl moieties affected anti-bacterial activity [16].In another study, some 1,2,4-trisubstituted imidazolin-5-one derivatives were synthesized and tested for their ability to inhibit carbonic anhydrase (CA) enzymes. Compound 4a showed the best inhibitory efficacy with Ki values of 95.0, 0.83, 6.90, and 12.4 nM, respectively, against all the carbonic anhydrase isoforms tested (CA I, II, IV, and IX) when compared to acetazolamide (Figure 2) [17].

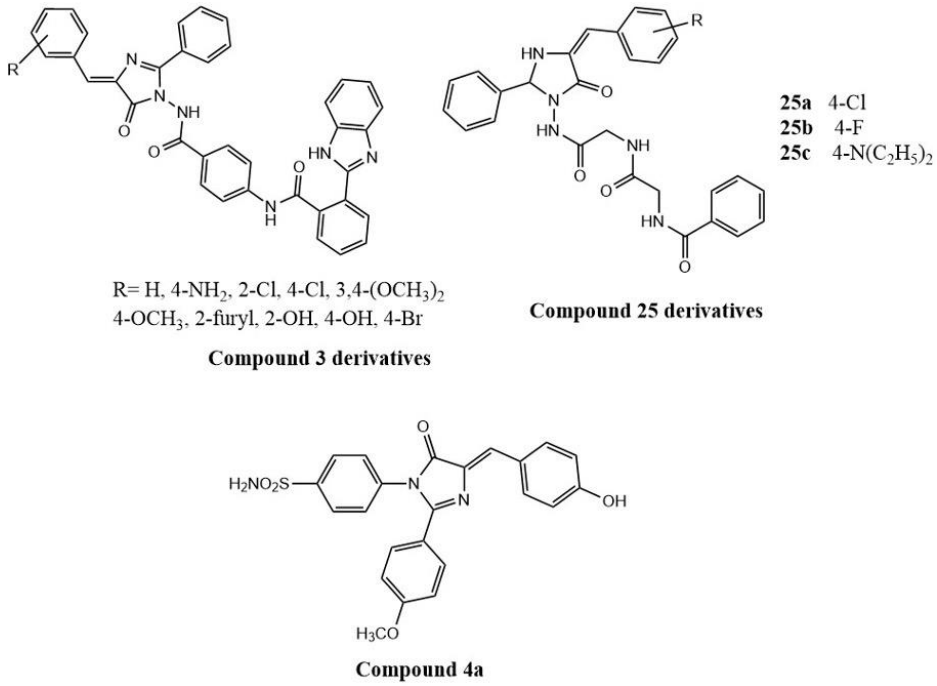
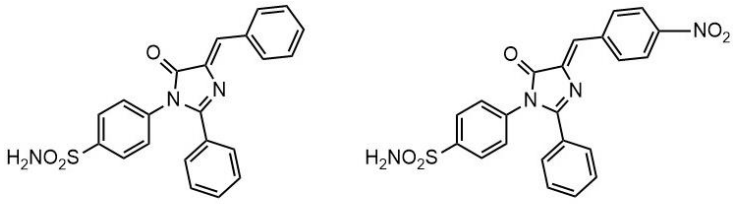


Figure 2. Chemical structures of bioactive imidazolinone derivatives

Sulfonamides contain the SO_2NH_2 moiety in their structure [18]. They are popular in drug design due to significant biological activities including antibacterial [19], antidiabetic [20], carbonic anhydrase inhibitory [21, 22], anti-inflammatory [23], antitumor [24], antioxidant [25], and anticancer [26] agent.

In a study, our research group generated a series of imidazolidinone derivative compounds (Figure 3). Following spectral confirmation of their structures, the compounds were tested against carbonic anhydrase (CA) and acetylcholinesterase (AChE) enzymes. Series 4 were found more potent CAs

inhibitors than series 3. Nitro-containing compounds in series 4 were 3.3-4.8 times more selective inhibitors than their series 3. Compounds 3c and 4c having the lowest Ki values, which contain the benzenesulfonamide moiety, were considered as the leaders in terms of AChE inhibition, [27].

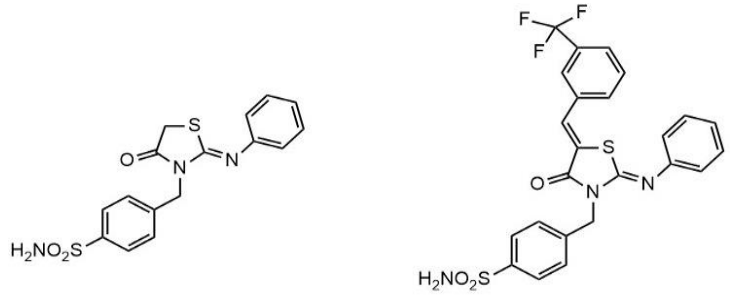


Compound 3c **Compound 4c**

Figure 3. Chemical structures of imidazolinone derivatives reported our research group

Carbonic anhydrase is a metalloenzyme that contains zinc (Zn^{2+}) ions in its active site and catalyzes the slow transformation of CO_2 to HCO_3^- and H^+. They have a role in a variety of physiological and pathological processes [28, 29]. The structures that represent the main inhibitory group of carbonic anhydrases are sulfonamide/sulfamate groups [30-32]. Carbonic anhydrase inhibitors (CAIs) have been used to treat glaucoma [33], diuretics [34], obesity [35], and cancer [36].

Zhang et al. synthesized compounds containing benzenesulfonamide residue and then tested their efficacy against the carbonic anhydrase enzymes. The profiles of the enzyme inhibition assays against *h*CA II were as good as the positive controls. Compounds 2 and 7 (Figure 4) efficiently bind in the active site cavity of an enzyme by generating adequate contacts with active site residues, according to docking studies [37].



Compound 2 **Compound 7**

Figure 4. Compounds with sulfonamide residues as CA inhibitors

The hydrolysis of acetylcholine (ACh) to choline and acetic acid by cholinesterases (ChEs) is a basic process for cholinergic neurotransmission [38]. At the presynaptic level, ACh molecules are produced from choline. Both the peripheral and central nervous systems (CNS) require them for cholinergic neurotransmission [38]. Alzheimer's disease (AD) is a neurodegenerative disease marked by cognitive decline, memory loss, and dementia [39]. In general, AD is treated with rivastigmine, tacrine, and donepezil acting as ChE inhibitors [40].

In a study, benzenesulfonamide derivatives were reported as acetylcholinesterase (AChE) enzyme inhibitors. The results revealed that the synthesized sulfonamide derivatives have potential inhibition properties for AChE with Ki constants ranging from 2.54±0.22–299.60±8.73 μM. The derivatives (S1, S1i, S3, and S3i) appeared a competitive inhibition effect, whereas others (S2, S2i, S4, and S4i) appeared mixed-type inhibition [41].

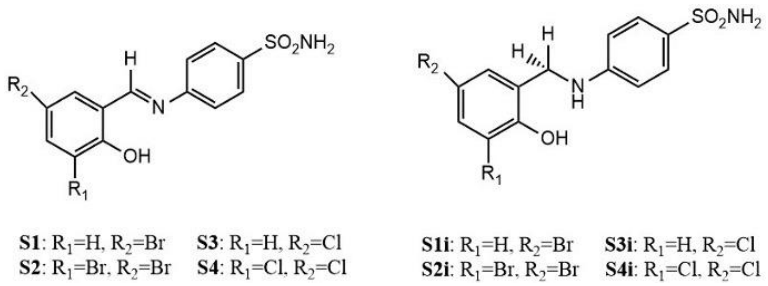
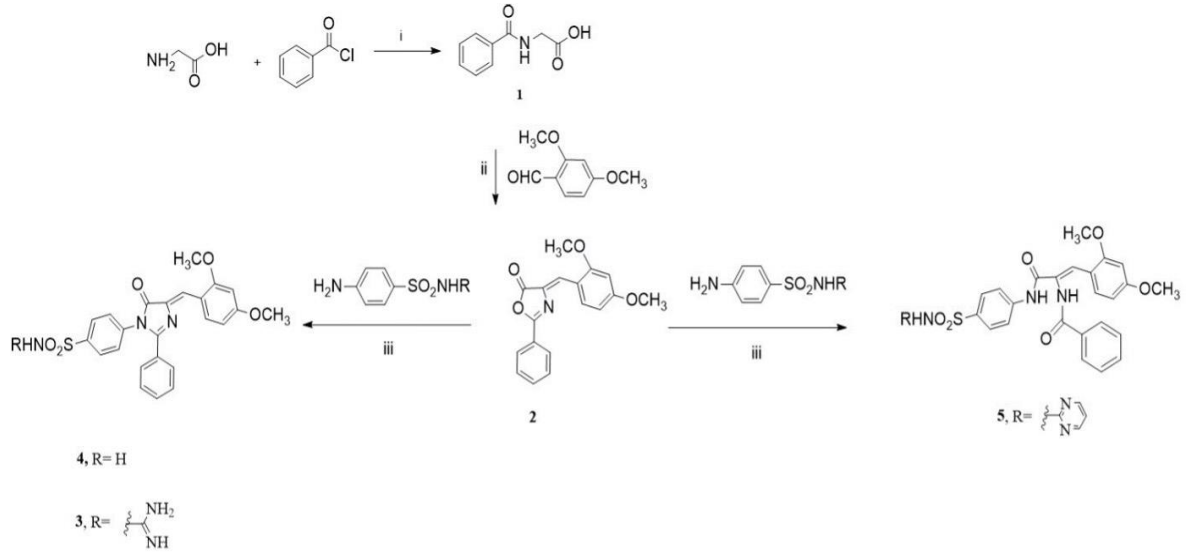


Figure 5. Chemical structures of the compounds (S1,S1i, S2, S2i, S3, S3i, S4, S4i)

Imidazolinone-based sulfonamide derivatives were designed and synthesized in this study (Scheme 1). Spectrometric methods (^{1}H, ^{13}C NMR, and HRMS) were used to characterize the chemical structures of the synthesized compounds. Compounds were investigated for their inhibitory effects on AChE, CA II, and CA I enzymes.



Scheme 1. Synthetic pathway of the target compounds **1-5 (**i: 10% NaOH, HCl, ii: acetic anhydride, sodium acetate, 100°C, iii: glacial acetic acid, sodium acetate, 100°C)

MATERIAL AND METHOD

Spectral techniques for ^{1}H and ^{13}C NMR (Varian, California, U.S.A), as well as HRMS (Shimadzu, Kyoto, Japan), were utilized for structural investigation. Coupling constants (*J*) are stated in hertz (Hz), and chemical shifts are reported in ppm. Electrothermal 9100/IA9100 (Bibby Scientific Limited in Staffordshire, U.K.) was used to determine melting points. To monitor the reaction process, silica gel 60 HF254 thin layer chromatography (TLC) was utilized. TLC solvent systems included a combination of chloroform and methanol (4.8:0.2).

Synthesis of 2-Benzamidoacetic Acid (1)

After 1 mmol of glycine was dissolved in sodium hydroxide solution (25 ml, 10%), benzoyl chloride (1 mmol) were added to the solution. Until the liquid is acidic (pH=4-5), strong hydrochloric acid was gently added to the mixture until pH=4-5. Then, benzoyl glycine was filtered and rinsed with ice-cold water. The solid was heated in carbon tetrachloride (100 ml, 10 min) to remove any benzoic acid. Then, cooled mixture was filtered and washed using carbon tetrachloride [27]. The following are the spectral and experimental results: White colour solid, mp: 192-193°C, yield 90%. ^{1}H NMR (DMSO-

d_6, ppm, 400 MHz), δ 8.76 (d, *J*= 5.3 Hz, 1H, Ar-H), 7.88 (d, *J*= 7.9 Hz, 2H, Ar-H), 7.57–7.46 (m, 3H, Ar-H, NH),3.94 (s, 2H, -CH_2); ^{13}C NMR (DMSO-d_6, ppm, 100 MHz) δ 171.3, 166.4, 133.8, 131.4, 128.3, 127.2, 41.2.

The General Method of Synthesis of 4-(2,4-Dimethoxybenzylidene)-2-phenyloxazol-5(4*H*)-one (2)

Dry sodium acetate, acetic anhydride, 2,4-dimethoxybenzaldehyde, and 4-benzoyl glycine were heated for one hour over a water bath. The resulting combination was let to stand at room temperature overnight. The solid was filtered and washed by cold water and dried at 60°C. The crude compound 2 was recrystallized using ethanol [27].

4-(2,4-Dimethoxybenzylidene)-2-phenyloxazol-5(4*H*)-one (2)

Cream colour solid, mp: 169-170°C, yield 76%. ^{1}H NMR (DMSO-d_6, ppm, 400 MHz) , δ 8.77-8.73 (m, 1H, Ar-H), 8.05 (t, *J*= 6.8 Hz, 2H, Ar-H), 7.69–7.57 (m, 3H, Ar-H, =CH-), 7.48 (d, 1H, *J*= 6.6 Hz, Ar-H), 6.74-6.71 (m, 1H, Ar-H), 6.64-6.61 (m, 1H, Ar-H), 3.91 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, ppm, 100 MHz) δ 167.2, 164.1, 161.3, 160.8, 133.6, 133.1, 129.5, 129.2, 127.5, 125.3, 124.2, 114.7, 97.7, 56.03, 55.6.

General Synthesis Method of the 3, 4 and 5

Sodium acetate (15 mmol), a suitable sulfonamide derivative (12 mmol) [sulfaguanidine (3), sulfanilamide (4), and sulfadiazine (5)], and compound 2 were refluxed in gl. acetic acid (10 ml) for 17-19 hours at 100°C. The crude was removed, washed with water, and dried. DMF/C_2H_5OH/H_2O mixture was used for crystallization. The isolated open-chain product 5 compound's spectral and experimental data are given below [17,27].

***N*-Carbamimidoyl-4-(4-(2,4-dimethoxybenzylidene)-5-oxo-2-phenyl-4,5-dihydro-1*H*-imidazol-1-yl)benzenesulfonamide (3)**

White colour solid, mp: 294-296°C, yield 55%. ^{1}H NMR (DMSO-d_6, ppm, 400 MHz) , δ 8.95–8.92 (m, 1H, Ar-H), 7.82–7.80 (m, 2H, Ar-H), 7.56 (s, 1H, =CH-), 7.50–7.48 (m, 3H, Ar-H), 7.43–7.36 (m, 4H, Ar-H, NH), 6.77–6.74 (m, 4H, Ar-H, NH), 6.69 (bs, 2H, NH_2), 3.93 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3); ^{13}C NMR (DMSO- d_6, ppm, 100 MHz) δ 169.2, 163.5, 160.7, 158.3, 158.1, 143.9, 136.8, 135.3, 133.9, 131.2, 128.8, 128.6, 128.4, 127.8, 126.5, 121.6, 115.4, 107.1, 97.8, 55.9, 55.6; HRMS (ESI-MS) $C_{25}H_{23}N_5O_5S$, Calculated $[M+H]^+$: 506.1493; Found $[M+H]^+$: 506.1493.

4-(4-(2,4-Dimethoxybenzylidene)-5-oxo-2-phenyl-4,5-dihydro-1*H*-imidazol-1-yl)benzenesulfonamide (4)

Dark cream colour solid, mp: 295-296°C, yield 65%. ^{1}H NMR (DMSO-d_6, ppm, 400 MHz) , δ 8.95-8.92 (m, 1H, Ar-H), 7.88 (d, *J*= 8.5 Hz, 2H, Ar-H), 7.57 (s, 1H, =CH-), 7.51-7.40 (m, 7H, Ar-H), 6.76 (d, *J*= 8.8 Hz, 2H, Ar-H), 6.68 (s, 2H, NH_2); 3.93 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3); ^{13}C NMR (DMSO- d_6, ppm, 100 MHz) δ 169.2, 163.5, 160.8, 158.3, 143.4, 137.4, 135.3, 133.9, 131.2, 128.8, 128.6, 128.5, 128.1, 126.6, 121.7, 115.3, 107.2, 97.8, 56.0, 55.6; HRMS (ESI-MS) $C_{24}H_{21}N_3O_5S$, Calculated $[M+H]^+$: 464.1275; Found $[M+H]^+$: 464.1274.

***N*-(1-(2,4-dimethoxyphenyl)-3-oxo-3-((4-*(N*-(pyrimidin-2-yl)sulfamoyl)phenyl)amino)prop-1-en-1-yl)benzamide (5)**

Light cream colour solid, mp: 228-229°C, yield 65%. ^{1}H NMR (DMSO-d_6, ppm, 400 MHz), δ 10.43 (s, 1H, NH), 9.98 (s, 1H, NH), 8.51-8.49 (m, 2H, Ar-H), 8.00 (d, *J*= 7.5 Hz, 2H, Ar-H), 7.94–7.88 (m, 4H, Ar-H), 7.57–7.49 (m, 4H, Ar-H), 7.28 (s, 1H, =CH-), 7.04–7.01 (m, 1H, Ar-H), 6.63 (s, 1H, NH), 6.54 (d, *J*= 8.6 Hz, 1H, Ar-H), 3.83 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, ppm, 100 MHz) δ 166.4, 165.5, 161.9, 159.1, 158.8, 157.5, 157.3, 143.8, 134.6, 133.9, 132.2, 130.3, 129.1, 129.0, 128.8, 128.3, 123.8, 119.7, 115.6, 105.9, 98.7, 56.2, 55.8; HRMS (ESI-MS) $C_{28}H_{25}N_5O_6S$, Calculated $[M+H]^+$: 560.1598; Found $[M+H]^+$: 560.1602.

Pharmacological/Biological Assays

AChE and *h*CAs Inhibition Assay

Human erythrocytes' CA isoenzymes (I and II) were purified in accordance with published studies [42,43]. The *p*-nitrophenylacetate is utilized as a substrate and converted to the *p*-nitrophenolate ion by both isoforms [44,45]. Acetylthiocholine iodide and 5,5-Dithiobis(2-nitrobenzoic) acid were used as the substrates and inhibitory effects of the novel imidazolinone-based sulfonamide derivatives (**1**-**5**). The AChE activity of the compounds (**1**–**5**) tested was measured at 412 nm spectrophotometrically [46-48].

AChE *and h*CAs Kinetic Assay

At least five distinct inhibitor concentrations were used to test the inhibitory effects of the new compounds (**1-5**) on *h*CAs and AChE. According to other studies [27,49], the IC_{50}s of the synthesized derivatives were determined from Activity (%) [Derivative] graphs for each derivative. Lineweaver - Burk's curves were graphed to identify the types [50] of inhibition and Ki values [51].

Table 1. The inhibitory effects of the compounds **1-5** on CAs and AChE enzymes

	IC_{50} (nM)						K_i (nM)		
Compd	***h*CA I**	**r^2**	***h*CA II**	**r^2**	**AChE**	**r^2**	***h*CA I**	***h*CA II**	**AChE**
1	49.50	0.9891	63.00	0.9771	31.50	0.9858	40.97±7.06	83.50±14.27	30.15±1.62
2	34.65	0.9818	49.50	0.9824	30.13	0.9941	50.64±12.15	31.56±1.96	27.34±4.03
3	27.72	0.9812	22.35	0.9832	24.75	0.9915	23.61±2.34	16.49±2.20	20.14±1.21
4	26.65	0.9769	28.87	0.9803	23.10	0.9823	19.53±1.23	25.30±1.47	21.71±4.04
5	29.85	0.9842	30.13	0.9945	23.90	0.9819	20.27±3.44	20.85±2.33	11.68±1.45
AZA	46.75	0.9932	38.25	0.9890	-	-	30.74±3.52	22.27±1.56	-
TAC	-	-	-	-	25.78	0.9878	-	-	18.45±2.12

AZA: Acetazolamide, **TAC:** Tacrine

RESULT AND DISCUSSION

Synthesis

Scheme 1 shows the synthesis of imidazolinone-based sulfonamide derivatives (**3-5**). In the presence of glacial acetic acid, the reaction of 4-(2,4-dimethoxybenzylidene)-2-phenyloxazol-5(4*H*)-one substituted with various sulfonamide derivatives and sodium acetate resulted in the desired novel compounds (**3–5**). Their chemical structures were validated using spectroscopies such as HRMS, ^{1}H, and ^{13}C NMR.

NMR data confirmed the ring closure of compound 2. The increase of proton integration in the aromatic area and aromatic carbons confirmed that compounds **3** and **4** were obtained starting from compound **2**. Interestingly, the targeted cyclic compound **5** could not be obtained under the same experimental conditions. The cyclic target product did not form in 18 hours when we utilized sulfadiazine by using starting compound **2**; instead, compound **5** was produced given in Scheme 1. According to NMR data, δ 10.43 (s, 1H, NH) and 9.98 (s, 1H, NH) peaks confirmed that the imidazolinone ring was not closed for the compound 5.

Biochemistry

AChE inhibition potential of the new compounds (**3-5**) synthesized in this study is given in Figure 6**E-F** and Table 1. Inhibition type and Ki parameters were defined using Lineweaver-Burk plots. The order of the Ki for compounds (**3-5**) was discovered to be between 11.68±1.45 and 21.71±4.04 nM against AChE (Table 1). Results clearly demonstrate that the Ki values of these compounds are rather close to one another. But the greatest inhibition was found in *N*-(1-(2,4-dimethoxyphenyl)-3-oxo-3-((4-(*N*-(pyrimidine-2-yl)sulfamoyl)phenyl)amino)prop-1-en-1-yl)benzamide (**5**) with low nanomolar Ki value of 11.68±1.45 nM. When compared to the reference compound (Tacrine Ki: 18.45±2.12 nM), compounds (**3-5**) demonstrated close inhibitory capacity based on their Ki values.

New imidazolinone compounds (3-5) were tested towards physiologically significant CA I and II isoforms. The inhibition results and graphs are shown in Figures 6A-6D and Table 1. CA isoenzymes are physiologically important enzymes. Dysfunction of the carbonic anhydrase isoenzyme is typically associated with many diseases such as cancer, glaucoma, epilepsy, osteoporosis, and so on. Therefore, the clinical use of CAIs is critical for treating these disorders. The cytosolic isoform *h*CA I was tested for imidazolinone derivatives (**3**–**5**) and Ki values were calculated in the range of 19.53±1.23–23.61±2.34 nM (Table 1). As a well-known *h*CA I inhibitor, AZA had a Ki of 30.74±3.52 nM. Among the compounds, **4** had the highest inhibition potency (Ki: 19.53±1.23 nM) towards the *h*CA I enzyme.

For *h*CA II, new imidazolinone derivatives (**3**–**5**) had Ki values in the range of 16.49±2.20–25.30±1.47 nM. Compound **3**, with a Ki value of 16.49±2.20 nM against cytosolic CA II, displayed the best inhibitory profile.-In addition, AZA exhibited inhibition potency at 22.27±1.56 nM.

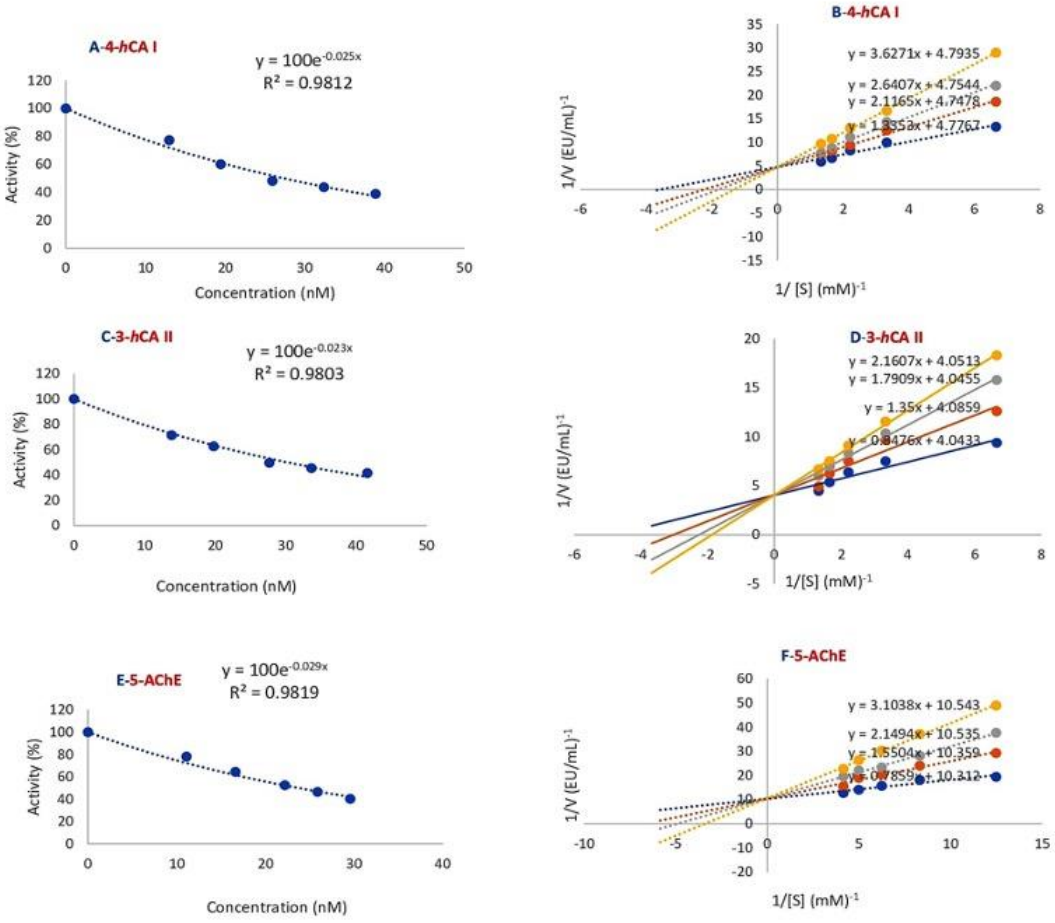


Figure 6. Lineweaver-Burk graphs for the compounds against CA I, II, and AChE enzymes

Lipinski's rule of five (RO5) is used to identify the drug-likeness characteristics of oral medicines [52]. Based on the analysis [53] (Table 2), the following values were determined: logP (lipophilicity, 2.35–3.16), number of H-bond donors (1–3), number of H-bond acceptors (7,8) and molecular weight (463.51–559.59). Lipinski's RO5 was shown to be compatible with compound 4. While the other two compounds (compounds **3** and **5**) were compatible with Lipinski's rule of 4, deviations in molecular weight were observed.

Table 2. Druglikeness properties of compounds **3-5** based on Lipinski's RO5

Compd No	Formula	Molecular Weight (g/mol)	H-bond acceptors	H-bond donors	TPSA	MLog P
3	$C_{25}H_{23}N_5O_5S$	505.55 g/mol	7	3	155.55 Å²	2.35
4	$C_{24}H_{21}N_3O_5S$	463.51 g/mol	7	1	119.67 Å²	2.76
5	$C_{28}H_{25}N_5O_6S$	559.59 g/mol	8	3	156.99 Å²	3.16

Lipinski filter: MW < 500, MlogP < 4.15, N or O <10, NH or OH <5 [52]

In conclusion, in the present research, the new imidazolinone derivatives (**3-5**) have shown effective inhibitory profiles against both *h*CA isoenzymes and the AChE enzyme. In this study, Ki and IC_{50} values were calculated for new compounds (**3-5**) against metabolic enzymes associated with some global disorders such as glaucoma, epilepsy, and Alzheimer's disease, among others. As a result, these substances (**3-5**) showed inhibitory potency in both *h*CA isoenzymes and the AChE enzyme in treating the aforementioned illnesses. Inhibition studies further demonstrated that compounds **4** and **3** have a strong inhibitory impact on *h*CA I and *h*CA II isoenzymes, respectively. In addition, compound **5** has a considerable inhibitory effect on the AChE enzyme. Based on Ki values, it could be concluded that the final compounds **3-5** have more favorable inhibitory potency than intermediates **1** and **2**. So, incorporating benzenesulfonamide moiety and imidazolinone ring in the structure increased inhibition against AChE and CA enzymes. Therefore, these benzenesulfonamide-based compounds could be used as lead compounds in future pharmaceutical research.

ACKNOWLEDGEMENTS

Dr. C. Kazaz and Dr. B. Anil (Ataturk University) are acknowledged for providing NMRs.

AUTHOR CONTRIBUTIONS

Concept: M.T.S., H.I.G; Design: M.T.S.; Control: M.T.S., C.Y., H. I. G.; Sources: M.T.S., H.I.G., I.G.; Materials: M.T.S., Y.D., I.G.; Data Collection and/or Processing: M.T.S.; Analysis and/or Interpretation: M.T.S., H.I.G., C.Y., Y.D., I.G.; Literature Review: M.T.S.; Manuscript Writing: M.T.S., C.Y.; Critical Review: M.T.S., C.Y., H.I.G., I.G., Y.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., 47(3): 927-934, 2023
Doi: 10.33483/jfpau.1329821

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANALYSIS OF ABACAVIR LAMIVUDINE AND ZIDOVUDINE BY SPECTROPHOTOMETRIC METHOD IN TRIPLE MIXTURE

ABAKAVİR, LAMİVUDİN VE ZİDOVUDİN'İN ÜÇLÜ KARIŞIMDA SPEKTROFOTOMETRİK ANALİZİ

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ABSTRACT

Objective: *The simultaneous determination of abacavir (ABV), lamivudine (LMV) and zidovudine (ZDV) were applied by dual amplitude difference method coupled with ratio difference spectrophotometric methods.*
Material and Method: *The LMV was quantified by selected the 226.0 nm and 235.0 nm in the dual amplitude difference method. For ratio difference method 297.0 nm and 268.0 nm wavelengths and 266.0 nm, 245 nm wavelengths were chosen to quantify respectively ABV and ZDV. Accuracy studies have been carried out with percent recovery.*
Result and Discussion: *The proposed study, three active substances used in Human immunodeficiency virus (HIV) treatment were quantified. These active ingredients are used in combination to provide effective treatment. With the applied methods, firstly LMV was determined by dual amplitut difference method, then ABV and ZDV were determined by ratio difference. The three active ingredients were studied in the concentration range of 3-21 µg/ml. Correlation coefficients were found to be between 0.9985 and 0.9996. Recovery results range from 95.2 to 106.2. In the method, it was only dissolved in the solvent and measured, and the analysis was carried out without pre-preparation and expensive equipment.*
Keywords: *Abacavir, determination, lamivudine, zidovudine*

ÖZ

Amaç: *Çalışmada abacavir (ABV), lamivudine (LMV) ve zidovudine (ZDV) etken maddelerinin aynı*

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Submitted / Gönderilme : 19.07.2023
Accepted / Kabul : 09.08.2023
Published / Yayınlanma : 20.09.2023

anda tayini ikili amplitüd farkı ile birlikte oran farkı spektrofotometrik yöntemleri uygulanarak yapılmıştır.

Gereç ve Yöntem: *İkili amplitüd farkı yönteminde 226.0 nm ve 235.0 nm seçilerek LMV için miktar tayini yapılmıştır. Oran farkı yöntemi için 297.0 nm ve 268.0 nm dalga boyları ve 266.0 nm, 245 nm dalga boyları sırasıyla ABV ve ZDV'nin miktar tayini için seçilmiştir. Yöntemlerin doğruluğu laboratuvar karışımlarına yüzde geri kazanım çalışmaları uygulanarak belirlenmiştir.*

Sonuç ve Tartışma: *Önerilen çalışmada, HIV tedavisinde kullanılan üç farklı etken maddenin aynı anda miktar tayini yapılmıştır. Yöntemler antiviral ilaçların aynı anda tayini için başarı ile uygulanmıştır. Bu etken maddeler, etkili tedavinin sağlanması için kombinasyon olarak kullanılmaktadır. Uygulanan yöntemler ile, ilk olarak dual amplitut difference yöntemi ile LMV tayin edilmiş sonrasında oran farkı ile ABV ve ZDV tayin edilmiştir. Üç etken madde 3-21 µg/ml konsantrasyon aralığında çalışılmıştır. Korelasyon katsayısı 0.9985 ile 0.9996 olarak bulunmuştur. Geri kazanım sonuçları 95.2 ile 106.2 arasındadır. Yöntemde sadece çözücüde çözünüp ölçülmüştür ön hazırlama işlemi ve pahalı cihazlar olmadan analiz yapılmıştır.*

Anahtar Kelimeler: *Abacavir, kantitatif belirleme, lamivudin, zidovudin*

INTRODUCTION

Viruses are the infectious agents that have a simple structure and can only multiply in their more developed cells in human, animal, plant, bacteria and similar organisms. Viruses have to use the biochemical mechanisms of the cells they enter to synthesize their own genetic material and new viral proteins. Human immunodeficiency virus (HIV) is an RNA virus of the retrovirus type. It retains T lymphocytes in blood and lymphoid tissue.

There are many different classes of antiretroviral used in HIV treatment. Antiretroviral drugs are classified as nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and protease inhibitors according to their mechanism of action. The purpose of these active substances used in combination is to prevent HIV replication.

Abacavir (ABV), (4-(2-amino-6-(cycloproylamino)-9*H*-purin-9-yl)cyclopent-2-enylmethanol (Figure 1A) is an antiretroviral drug used orally. It is a guanosine nucleoside reverse transcriptase inhibitor that can be used in combination with other antiretroviral active substances. Lamivudine (LMV), 4-amino-1-((2R,5S-2-(hydroxymethyl)-1,3-oxathiolan-5-yl) pyrimidin-2-(1*H*)-one (Figure 1B) is an antiviral drug used in combination with other drugs in the treatment of HIV infection. Zidovudine (ZDV), 3-azido-3-deoxythymidine (Figure 1C) is used in the treatment of HIV infection in adults and children [1].

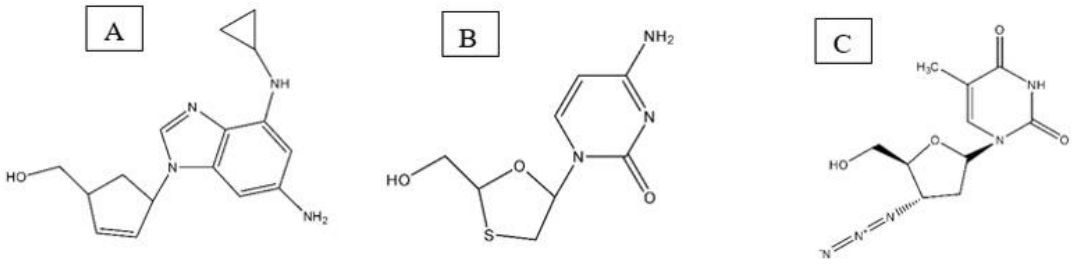

Figure 1. Chemical structure of ABV, LMV and ZDV

In literature, the drugs have been determined using different techniques. Several techniques have been studied quantification for three drug, ABV, LMV and ZDV, chemometric method [2], LC-MS/MS [3-5], high-performance liquid chromatography (HPLC) [6-11]. Analyzes were made in different matrices, including pharmaceutical preparation, wastewater, and plasma.

Due to the role of drugs in the form of combination in therapy, simultaneous quantification of the active substances is important and there is a lack of such a spectrophotometric method in the literature.

In this study, we present a simple, not need a special program, cost reduced, and accuracy validated analytical method to determine ABV, LMV and ZDV in prepared laboratory solution by novel spectrophotometric methods without any pretreatment step. The method is dual amplitude difference method coupled with ratio difference spectrophotometric method. The method can be applied in quality control laboratories for the simultaneous quantification of three active substances. The goal we want to reach in this study is to analyze simultaneously three active substances, which are important to analyze simultaneously, simply, using simple devices. To apply the experimental used device was consumed lower energy. Experiments were carried out by dissolving the active ingredients directly in a single solvent. The effects of waste on the environment have been minimized due to their direct and indirect effects on our health. Applied experiment, three active substances used in the treatment of HIV were determined simultaneously. Spectrophotometer, which can be found in every laboratory, and software were used in the quantitative determination study. This allowed the method to be applied easily and economically.

MATERIAL AND METHOD

Instrument and Software

The spectra of the active substances were taken in the spectrophotometer device, in the UV region, between 200-400 nm. A quartz cuvette was used for measurements. The brand and model of the dual-beam spectrophotometer device used is Shimadzu UV 1800. UV probe 2.52 was used as software for the spectra. Excel program was used to create calibration curves and apply data.

Used Chemicals

Used all materials were of analytical grade. Reference standards were kindly supplied by Abdi Ibrahim Pharmaceutical Industry, Turkey. Liquid chromatography grade methanol was purchased from Merck (Darmstadt, Germany).

Preparation of Standard Solutions

By transferring the 15.0 mg of active substances into 50.0 ml volumetric flasks containing methanol, standard stock solutions of each of the three analytes, equal to (0.3 mg/ml), of ABV, LMV and ZDV were prepared separately. Active substances solutions containing 3.0-21.0 µg/ml of ABV, LMV and ZDV were solved separately in methanol. Laboratory mixtures were prepared three replicates in certain proportions and measurements were made and percent recovery values were calculated.

RESULT AND DISCUSSION

Three active ingredients were analyzed by dual amplitude difference method coupled with ratio difference spectrophotometric technique. The spectrum of ABV, LMV and ZDV between 200-400 nm are given in Figure 2. The methods are applied direct prepared laboratory mixture.

Dual Amplitude Difference Method Coupled with Ratio Difference

In the dual amplitude method, two wavelengths were selected and a factor spectrum was constructed [12].

Using ZDV as a divisor, the dual amplitude difference method enables the elimination of the interfering component. ZDV will thus cancel out when the subtract between two chosen wavelengths is obtained since it is a constant whereas the ratio of ABV to ZDV has the same amplitudes. This enables the easy measurement of LMV in different synthetic-prepared combinations with varied ratios of ABV and ZID.

The spectrum of the laboratory mixture was divided by the ZDV' spectra (6.0 µg/ml) as a divisor in the proposed approach to obtain a ratio spectra. For the LMV's finding of a zero amplitude difference for ABV and ZID, the amplitudes at 226.0 and 235.0 nm were selected, Figure 3.

The ternary mixture is divided into the ZDV spectrum. Amplitude difference was found in the obtained ratio spectrum. The amplitude difference (226.0 and 235.0 nm) was multiplied by the factorized

spectra. The ratio spectra of LMV to ZDV was constructed. To obtain the factorize spectrum, the LMV spectrum was divided ZDV spectrum and the obtained spectrum was divided into selected wavelengths (226.0 and 235.0 nm) difference. The constructed of factorize ratio spectrum is shown in Figure 4.

The zero order spectrum of LMV were created by multiplying the resulting ratio spectra by the ZDV' (divisor) (6.0 µg/ml). Thus, the LMV compound was determined at its maximum absorbance. The maximum absorbance of LMV was determined at 272.0 nm over the 3.0–21.0 µg/ml calibration range.

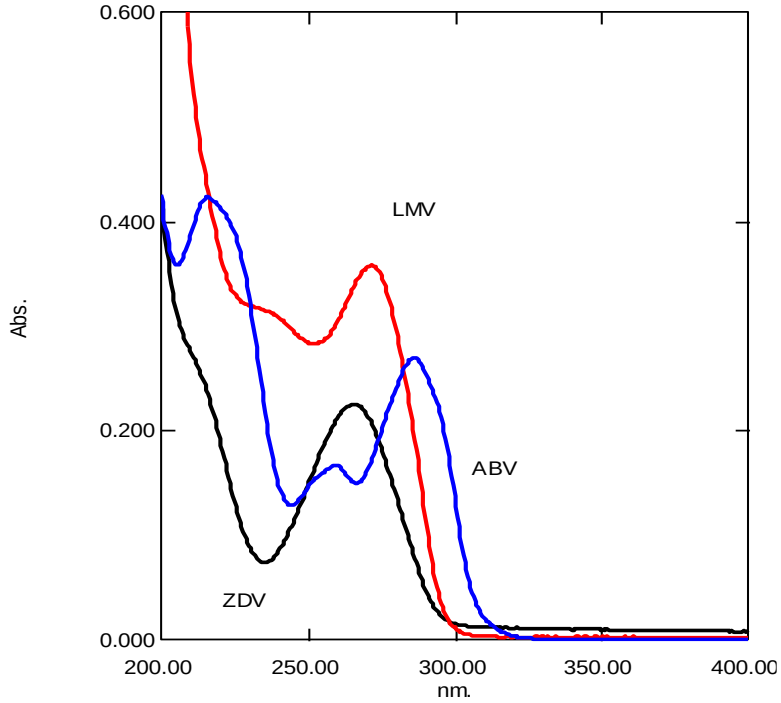

Figure 2. Zero order spectrum of ABV, LMV and ZDV

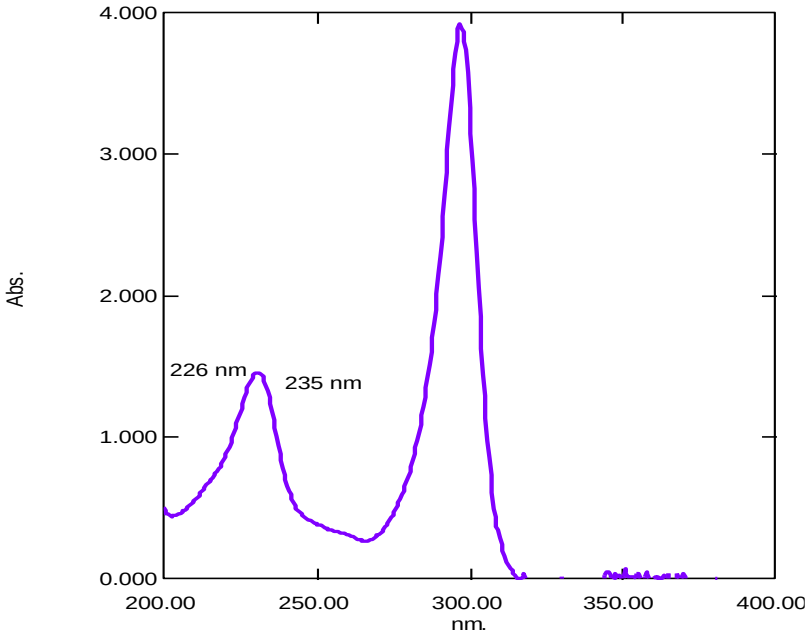

Figure 3. Ratio spectrum of ABV (3.0 µg/ml) to ZDV (6.0 µg/ml)

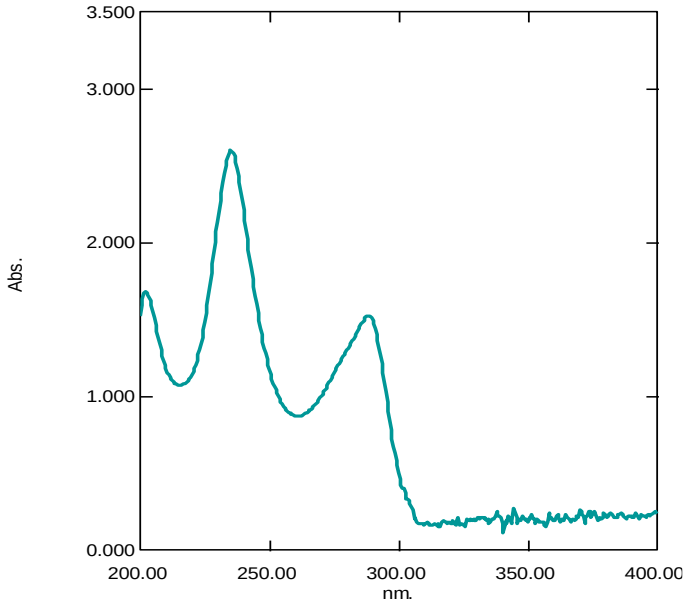

Figure 4. The obtain factor spectrum of LMV

ABV and ZDV mixture was obtained by subtracting the LMV spectra (D^0) from the relevant mixture.

For a mixture of ABV and ZDV was applied ratio difference method. For this method different ZDV's spectrums were divided by constant ABV's spectrum. Two different wavelengths were selected to obtain the ratio spectrum. A calibration graph was drawn by plotting the difference between the selected wavelengths (266.0 nm and 255.0 nm) against the concentration. To determine the ABV, a ratio spectrum was constructed. While creating the ratio spectrum, ABV spectrums were divided into ZDV spectrums. The selected wavelengths were 297.0 and 268.0 nm. This ratio spectrums were shown in Figure 5.

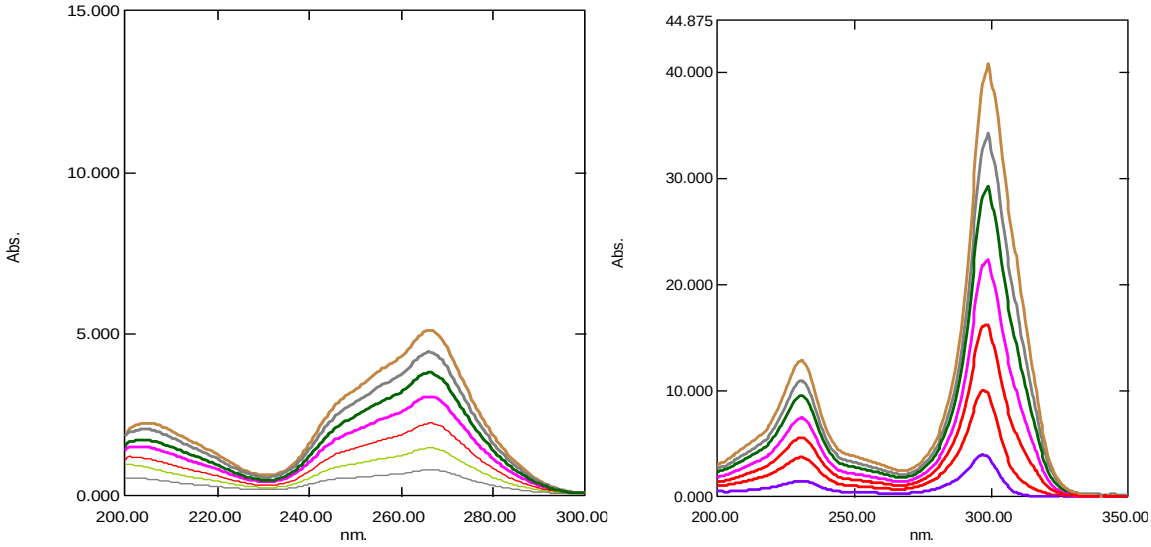

Figure 5. The ratio spectrum of ZDV and ABV

A calibration graph was drawn against concentration versus difference wavelengths for ABV and ZDV. Calibration graph parameters were given in Table 1.

Table 1. The parameter of the spectrophotometric method

Parameters	ABV	LMV	ZDV
Concentration range (µg/ml)	3.0-21.0	3.0-21.0	3.0-21.0
Wavelength, nm	297.0-268.0	272.0	266.0-255.0
Intercept value	-1.531	-0.012	-0.009
Slope value	1.808	0.040	0.062
Correlation coefficient, R^2	0.9996	0.9987	0.9985

Recovery Results for Methods

Recovery experiments were made from the laboratory mixture. These mixtures were prepared in different concentration series. The recovery values were illustrated in table 2.

Table 2. The recovery results of spectrophotometric method

Sample	Added concentration			Recovery %		
	ABV	LMV	ZDV	ABV	LMV	ZDV
Dual amplitude difference coupled with ratio difference method						
Sample I	3.0	3.0	6.0	106.2	102.3	96.4
Sample II	3.0	3.0	12.0	104.3	98.7	95.2
Sample III	3.0	6.0	3.0	103.2	99.4	97.2
Sample IV	9.0	9.0	18.0	95.4	97.2	96.1
Sample V	18.0	9.0	18.0	97.2	98.9	98.2

Precision Results for Methods

Precision studies were carried out to demonstrate the reproducibility of the methods. The calculated results are given in Table 3.

Table 3. The precision results of dual amplitude difference coupled with ratio difference method

Sample	Added concentration			Found concentration %		
	ABV	LMV	ZDV	ABV	LMV	ZDV
1. day	12.0	12.0	12.0	101.2	103.3	100.5
2. day	12.0	12.0	12.0	100.4	97.8	104.1
3. day	12.0	12.0	12.0	102.4	99.2	103.2

In the study was applied directly by spectrophotometer and without derivatization procedure (Table 4).

Table 4. The comparison of other methods

Analyte	Linear range µg/ml	Correlation coefficient	Recovery %	Reference
ABA, LAM and ZID	3.0-21.0	0.9996, 0.9987 and 0.9985	95.2-106.2	Proposed method
ABA, LAM and ZID	0.015-5.0	-	92.0-102.0	8
ABA, LAM and ZID	4.9-306.0	0.9999, 0.9999 and 0.9997	98.7-103.7	9

Assessment of Greenness for Methods

When the method is evaluated for the environment, it is superior to other applied methods in that it was used a simple device, easy processes and did not have an extraction step.

A green analytical procedure index (GAPI) evaluation was made for the method. According to the GAPI, the methods are examined according to 15 different parameters such as sample preparation, solvents used, and energy consumption [13]. The applied method was examined according to the GAPI and the GAPI results for the method we applied are shown in Figure 7.

Figure 7. GAPI result of applied methods

In conclusion, the applied spectrophotometric method was applied active ingredients of ABV, LMV and ZDV. The spectrophotometric method is dual amplitude difference method coupled with ratio difference. The method was applied to the active ingredients in two steps. First, LMV was obtained by dual amplitude difference method and then ABA and ZID were determined by ratio difference method. The method applied as fast, practically and without pre-preparation step the comparison of the other method. The active ingredients were solved simple and were measured directly. Thanks to the applied methods three drugs were determined simultaneously without expensive devices. Therefore, the energy consumption is reduced. The chemical reagents were not used because of pretreatment procedure. The applied methods were greenness for environmental for this reason. These methods can be applied easily for determination of three active substances and it is an easily applicable method in the laboratory.

AUTHOR CONTRIBUTIONS

Concept: G.T., N.E.; Design: G.T., N.E.; Control: G.T., N.E.; Sources: G.T., N.E.; Materials: G.T., N.E.; Data Collection and/or Processing: G.T., N.E.; Analysis and/or Interpretation: G.T., N.E.; Literature Review: G.T., N.E; Manuscript Writing: G.T., N.E.; Critical Review: G.T., N.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 declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 935-943, 2023
Doi: 10.33483/jfpau.1330731

ORIGINAL ARTICLE / ÖZGÜN MAKALE

EVALUATION OF KNOWLEDGE, ATTITUDES, AND PRACTICES OF COMMUNITY PHARMACISTS TOWARD CELIAC DISEASE

SERBEST ECZACILARIN ÇÖLYAK HASTALIĞINA YÖNELİK BİLGİ, TUTUM VE UYGULAMALARININ DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *The knowledge and attitude of pharmacists play an essential role in the patient counseling services of pharmacists. Celiac disease is one of the diseases in which patient follow-up and counseling are essential, and the number of studies dealing with the roles of pharmacists in this disease is very limited. This study aims to fill this gap and contribute to public health by evaluating community pharmacists' knowledge, attitudes, and practices for celiac disease.*
Material and Method: *Based on the literature, a measurement tool including the knowledge, attitudes, and practices of community pharmacists for celiac disease has been developed. The measurement tool was applied online to community pharmacists in Türkiye in 2021. The obtained data were subjected to explanatory factor analysis (EFA).*
Result and Discussion: *The number of pharmacists participating in this study is 408. A four-factor structure was obtained: knowledge of celiac disease, attitude towards celiac disease, counseling practices for celiac patients, and professional development practices. The Cronbach's alpha values of the factors were calculated between 0.794 and 0.935, which shows high reliability. These factors explained 70.343% of the total variance. The community pharmacists had positive attitudes toward counseling for celiac disease. Still, there were some deficiencies in terms of knowledge and practice. It is thought that the knowledge and awareness of pharmacists on celiac diseases can be increased by including issues related to celiac disease in both undergraduate education and vocational training programs.*
Keywords: *Attitude, celiac disease, community pharmacist, knowledge, practice*

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Submitted/ Gönderilme : 21.07.2023
Accepted /Kabul : 16.08.2023
Published/Yayınlanma : 20.09.2023

ÖZ

Amaç: *Eczacıların bilgi ve tutumları, eczacıların hasta danışmanlığı hizmetlerinde önemli bir rol oynamaktadır. Çölyak hastalığı, hasta takibi ve danışmanlığının gerekli olduğu hastalıklardan biridir ve bu hastalıkta eczacıların rollerini ele alan çalışma sayısı oldukça sınırlıdır. Bu çalışma, serbest eczacıların çölyak hastalığı konusundaki bilgi, tutum ve uygulamalarını değerlendirerek bu boşluğu doldurmayı ve halk sağlığına katkıda bulunmayı amaçlamaktadır.*

Gereç ve Yöntem: *Literatüre dayalı olarak serbest eczacıların çölyak hastalığına yönelik bilgi, tutum ve uygulamalarını içeren bir ölçüm aracı geliştirilmiştir. Ölçme aracı 2021 yılında Türkiye'deki serbest eczacılara online olarak uygulanmıştır. Elde edilen veriler açıklayıcı faktör analizine (EFA) tabi tutulmuştur.*

Sonuç ve Tartışma: *Çalışmaya katılan eczacı sayısı 408'dir. Çölyak hastalığı bilgisi, çölyak hastalığına yönelik tutum, çölyak hastalarına yönelik danışmanlık uygulamaları ve mesleki gelişim uygulamaları olmak üzere dört faktörlü bir yapı elde edilmiştir. Faktörlerin Cronbach alfa değerleri 0.794 ile 0.935 arasında hesaplanmıştır ve bu yüksek güvenilirlik göstermektedir. Bu faktörler toplam varyansın %70.343'ünü açıklamaktadır. Serbest eczacılar, çölyak hastalığı danışmanlığına karşı olumlu tutumlara sahiptir. Yine de bilgi ve uygulama açısından bazı eksiklikler bulunmaktadır. Hem lisans eğitiminde hem de mesleki eğitim programlarında çölyak hastalığı ile ilgili konulara yer verilerek eczacıların çölyak hastalıkları konusundaki bilgi ve farkındalıklarının artırılabileceği düşünülmektedir.*

Anahtar Kelimeler: *Bilgi birikimi, çölyak hastalığı, serbest eczacı, tutum, uygulama*

INTRODUCTION

Celiac disease, defined by the Anatolian physician Aretaus in the second century, is known as wheat allergy and gluten sensitivity [1]. The incidence of celiac disease in adults is 0.1%, which is a disease that generally presents with findings such as urticaria, angioedema, nausea, and abdominal pain in children and gastrointestinal symptoms in adults [2]. It can also be described as an autoimmune disease that causes damage and inflammation in the small intestine in individuals with hypersensitivity to gluten [3]. Celiac disease can be congenital, or it can be seen especially in the 30s and 40s, depending on the individual's diet [4]. Gottlieb et al. mentioned new treatment approaches with drugs being developed recently, although a gluten-free diet is the only known and effective treatment for celiac disease [5].

According to Rajput and Molder et al., the worldwide prevalence of celiac is nearly 1 %, but the number of undiagnosed patients cannot be ignored [6,7]. Similarly, the incidence of celiac disease in Turkey ranges from 0.003 to 0.01, according to the findings of the Ministry of Health of the Republic of Türkiye. As Lerner et al. mentioned, it is necessary to increase awareness of primary care sectors like physicians and dietitians to improve the detection rate of celiac disease and patient compliance [8]. In this regard, accurately informing and accurately directing individuals with celiac disease is also closely related to pharmacists, who are accepted as the closest and most accessible healthcare providers, and awareness of the disease's signs and symptoms. Therefore, pharmacists are expected to primarily provide healthy life support related to gluten-free nutrition, drugs, and nutritional supplements [9]. It is known that long-term vitamin and mineral supplements are recommended for celiac patients. Patients should be carefully monitored to determine whether existing nutritional deficiencies are being resolved and whether new deficiencies do not develop. Pharmacists should be able to cooperate with nutritionists in selecting the gluten-free nutritional supplements needed [10]. A study on pharmaceutical companies concluded that five out of 100 companies have a policy that provides gluten-free status for their drugs and that most companies believe their products are gluten-free [11].

Community pharmacists must know about drug absorption and gluten content of pharmaceutical products, especially in celiac patients, and provide consultancy services to patients on these issues and increase their quality of life. In the literature, the number of studies dealing with pharmacists' knowledge, attitude, and practices in celiac disease is quite limited. Some studies discuss other health professionals' knowledge and awareness levels. Assiri et al., Dembinski et al., and Riznik et al. determined that the knowledge level of young physicians about celiac disease was higher than seniors

[12-14]. In contrast, Bargezar et al. revealed that the experience positively affects healthcare providers' knowledge about the diagnosis and treatment of celiac [15]. Thus, it can be seen that the physicians' knowledge level was not satisfactory. Jinga et al. surveyed physicians' ability to define celiac disease and their attitudes and suggested that more efforts should be made to increase awareness of celiac disease among physicians in medical specialties [16]. Karaoğlu aimed to examine the awareness level of physicians about celiac disease and the contribution of education to the awareness level. A statistically significant difference was found in the 90% confidence interval between the pre-training and post-training tests. Thus, it has been concluded that celiac education given in the first steps of education will increase the correct diagnosis rate of this disease [17]. According to Avena-Woods et al., only 27% of pharmacists could define celiac disease as both autoimmune and chronic. The majority of respondents (60%) correctly state that there is no regulation requiring manufacturers to specify drugs as gluten-free, with 20% saying that they recommend dietary changes to people suspected of having celiac disease before diagnosis [18].

This study's motivation comes from evaluating community pharmacists' knowledge, attitudes, and practices toward celiac disease to close the gap in the literature and contribute to public health. A measurement tool containing community pharmacists' knowledge, attitude, and practices has been developed in line with this aim. Then, the validity and reliability of the measurement tool were evaluated.

MATERIAL AND METHOD

Measurement Tool

Within the scope of the study, a measurement tool was designed to determine the knowledge, attitudes, and practices of community pharmacists in Türkiye toward celiac disease by adhering to the KAP (Knowledge-Attitude-Practice) survey structure. KAP survey style is frequently preferred due to its advantages, such as providing measurable data, straightforward design and interpretation, and ease of application [19]. Such surveys provide systematic data about what is known, exhibited, and done about a particular subject, enabling individuals to identify knowledge gaps, cultural beliefs, or behavioral patterns [20]. Items of the tool were adapted from Mehralian et al., Bastani et al., and Avena-Woods et al. in which the importance of demonstrating the pharmacist's knowledge of celiac disease, as well as their attitude toward helping these patients, to optimize care for celiac patients was emphasized [18,21,22].

While preparing the measurement tool, an item pool consisting of 40 statements was first created. A 5-point Likert-type scale was used in the evaluation of the items. Out of 40 items, 13 are knowledge (1 (Very little) - 5 (Very good)), 10 are attitude ((1) Strongly disagree-(5) Strongly agree, and 17 are practice ((1) Never - (5) Always) statements. It is aimed to determine community pharmacists' knowledge of celiac disease with the knowledge expressions in the measurement tool. The attitudes of community pharmacists towards celiac disease have been tried to be discussed with the attitude statements. In the last part, it was tried to determine the practices of community pharmacists for celiac patients and the frequency of their practices for their professional development. Finally, questions that will determine the demographic characteristics of the participants in light of the relevant literature were added to the questionnaire to be applied. Before applying the questionnaire to the target audience, a pilot application was conducted based on the assumption that a spelling, expression, or form problem might be related to the items in the measurement tool. As a result of the pilot application conducted on a group of 14 people with similar characteristics to the target population, it was determined that 3 of the statements related to attitude and 1 of the statements on practice were not fully understood. Therefore, these items were removed from the measurement tool, and 36 statements were included.

Sample Size and Data Collection

The population of this study consists of nearly 25000 pharmacists working as community pharmacists in Türkiye. The sample size of this study was calculated using the acceptable error level method under the assumption that the sample statistics are normally distributed. The sample size was calculated as 379 by taking 0.05 confidence level, z=1.96, d (sensitivity)=0.05, and p and q values as

0.50. The questionnaires were applied online because of the Coronavirus pandemic via Google Forms between 20 November 2020 - 15 March 2021. The questionnaire links were first shared with managers of local pharmacists' chambers, and the managers shared the link with their members working as community pharmacists. To increase the reliability of the results obtained from the study, it aimed to reach the maximum number of people, and the participation of 410 community pharmacists was ensured. The data obtained from two participants were eliminated due to the detected errors and deficiencies. The analyzes were continued with the data from 408 participants.

Data Analysis

The data obtained from the measurement tool were first subjected to descriptive statistical analysis with the help of the IBM SPSS Statistics 22.0 package program, and then explanatory factor analysis (EFA) was applied using the principal component analysis technique.

RESULT AND DISCUSSION

The demographic characteristics of the participants (n=408) are presented in Table 1.

Table 1. Demographic characteristics of the participants

Characteristics of the Participants	Frequency (%)
Gender	
Female	55.9
Male	44.1
Ages	
Under 25	5.0
26-50	82.0
Older 50	13.0
Working Year	
<10 years	33.0
10-20 years	52.0
>20 years	15.0
Number of celiac patients per year	
<5	47.0
6-19	25.0
>20	28.0

According to Table 1, it is seen that the pharmacists participating in the study are generally experienced, and more than half of them fill more than five celiac patient prescriptions in a year. Additionally, participants were asked whether they paid attention to product ingredients when presenting prescribed and non-prescribed medicines to a celiac patient. When the answers to this question were examined, it was determined that 39 pharmacists checked whether they contained gluten. In addition, it was determined that 156 participants looked for wheat, 150 for starch, 117 for rice flour, 78 for oats, 96 for rye, 46 for barley, and 16 for malt.

Kaiser-Meyer-Olkin (KMO) and Bartlett sphericity tests were used to determine the suitability of the obtained data for EFA, and the KMO value was calculated as 0.898. This value above 0.8 indicates that the sample size is sufficient for EFA [23]. With the Bartlett test of sphericity, the hypothesis that the correlation matrix equals the unit matrix for $p<0.05$ was rejected. As a result of Varimax rotation, 13 items in the measurement tool were excluded from the analysis because they were not included in any factor or had low factor loadings (less than 0.50), and it was determined that the remaining 23 items were grouped under four factors. This four-factor structure explained 70.343% of the total variance, which denotes a strong factor structure [24,25]. The four factors were labeled as (i) knowledge about celiac disease (K), (ii) attitude towards celiac disease (A), (iii) counseling practices for celiac patients (CP), and (iv) professional development practices for celiac disease (PDP). Details of the EFA results are given in Table 2.

Table 2. EFA results

	Mean	Factor Loadings			
Factors and items	**($\bar{x}$)**	**K**	**A**	**CP**	**PDP**
Knowledge about celiac disease (K)					
I have knowledge of the symptoms of celiac disease	3.076	0.880			
I have general knowledge of celiac disease	3.172	0.878			
I have information about what celiac patients should pay attention to	3.194	0.869			
I have general knowledge of gluten sensitivity	3.226	0.837			
I have knowledge about a gluten-free diet	3.289	0.819			
I know about gluten-free products	3.167	0.780			
Attitude towards celiac disease (A)					
Pharmacist and patient communication increases success in the treatment	3.654		0.842		
of celiac disease	3.412		0.800		
Pharmacists play an essential role in the treatment of celiac disease	3.256		0.793		
Pharmacists should review the ingredients of non-pharmaceutical products	3.772		0.784		
used by celiac patients	3.098		0.772		
Pharmacist and doctor communication increases success in the treatment	3.564		0.744		
of celiac disease	3.012		0.744		
Pharmacies must have particular areas where gluten-free products are served					
Pharmacists play an essential role in the follow-up of celiac disease					
Gluten-free products should only be sold in pharmacies					
Counseling practices for celiac patients (CP)					
I follow the food supplement intake of my patients with celiac disease	2.042			0.855	
I follow the life habits of my patients with celiac disease	2.032			0.843	
I follow the vitamin intake of patients with celiac disease	2.137			0.836	
I follow the nutritional habits of my patients with celiac disease	2.083			0.830	
I examine whether non-drug products contain gluten.	2.483			0.601	
I follow the drugs used by my patients with celiac disease	2.576			0.569	
Professional development practices for celiac disease (PDP)					
I attend training on celiac disease	2.120				0.831
I ensure that my pharmacy staff attend training for celiac disease	1.958				0.764
I follow current developments in the treatment of celiac disease	2.767				0.600
I inform my pharmacy staff about celiac disease	2.512				0.497
Variance Explained (%)		21.724	19.588	18.744	10.287
Cumulative Variance Explained (%)		21.724	41.312	60.056	70.343
Cronbach's alpha (α)		0.935	0.904	0.902	0.794

For the validation and reliability of the measurement tool, factor loadings and Cronbach's alpha (α) values were used [26]. According to Table 2, factor loadings are over 0.70 in general, and it is considered sufficient if this value is above 0.40 [27]. In addition to the factors' Cronbach's alpha values, the overall Cronbach's alpha reliability coefficient of the measurement tool was calculated as 0.894. Therefore, it is seen that the reliability level of the factors obtained and the measurement tool is high.

Counseling services regarding pharmacological and non-pharmacological (diet, lifestyle, etc. change) treatments to be offered to celiac patients positively affect their treatment processes and quality of life. Counseling services provided by pharmacists to celiac patients are of great importance since the closest health institutions where patients can access information about drugs and treatment are community pharmacies. The delivery of these services is affected by factors such as the knowledge and attitude of pharmacists, as stated in many psychometric theories. This study aimed to evaluate community pharmacists' knowledge, attitudes, and practices toward celiac disease. For this purpose, a measurement tool was designed for pharmacists' knowledge, attitudes, and practices toward celiac disease by considering the studies in the relevant literature in detail. The data obtained from the measurement tool were subjected to EFA, and a 4-factor structure was obtained. Cronbach's alpha coefficients of these factors were found to be high, so the reliability of the measurement tool was ensured. In this context, it is possible to say that this measurement tool will fill the literature gap in measuring pharmacists' knowledge, attitudes, and practices toward celiac disease. This paper has clearly shown that the developed measurement tool is a reliable guide in filling the deficiency in the literature

and revealing pharmacists' views on celiac disease. To the best of the authors' knowledge, this is the first study that deals with celiac disease from the perspective of community pharmacists.

It has been revealed that most of the pharmacists participating in the study have provided services to individuals with celiac disease, and more than half have provided services to 6 or more celiac patients until now. In the study conducted by Karaoğlu to determine physicians' awareness of celiac disease, it was determined that more than 70% of the physicians served patients previously diagnosed with celiac disease. Additionally, it was observed that 39 pharmacists looked at the gluten content of the products [17]. Mangione and Patel emphasized that celiac patients who adhere to a gluten-free diet for life should avoid wheat, barley, rye, and derivatives of these products in pharmaceutical products [10]. Shah et al. stated that wheat, rye, and barley starch should be considered in pharmaceutical products because it contains gluten [28]. When the average of the responses given to the statements related to the information statements in the measurement tool is considered, it is seen that this value is generally above 3 (minimum 1 - maximum 5). This situation indicates that pharmacists have a moderate level of knowledge about celiac disease.

Similarly, Jinga et al. evaluated physicians' attitudes and perceptions toward celiac disease and found their awareness about it poor [16]. Riznik et al. determined that the knowledge level of healthcare professionals about celiac disease is not satisfactory [14]. Avena-Woods et al. revealed that community pharmacists serving in chain pharmacies in New Jersey and New York have knowledge about celiac disease, but their knowledge level is insufficient [18]. Mangione and Petal emphasized the importance of community pharmacists' knowledge of celiac disease and stated they could increase their quality of life by providing counseling services to patients [10].

Mercan stated that the cooperation of pharmacists and physicians in preventive health services would have positive health outcomes [29]. Narmansoy explained that pharmacists' communication with physicians during patient follow-up also undertakes teaching to convey drug information to patients. Similarly, this study stated pharmacists had a positive attitude toward communicating with the doctors of celiac patients [30]. Another critical and addressed point in the counseling services pharmacists offer is pharmacist-patient communication. Effective communication that can be established between the pharmacist and the patient is an element that affects the patient's compliance with the treatment and recovery process [31-33]. In studies dealing with gluten-free diets and treatment compliance of celiac patients, it has been revealed that patients with high knowledge and awareness of celiac disease and their relatives have higher compliance [15,34].

For this reason, pharmacists' counseling on gluten-free nutrition, drugs, vitamins, and nutritional supplements to celiac patients and their relatives, apart from clinical outcomes, plays a vital role in guiding and informing patients correctly [9,29]. It is thought that patients' desire to get information from pharmacists on this subject will also increase with activities such as preparing brochures and presenting seminars to raise awareness about celiac disease from a social point of view. In this context, the high average of the answers given by pharmacists to the statements about pharmacist counseling within the factor of attitude towards celiac disease shows that pharmacies are a sociologically important place for communication and that community pharmacists have an attitude towards taking on the task as healthcare providers.

Contrary to the positive attitude of the participant pharmacists in the study, the fact that the average responses given in the statements about nutrition and life follow-up of the medication, vitamin, and food supplement intake of the patients considered in the CP factor is around 2, which reveals that the pharmacists are deficient in the point of application. Studies by Green and Cellier, Akkelle and Ertem, and Mangione and Patel discussed the importance of vitamin and mineral follow-up in these patients [10,35-36]. Haines et al., See and Murray, and Avena-Wood mentioned the importance of monitoring the nutritional habits of celiac patients [18,37-38]. The practices of pharmacists regarding celiac disease were also discussed regarding the possibility of nutritional complications such as iron deficiency anemia, lactose intolerance, and osteoporosis. Considering this possibility, approximately half of the participants referred the patients to the doctor. As stated in many studies in the literature [39-42], nutritional complications are among the factors to be considered in terms of celiac disease. Pharmacists' responses to the practices specified in the CP factor also indicate their awareness of this situation.

Within the scope of this study, the practices of pharmacists for celiac disease are discussed not only in terms of the consultancy services they provide but also in terms of professional development. It is seen that the averages of the expressions under this factor are generally around 2. In the literature, many studies deal with the fact that the training received by healthcare professionals in providing counseling services for chronic diseases improves their knowledge and practices. Karaoğlu researched to determine the awareness of physicians about celiac disease, and a statistically significant difference was found in the awareness level of celiac disease with the pre-and post-training questionnaire about celiac disease ($p<0.001$) [17].

Additionally, under the PDP factor, the rate of pharmacists following current developments in the treatment of celiac disease was also discussed, and it was determined that this rate was low. Different treatment approaches are discussed in various studies in the literature, and it is important to follow current developments and to have information about alternative treatment methods for celiac disease. In this context, it is thought that by integrating current treatment approaches with systems such as electronic drug information resources, which are frequently used in pharmacies, it will be easier for pharmacists to access this information and the frequency of counseling practices related to celiac disease can be increased.

As a result of the study, it has been seen that most community pharmacists providing service throughout Türkiye think that pharmacists play an essential role in managing celiac disease. It has been determined that the participants have a positive attitude toward counseling in celiac disease, but there are some deficiencies in knowledge and practice. Therefore, pharmacists for celiac disease can be supported and developed to take an active role by providing regular on-the-job training and increasing communication with other health professionals. In addition, it is thought that giving more space to autoimmune diseases such as celiac and counseling services for these diseases in the curriculum of pharmacy faculties will contribute to eliminating these deficiencies.

Limitation

There may be some possible limitations in this study. Although the coincident of the survey implementation process with the Covid-19 pandemic process reduced the chance of conducting a face-to-face survey, causing the participation rate to be below the expected level, the smallest sample targeted could be reached. In addition, only community pharmacists were included in the study. For this reason, in order to ensure that the subject is evaluated from a broader perspective, validity, and reliability studies should be carried out in different groups of health professionals who are involved in celiac diseases, such as hospital pharmacists, dietitians, nurses, endocrinologists, and internal medicine specialists working in the public and pharmaceutical industry by expanding the topics covered in the study and celiac disease. Additionally, the authors want to state their future research plan on modeling the practices of pharmacists regarding celiac disease from a psychometric point of view.

ACKNOWLEDGEMENTS

This study was created by expanding the master of science thesis that the first author completed at Van Yüzüncü Yıl University Faculty of Pharmacy, Department of Basic Pharmacy Sciences, under the supervision of the corresponding author.

AUTHOR CONTRIBUTIONS

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

This study was performed strictly following good research practices and approved by the Van Yüzüncü Yıl University Ethical Committee decision dated 16/10/2020 and numbered 2020/07-03.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 944-956, 2023
Doi: 10.33483/jfpau.1341561

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PHARMACIST-LED ASSESSMENT OF ANXIETY LEVELS AND ASSOCIATED FACTORS: A PROSPECTIVE OBSERVATIONAL SURVEY

ECZACI TARAFINDAN YÖNETİLEN ANKSİYETE DÜZEYLERİNİN VE İLİŞKİLİ FAKTÖRLERİN DEĞERLENDİRİLMESİ: PROSPEKTİF GÖZLEMSEL BİR ÇALIŞMA

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ABSTRACT

Objective: *Mental illnesses constitute a significant contributor to the overall burden of illness, affecting a substantial number of individuals worldwide. The aim of the study is to to measure anxiety levels, along with a questionnaire to assess the pharmacist possible role in anxiety.*
Material and Method: *A prospective study with onlineonline questionnaire held between November 2020 and February 2021. The questionnaire was sent to the general public. The questionnaire consists of 2 sections, demographics and anxiety levels. Anxiety levels were determined by the Generalized Anxiety Disorder (GAD-7) scale. Assessment of internal consistency, reliability, binary logistic regression and correlation analysis, group comparisons were conducted utilizing the Mann-Whitney U-test.*
Result and Discussion: *A total of 398 responses were gathered. The majority of the participant were female (256, 64.3%) and the mean number of age was 29.7±10.7. The mean GAD-7 score was 6.31±4.21. The interclass correlation coffecient of the questionnaire was 0.671 (95% CI: 0.503-0.794, F: 4.55, p<0.001) and the Cronbach's alpha test was found as 0.854. Feeling anxious or stressed lately (OR: 2.358, %95 CI:1.321-4.210,p<0.005) and the level of education (OR:5.618, %95 CI 1.332-23.698 p<0.05) were statistically significant factors for anxiety. The importance of involving pharmacists in anxiety disorder screenings were pointed out by this study. Pharmacists'*

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Submitted / Gönderilme : 11.08.2023
Accepted / Kabul : 17.08.2023
Published / Yayınlanma : 20.09.2023

screenings effectively identify anxiety, and aids referrals to appropriate healthcare providers.
Keywords: *Anxiety, clinical pharmacy, mental health sceerining, patient monitoring, pharmaceutical care*

ÖZ

Amaç: *Ruh ve sinir hastalıkları, dünya çapında önemli sayıda bireyi etkileyerek genel hastalık yüküne önemli bir katkıda bulunan hastalık gruplarındadır. Çalışmanın amacı, bireylerin anksiyete düzeylerini ölçmek ve eczacının rolünü araştırmaktır.*
Gereç ve Yöntem: *Kasım 2020 ile Şubat 2021 arasında prospektif bir çevrimiçi anket düzenlenmiştir. Anket, demografik bilgiler ve anksiyete düzeyleri olmak üzere 2 bölümden oluşmaktadır. Anksiyete düzeyleri Yaygın Anksiyete Bozukluğu (YAB-7) ölçeği ile belirlenmiştir. İç tutarlılık, güvenilirlik, ikili lojistik regresyon ve korelasyon analizleri ve grup karşılaştırmaları Mann-Whitney U-testiyle anksiyeteyle düzeyleri ve ilişkili faktörler belirlenmiştir.*
Sonuç ve Tartışma: *Toplam 398 yanıt toplanmıştır. Yaş ortalamasının 29,7±10,7 olduğu katılımcı profilinin çoğunu kadınlar oluşturmuştur (256, %64,3). Ortalama YAB-7 puanı 6.31±4.21 olarak belirlenmiştir. Anketin iç tutarlılığı 0.671 (%95 GA: 0.503-0.794, F: 4.55, p<0.001) ve Cronbach alfa testi 0.854 idi. Son zamanlarda endişeli veya stresli hissetme (OR: 2.358, %95 GA:1.321-4.210, p<0.005) ve eğitim düzeyi (OR:5.618, %95 GA 1.332-23.698, p<0.05) istatistiksel olarak anlamlı faktörler olarak belirlenmiştir. Bu çalışma, eczacıların anksiyete bozukluğu taramalarına dahil edilmesinin önemine işaret etmektedir. Eczacıların taramaları anksiyeteyi etkili bir şekilde tanımlamakta ve uygun sağlık hizmeti sağlayıcılarına yönlendirmeye yardımcı olmaktadır.*
Anahtar Kelimeler: *Anksiyete, farmasötik bakım, hasta izlemi, klinik eczacılık, ruh sağlığı taraması*

INTRODUCTION

Anxiety disorders refer to a set of mental disorders characterized by the presence of anxiety and fear, with anxiety being a feeling of apprehension about future events and fear being a response to current events. These emotions may manifest as physical symptoms like rapid heartbeat and tremors. Chronic and excessive anxiety and worry about a variety of everyday situations or events that persist for a minimum of six months is categorized as generalized anxiety disorder (GAD) [1].

Mental disorders are a major cause of disease burden, with a global prevalence of 970 million people in 2019, or 12.6% of the world's population. They account for 13% of disability-adjusted life years and cause more burden than cardiovascular and circulatory disorders. Consequently, mental disorders impose an enormous economic burden, with depression and anxiety disorders alone costing the global economy $1 trillion annually [2,3].

Anxiety is a typical response to stress that can have beneficial effects in certain circumstances. It serves as a warning mechanism against potential threats and can help individuals remain vigilant and attentive. Anxiety disorders are distinct from normal feelings of irritability or anxiety and are characterized by excessive and irrational fear or anxiety. These disorders are among the most prevalent of mental health conditions and impact roughly 30% of adults at some point in their lives. Anxiety disorders may prompt individuals to avoid situations that trigger or exacerbate their symptoms, which can negatively impact performance in business, school, and personal relationships. However, effective treatments are available for anxiety disorders, and the majority of individuals who receive treatment are able to achieve a normal and productive life [3,4].

Clinicians encounter numerous challenges when treating patients with anxiety disorders on a daily basis. To address these obstacles, collaborative medical teams are needed, with pharmacists playing a significant role. Within such teams, pharmacists assume a major responsibility in enhancing patient compliance, optimizing dosage schedules, promptly identifying the onset of the illness, averting drug-drug interactions, and mitigating side effects [5].

Pharmacists have a unique opportunity to enhance the outcomes of patients suffering from mental illnesses in community pharmacies. According to the International Pharmacy Federation Report, pharmacists have been shown to improve the care and well-being of individuals with mental health issues through various means, such as education, prompt diagnosis, triage, collaboration, and healthcare

services [6].

Community Health Management refers to a targeted intervention process aimed at identifying patients and optimizing their well-being, while minimizing negative outcomes and reducing healthcare costs [5,6]. Various healthcare professionals, including clinical pharmacists, participate in community health management in different capacities. With the involvement of clinical pharmacists, drug management is conducted accurately to prevent uncontrolled treatment of diseases. Clinical pharmacists may refer patients to their primary healthcare providers for disease management, and they can provide drug management to patients until chronic disease control is achieved pharmacists can play a pivotal role in providing an effective community health approach by providing patient education and optimizing patient health outcomes. Studies have revealed that integrating pharmacists into primary healthcare institutions has led to improvements in the utilization of evidence-based treatments and patient outcomes for many chronic diseases [7].

The clinical pharmacist plays a crucial role in providing mental health diagnoses and training related to psychotropic drugs for both patients and staff. Besides mental health problems, clinical pharmacists also manage metabolic disorders such as hypertension, hyperlipidemia, and diabetes by making appropriate referrals for dietary interventions and further treatment. For outpatient care, clinical pharmacists participate in interdisciplinary team meetings comprising of psychiatrists, nurses, clinical psychologists, and social workers. They provide expertise in pharmacotherapy to optimize patient care and help evaluate patients who require complex drug therapies. Clinical pharmacists have significant responsibilities in ensuring the continuity of care in mental health services for inpatient or outpatient psychiatric unit patients, including the appropriate dosage of long-acting injectable antipsychotics [8].

Pharmacists can perform mental health assessments for patients, assess drug suitability and side effects, review drug treatment and monitoring. Clinical pharmacists should take part of the treamment of mental health disorders in outpatient settings. Clinical pharmacists should not be limited to contributing to the patients with diagnoses. CP can participate in patient assessments, based on mental health symptoms, and refer patients to psychiatrists or therapy accordingly [8].

In our study, we utilized the Generalized Anxiety Disorder-7 (GAD-7) to measure anxiety levels, along with a questionnaire to assess the individual's knowledge of anxiety.

MATERIAL AND METHOD

Study Design and Sample Size

From November 2020 to February 2021, a descriptive, observational study was conducted in Turkey. Participants were restricted by age (18-65 years old has been included) only and chosen based on their willingness to participate in the study. The study was approved by the Bezmialem Vakif University local Ethics Committee with a decision number of 18/345. The exponential non-discriminative snowball sampling method was used to select participants. The study is reported according to the CROSS (A Consensus-Based Checklist for Reporting of Survey Studies) standards [9].

$$\boldsymbol{n = \frac{Z^2\, p(1-p)}{d2}}$$

The formula presented above is used to calculate the sample size. In the formula, n represents the sample size. A p-value of 0.5 is used for the proportion. The margin of error for sampling is set to 0.05, and Z represents the standard normal value at a 95% confidence interval, which is equal to 1.96. Using these values and considering similar studies in the literature, the sample size determined to be 345. Considering a non-response rate, the total sample size is set to 398 [10, 11].

Questionnarie, Survey Distribution and Data Collection

To evaluate individuals' anxiety levels, a web based online questionnaire was developed using Google Forms. The survey was disseminated via various methods, including email, direct messages, and social media platforms, along with information about the study. To increase participation, participants were encouraged to share the survey link with their social circles. The survey consisted of three sections, including demographics, the GAD-7 scale, and an anxiety knowledge assessment test, with a total of 21

items. Participants electronically signed a written informative and approval form, and each response was cross-checked to ensure it was only provided once. A hyperlink of consent form has been embedded to an online survey for participant for personal use.

The Generalized Anxiety Disorder-7 (GAD-7) is a brief, self-reported questionnaire used to assess generalized anxiety disorder, which was developed based on DSM-IV criteria by Spitzer et al. It consists of seven items that are rated on a four-point Likert scale (0=never, 1=several days, 2=more than half the days, 3=nearly every day) and is completed using a paper-pencil format to evaluate symptoms experienced over the previous two weeks [4]. A total score of 5, 10, and 15 on the GAD-7 correspond to mild, moderate, and severe anxiety, respectively, with a cut-off score of 10 or higher indicating possible GAD. The sensitivity and specificity of the GAD-7 are 89% and 82%, respectively, when a cut-off score of 10 is used. The original article for the GAD-7 established cut-off scores of 0-4 for mild anxiety, 5-9 for moderate anxiety, 10-14 for high anxiety, and 15-21 for severe anxiety. The Turkish version of the GAD-7 has demonstrated high validity and reliability, and good psychometric properties in clinical settings, comparable to the original version. Therefore, the GAD-7 can be used as a screening tool for generalized anxiety disorder. The Turkish adaptation and validation of GAD-7 scale was conducted by Konkan et al [12]. A permission has been obtained by the the corresponding author of the GAD-7. Seven items rated on a scale ranging from 0 (never) to 4 (always). The scores indicated greater scores represent greater anxiety level. The threshold was 10, which indicated a critical level of anxiety which requires medical support and attention.

The dependent variable was the presence of anxiety, which was assessed using GAD-7 scores (GAD-7 scores higher than 10 were considered as presence of anxiety). The study incorporated various independent variables to assess their potential impact on the research outcome. These variables encompassed employment status, gender, marital status, level of education, presence of chronic illnesses and medications, as well as the Feeling anxious or stressed lately for any reason.

A team of experts consisting of four professionals was consulted to provide feedback on the survey's language design and the questions were revised based on their recommendations. Additionally, the questionnaire was pre-tested with two members (a clinical pharmacist and a public health specialist physician) who possess expertise in behavior change education. The pre-testing involved retrospective cognitive interviews with a focus on assessing the content, format, and wording of the survey. Pilot research was conducted with a group of fifteen individuals who did not participate in the initial evaluation, and feedback obtained was used to improve the survey's clarity and understandability. A group of twenty participants, who were not included in the original study data set, were asked to complete the questionnaire within a period of two weeks. The survey took approximately 15-20 minutes to complete. To assess the test-retest reliability of the questionnaire, the Spearman correlation coefficient, Wilcoxon test, and intraclass correlation coefficient (ICC) were evaluated using a sample of 20 participants. The results indicated that the questionnaire exhibited a statistically insignificant correlation of 0.619 ($p>0.05$) and an ICC of 0.671 (95% CI: 0.503-0.794, F: 4.55, $p<0.001$). The reliability of the GAD-7 scale was evaluated using Cronbach's alpha test, which yielded a value of 0.854 for the survey tool used in this study.

Statistical Analysis

For continuous variables, descriptive statistics such as mean, median, standard deviation, and interquartile range (IQR) were reported, while categorical variables were presented as frequency and percentage. The Kolmogorov-Smirnov, Shapiro-Wilk tests Q-Q plots, histogram and density analysis, skewness and kurtosis values was used to test for normality of continuous variables. To assess differences between groups, independent t-tests or Mann-Whitney U tests were performed, while chi-square tests were used to investigate the relationship between categorical variables. Univariate logistic regression analysis was conducted with a significance level of $p < 0.20$ to determine significant variables, which were then included in binary logistic regression analysis. Estimated risk values and confidence intervals were reported. The study aimed to identify variables that predict factors related to anxiety, and the Nagelkerke R square and Hosmer and Lemeshow tests were used to assess the explanatory power and fit of the model, respectively. Missing data were excluded from analysis, and Statistical Package for Social Science (SPSS) version 26® and Jamovi version 1.6 software were used

for statistical analysis. Univariate and multivariate logistic regression analyses were performed to identify factors associated with anxiety disorder. Statistical significance was defined as $p < 0.05$.

RESULT AND DISCUSSION

Sociodemographic Characteristics of Study Participants

This study involved 398 participants, with an average age of 29.7±10.7. The majority of participants (293, 73.6%) were under the age of 35. The mean GAD-7 score of participants were 6.31±4.21. Participants with GAD-7 scores above 10 were found to be younger (27.60±10.7). A large proportion of participants (330, 82.9%) were not experiencing anxiety (GAD-7 score below 10), which would require medical attention or professional support. The sample was primarily female (256, 64.3%), and the majority were single (256, 64.8%) and had a graduate or higher education level (334, 83.9%). Demographic characteristics of the sample are presented in Table 1. Most of the participants were employed (368, 92.5%) and did not have any chronic conditions (338, 85.4%).

Table 1. Sociodemographic characteristics of participants

Parameter	Total N (%)	GAD-7⩾10 N (%)	GAD-7<10 N (%)	p
	398 (%100)	68 (%17.1)	330 (%82.9)	NA
Age, Mean ±SD	**29.7±10.7**	**27.60±10.7**	**30.1±10.7**	*=0.035#*
18-24 years	203 (51.0)	45 (11.3)	158 (39.7)	*=0.033**
25-34 years	90 (22.6)	11 (2.8)	79 (19.8)	
35-44 years	48 (12.1)	4 (1.0)	44 (11.1)	
45-54 years	42 (10.6)	4 (1.0)	38 (9.5)	
54-65 years	15 (3.8)	4 (1.0)	11 (2.8)	
Gender				>0.001*
Male	142 (35.7)	20 (5.0)	122 (30.7)	
Female	256 (64.3)	48 (12.1)	208 (52.3)	
Marital Status				>0.001*
Married	140 (35.2)	17 (4.3)	123 (30.9)	
Single	258 (64.8)	51 (12.8)	207 (52.0)	
Level of Education				>0.001*
Primary School	10 (2.5)	1 (0.3)	9 (2.3)	
Secondary School	10 (2.5)	2 (0.5)	8 (2.0)	
High School	44 (11.1)	6 (1.5)	38 (9.5)	
Graduate	285 (71.6)	49 (12.3)	236 (59.3)	
Postgraduate	49 (12.3)	10 (2.5)	39 (9.8)	
Employment Status				>0.001*
Employed	368 (92.5)	66 (16.6)	302 (75.9)	
Unemployed	30 (7.5)	2 (0.5)	28 (7.0)	
Cormobidity				>0.001*
Yes	58 (14.6)	7 (1.8)	51 (12.8)	
No	338 (85.4)	61 (15.4)	278 (70.0)	
Feeling anxious or stressed lately for any reason				=0.003*
Yes	221 (55.5)	49 (12.3)	172 (43.2)	
No	177 (44.5)	19(4.8)	158 (39.7)	
GAD-7 Score	**6.31±4.21**	**13.4±2.91**	**4.85±2.65**	>0.001#

GAD-7: General Anxiety Disorder 7 Scale. * Chi Square test, # Mann Whitney U test

This study aimed to evaluate the prevalence and factors that are associated with anxiety levels among Turkish people. In the year 2019, the global count of individuals grappling with an anxiety disorder stood at 301 million, encompassing approximately 58 million children and adolescents [13]. Extensive community surveys carried out within the Eastern Mediterranean Region (EMR) indicate a range of psychological distress rates spanning from 15.6% to 35.5%, with the figures tending to be higher in nations contending with intricate emergency scenarios [14,15]. Moreover, the occurrence of mental disorders over a span of twelve months spans from 11.0% to 40.1%, as reported in these surveys. A German study the 5.9% of the total sample had GAD-7 scre 10 or higher. [16]. Our study results showed a prevalence 17.1% of the participants has GAD-7 score considered as anxiety disorder in Turkish people and it is more frequent in young adults (51.0%) although this prevalence rate is greater than the EMR [14, 15]. Recollection intervals, healthcare services, reimbursement strategies, the economic status, social and cultural variables might all contribute to the difference among the countries.

Among the participants, 60 (15.07%) individuals reported having a chronic disease, Specifically, 11 participants reported having hypertension, 6 reported having type 2 diabetes, and 4 reported having hypothyroidism. Notably, hypertension was the most frequently reported chronic disease among the participants. It was observed that among the participants, a total of 88 individuals had a history of drug usage. Specifically, 29% of the 88 individuals reported using antidepressant drugs, 19% reported using antihypertensive drugs, 15% reported using antidiabetic drugs, 9% reported using analgesic drugs, 9% reported using thyroid drugs, and 19% reported using other drugs.

The percentage of individuals exhibiting symptoms indicative of generalized anxiety disorder, with a cumulative score higher than the 10 points on the GAD-7scale, accounts for 17% of the total participants. Among this subset, 57% were identified as female, while 43% were identified as male. The findings underscore a higher prevalence of generalized anxiety disorder in the female demographic. Notably, the Turkey Mental Health Profile Survey of 2006 revealed a 12-month prevalence rate of 0.7% for GAD [17]. Correspondingly, an investigation conducted by Carter et al., involving 7,124 individuals revealed a 12-month GAD prevalence rate that was twice as high among women (2%) in comparison to men (1%) [14]. The collective evidence from these investigations, as well as our own study, signifies a heightened anxiety disposition among women.

In their investigation, Wittchen observed a greater prevalence of GAD among individuals who were divorced, widowed, or separated. Similarly, in a study undertaken by Özcan et al. in 2006, a notably elevated occurrence of GAD was identified in married individuals when contrasted with their single counterparts [18]. Within our own study, the potential for GAD diagnosis was evident in 20% of single participants, 12% of married participants, and 20% of those who were divorced or widowed. Notably, the anxiety levels of single and divorced or widowed participants surpassed those of married participants. However, it is important to acknowledge the non-uniform distribution of single, married, divorced, and widowed participants within our sample.

As reported by Wittchen, the prevalence of GAD demonstrates an increase with advancing age, with the highest incidence observed among individuals aged 45 years and older. This study reinforces the notion that GAD tends to emerge in the late twenties and is most frequently encountered during middle age. The mean age associated with GAD was approximately 32 years [19,20]. Another investigation examining age distribution found that mean anxiety scores exhibited a slight increment from the youngest age group to the 55-59 range, followed by a decline until the 65-69 category, and then a subsequent rise [16]. Within the scope of our own study, it was revealed that 22% of participants aged 18-24, 13% of those aged 25-34, 8% of individuals aged 35-44, 10% of those aged 45-54, and 24% of those aged 55-65 exhibited elevated levels of generalized anxiety disorder. Particularly noteworthy were the age cohorts of 18-24 and 55-65, displaying the highest levels of anxiety.

In the research conducted by Wittchen et al., the absence of employment or the role of a homemaker was identified as a risk factor for GAD. Their findings indicated a direct correlation between the prevalence of GAD and a lower income level, although no significant association was observed with educational attainment [19, 20]. Similarly, Özcan et al. observed that two-thirds of GAD-diagnosed individuals in their study were homemakers. Nonetheless, their study indicated that GAD was unrelated to income level, yet more prevalent among individuals with lower educational backgrounds [20]. Another study also provided support for the notion that GAD is more frequently encountered among

individuals with limited educational attainment. Notably, these investigations suggest a higher prevalence of GAD among those with lower educational levels, which is differs from international studies. In our study, it is noteworthy that individuals with secondary school, university, master's, and doctorate degrees exhibited elevated anxiety levels.

Feeling Anxious or Stressed Lately for Any Reason

Participants were asked whether they had been experiencing feelings of anxiety or stress recently, to which 221 (55.5%) participants answered "yes" and 177 (44.5%) participants answered "no". Of those who answered "yes", 56% reported experiencing a stressful situation. A follow-up question was posed to these participants, asking them to elaborate on the cause of their stress. A total of 92 participants provided an explanation, with Coronavirus disease 2019 (COVID-19) being the most frequently cited stressor (22%), followed by exam stress (19%), work stress (18%), family problems (17%), pandemic (13%), earthquake (3%), and other causes (9%). According to our findings a negative correlation has been observed between level of education and feeling stressed lately for any reason. Also the gender difference was statistically significantly correlated with feel of stressed lately due to any reason (r: -0.115, $p<0.05$).

Reasons for Anxiety and Stress

Participants who reported family problems as the cause of their stress mentioned a range of issues, including the separation of their parents, the entry of a new person into their father's life, the father's transient ischemic attacks, the death of a family member, a family traffic accident, divorce, home-related problems, and family incompatibility. Respondents who identified Covid-19 as the cause of their stress cited factors such as Covid-19 infection, being afraid of contracting the virus, quarantine, noncompliance with Covid-19 rules, and general anxiety about Covid-19. Participants who identified work stress as the cause of their stress mentioned problems such as not receiving their salaries on time, having difficulties in their workplace and sharing work, work intensity, new responsibilities, workplace conditions, unemployment, working in a pandemic hospital, and health personnel having stressful experiences. Exam-related stress was reported by participants who expressed concerns about the invigilation process, exam difficulty, time constraints, and fears of failure. Finally, other stressors mentioned included health problems, financial concerns, engagement pressure, restrictions on international travel, traffic congestion, adjusting to a new city or environment, and obsessions. The most common stressors illustrated in figure 1.

The reason for self-medication and not consulting a physician was found mostly because participants had an experience with the drug they used (94%). Meanwhile, the minority (6%) stated that the reason for self-medication was due to their lack of time. Most common reason for analgesic use was headache (46.3%) which is followed by a stomachache (13.8%), toothache (6.4%) and menstrual pain (5.8%). The distribution of causes of stress is illustrated in Figure 1.

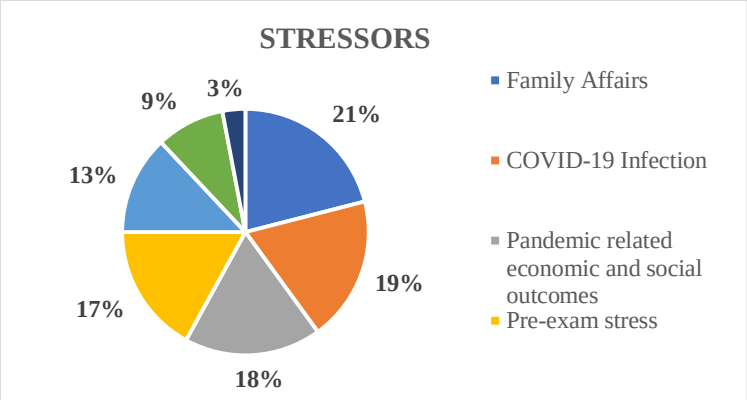

Figure 1. Most common stated stressors (n=398)

Generalized Anxiety Levels of The Participants

A survey on generalized anxiety disorders (GAD-7) was administered to the participants, wherein they were requested to mark the frequency with which they experienced certain conditions. The conditions that were desired to be marked included feelings of anger, anxiety, worry, inability to control or stop concerns, worrying about various topics, inability to relax, restlessness, feeling angry or restless, and fear of something terrible happening. Participants were required to mark their frequency of experiencing these conditions as never, more than half of the days, or every day. These scores were collected for each participant, and their level of anxiety was determined accordingly.

A total of 146 (36.68%) participants scored between 0-4 points, indicating either no anxiety or a low level of anxiety. Another 184 (46.23) participants scored between 5-9 points, indicating a moderate level of anxiety. Out of 398, 47 (11.81%) participants scored between 10-14 points, indicating a high level of anxiety. Lastly, 21 (5.28%) participants scored be-tween 15-21 points, indicating a severe level of anxiety. The majority of the participants have a moderate level of anxiety, and the mean and standard deviation of the GAD-7 score is 6.31±4.21. A statistically significant correlation has been established between the anxiety level and feeling stressed lately due to any reason (r: 0.151, $p<0.005$) and level of education (r: 0.106, $p=0.035$).

The survey was conducted with the aim of investigating the knowledge and attitudes of the participants towards anxiety as a medical condition. A total of 398 respondents participated in the survey, of whom 361 (90.73%) answered “yes” to the question of whether anxiety is a disease, while 39 (9.27%) responded “no”. It is noteworthy that 90% of the participants gave correct answers, indicating a relatively high level of awareness regarding anxiety as a medical condition. However, around 9% of the participants gave incorrect answers, suggesting that there is still room for improvement in terms of public education and awareness-raising campaigns.

Furthermore, the survey results showed that 372 people acknowledged the need for medical treatment under the supervision of a doctor, representing a response rate of 93.46%. In contrast, 28 respondents stated that there is no need for treatment for anxiety, accounting for 6.54% of the participants. It is worth noting that the vast majority of respondents acknowledged the importance of seeking medical treatment for anxiety, as it is a condition that requires professional care and monitoring.

Regarding medication for anxiety treatment, the survey results revealed that 299 participants believe that anxiety drugs should be taken regularly every day, while 101 individuals believed that anxiety drugs should only be taken when they feel bad. However, it should be emphasized that taking anxiety medication regularly is essential for its effectiveness in the treatment of anxiety. Therefore, the proportion of correct answers is 75.12%, while the rate of incorrect answers is 24.88%. Moreover, the survey investigated the possible causes of anxiety and found that 366 (91.95%) respondents attributed anxiety to stress, 193 (48.49%) to genetic/familial factors, 44 (11.05%) to old age, 54 (13.58%) to obesity, and 91 (22.86%) were unsure of the cause. The majority of participants associated anxiety with stress, suggesting that stress management techniques could be useful in the treatment of anxiety.

Additionally, the survey explored the various diseases caused by anxiety and found that 372(93.46%) respondents associated anxiety with sleep problems, 275(69.09%) with heart diseases, 149(37.43%) with diabetes, 144 (36.18%) with thyroid, and 98(24.62%) with paralysis/brain hemorrhage. These results highlight the importance of proper anxiety treatment to prevent the development of other medical conditions. Furthermore, the survey findings revealed that participants believed in the importance of adopting auxiliary approaches to anxiety treatment, such as avoiding stress factors, relieving stress, eating healthy, not smoking, regular exercise, and managing stress. Around half of the respondents (52%) believed that all options could help in the treatment of anxiety.

Finally, the survey explored the sources of information about anxiety available to the participants. The results showed that 92% of the respondents considered medical doctors as a source of information, while 57% identified pharmacists as an important resource. Other sources of information cited by the participants included the internet (23%), the environment (8.75%), newspapers (8.75%), television (8.5%), and magazines/brochures (14.25%). These results highlight the crucial role of healthcare professionals in educating the public about anxiety and its treatment options.

Factors Associated with Anxiety Disorder

In this study, the effects of various factors on anxiety were examined using binomial logistic regression model. Specifically, the study investigated the influence of employment status, gender, marital status, level of education, presence of chronic illnesses and medications, as well as the Feeling anxious or stressed lately for any reason on anxiety levels of individuals. Of the predictor variables examined, only two were found to be statistically significant: Feeling anxious or stressed lately for any reason and level of education. Individuals who felt anxious or stressed lately had 2.358 times higher odds of exhibiting anxiety disorder with GAD-7 scores higher than 10 (OR: 2.358, %95 CI: 1.321-4.210, p<0.005). Additionally, level of education was found to be associated with an increased likelihood of having anxiety disorder. Individuals with a secondary school education were more prone to exhibit anxiety in comparison to those with post-graduate education (OR: 5.618, %95 CI: 1.332-23.698 p<0.05). These findings underline to consider anxious feelings and educational level in the early detection of general anxiety disorder (Table 2).

Table 2. Binary logistic regression analysis of factors related with presence of general anxiety disorder

Variables	**Presence of General Anxiety Disorder (GAD-7 score≥10)**					
	Univariate Analysis			**Multivariate Analysis**		
	OR	**95% CI for Odds Ratio**	***p***	**OR**	**95% CI for Odds Ratio**	***p***
Marital Status						
Single	0.561	0.310 -1.010	0.054	0.688	0.361-1.310	0.256
Married	*Reference*					
Gender						
Female	0.710	0.403-1.250	0.197	0.826	0.450-1.520	0.538
Male	*Reference*					
Employment Status						
Employed	3.06	0.711-13.200	0.115	2.701	0.591-1.350	0.200
Unemployed	*Reference*					
Feeling anxious or stressed lately for any reason						
Yes	2.370	1.340-4.20	0.003	2.358	1.321-4.210	0.004
No	*Reference*					
Comorbidity						
Present	0.626	0.271-1.44	0.268			
Not Present	*Reference*					
Level of Education		-				
Primary School	1.932	0.474-7.870	0.358	1.922	0.483-7.638	0.354
Secondary School	5.599	1.322-23.712	0.019	5.618	1.332-23.698	0.019
High School	2.243	0.972-5.175	0.058	2.224	0.970-5.098	0.059
Graduate	1.615	0.843-3.093	0.148	1.640	0.867-3.105	0.129
Postgraduate	*Reference*					
Age	1.004	0.976-1.032	0.789			

The logistic regression model yielded a statistically significant result, with a $\chi 2(5) = 16.534$ and $p < 0.05$. The model accurately classified 64.8% of the cases. Sensitivity was found to be 39.9%,

specificity was 76.9%, positive predictive value was 61.78% and negative predictive value was 58.4%. The model reliability was assessed using an omnibus ANOVA test for model coefficients ($p<0.05$) and Hosmer and Lemeshow Test ($p=0.644$).

Of the predictor variables examined, only two were found to be statistically significant: Feeling anxious or stressed lately for any reason and level of education. Individuals who felt anxious or stressed lately had 2.358 times higher odds of exhibiting anxiety disorder with GAD-7 scores higher than 10 (OR: 2.358, %95 CI: 1.321-4.210, $p<0.005$). Additionally, level of education was found to be associated with an increased likelihood of having anxiety disorder. Individuals with a secondary school education were more prone to exhibit anxiety in comparison to those with post-graduate education (OR: 5.618, %95 CI: 1.332-23.698 $p<0.05$). These findings underlines to consider anxious feelings and educational level in the early detection of general anxiety disorder.

The primary interventions for individuals suffering from anxiety encompass the administration of antidepressants such as selective serotonin reuptake inhibitors (SSRIs) or serotonin and norepinephrine reuptake inhibitors (SNRIs), as well as cognitive behavioral therapies [20]. These therapeutic approaches have been integrated into collaborative care models, yet many of their components are adaptable to clinical environments. Commencing anxiety treatment with readily accessible, cost-effective, and safe interventions is a logical approach. Among these are physical exercise and mindfulness-based stress reduction techniques, which can be accessed through applications or web-based programs. Moreover, patients should be guided toward authoritative sources of information regarding their condition and its management [20]. Consequently, apart from pharmacological interventions, supplementary treatment modalities play a pivotal role in addressing anxiety. The quality of information available on the internet, along with numerous websites, was not adequate enough. While reliable sources of high-quality information may enhance patients' understanding of their condition, it is plausible that certain online resources, particularly unvetted ones, might exacerbate anxiety.

Pharmacists can enhance patient-centered care to promote positive outcomes in an underserved population by improving communication between healthcare professionals, coordinating the treatment of patients, improving access to and safety of medications, and optimizing medication regimens [8,21-23]. On the other hand, in the study completed by Samorinha et al, pharmacists may encounter barriers in providing mental health services [24]. It was stated that the most important of these barriers are emotional discomfort with the patient's current situation and lack of practice. Samorinha et al. reported that pharmacists who provided a higher number of mental health counselling were younger pharmacists and pharmacists who had participated in continuing professional education in the last two years. Considering the studies in the literature and the results obtained in our study, it is obvious that pharmacists have an important role in anxiety disorders within the health team and in early diagnosis and diagnosis.

Nevertheless, this study is subject to certain limitations despite its inclusion of participants from various regions of the country. One limitation is the relatively small sample size, which should be considered interpreting the findings. Furthermore, the generalizability of the results may be constrained due to the overrepresentation of highly educated, employed women in our sample. This bias may have arisen from the accessibility of online surveys, potentially excluding individuals with lower educational backgrounds who have limited internet usage. Therefore, future investigations should aim to explore the behaviors and perspectives of individuals with recurring feelings of anxiety and those with lower levels of education to obtain a more comprehensive understanding, as our findings may not accurately reflect their experiences. It is also important to note that the study was conducted during the fall-winter seasons and did not account for potential variations in anxiety or stress behaviors across different seasons. Additionally, the data collected relied on self-reported responses, which may be subject to personal biases or reluctance to report inaccurate opinions. However, efforts were made to assure participants of confidentiality, which may have mitigated this potential effect. Furthermore, the study focused on the three months leading up to the interviews, which may have introduced recall bias and influenced participants' perceptions. Moreover, it is worth noting that the study was conducted during a period of stringent COVID-19 containment measures, which could have significantly influenced anxiety levels due to the unique challenges posed by the pandemic compared to normal circumstances. Therefore, caution should be exercised when generalizing the findings of this study to anxiety levels in non-

pandemic periods.

Our research findings highlight the prevalence of the GAD among society and the involvement of pharmacist in conducting GAD screenings, which can effectively identify individuals with anxiety. Thus, could enable timely referrals to appropriate healthcare providers. This proactive approach facilitates early diagnosis and treatment of anxiety disorders, ultimately leading to enhanced management of psychiatric conditions, improved treatment outcomes, and an overall better quality of life for individuals affected by these disorders.

ACKNOWLEDGEMENTS

The authors would like to thank Bezmialem Vakif University for the given opportunity. This research received no external funding.

AUTHOR CONTRIBUTIONS

Concept: M.Y.B., B.T., F.V.I.; Design: M.Y.B., B.T., F.V.I.; Control: D.S., M.A.; Sources: M.Y.B., B.T., F.V.I.; Materials: M.Y.B., B.T., F.V.I.; Data Collection and/or Processing: M.Y.B., B.T.; Analysis and/or Interpretation: M.Y.B., B.T., D.S., M.A., F.V.I.; Literature Review: M.Y.B., B.T., D.S., M.A., F.V.I.; Manuscript Writing: M.Y.B., B.T.; Critical Review: M.Y.B., B.T., D.S., M.A., F.V.I.; 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 study has been approved by the Bezmialem Vakif University, local Ethics Committee with the decision number of 18/345. An electronically signed informed consent was obtained from all individual participants included in the study. All procedures performed in the study were in accordance with the ethical standards of the University of Siena and with the 1964 Helsinki declaration and its later amendments. An electronically signed informed consent was obtained from all individual participants included in the study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 957-966, 2023
Doi: 10.33483/jfpau.1247781

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREDICTIVE BIOMARKERS OF LIVING DONOR LIVER TRANSPLANTATION

CANLI DONÖR KARACİĞER NAKLİNİN ÖNGÖRÜSEL BİYOBELİRTEÇLERİ

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ABSTRACT

Objective: *Liver transplantation using a living donor (LDLT) is currently the most popular method used in the worldwide. Appropriate biomarkers that predict graft status should be used to detect early post-transplant complications that may lead to a rejection reaction.*
Material and Method: *The study involved a total of 44 liver recipients and 44 liver donors, from whom preoperative blood samples were taken and immunoassay and spectrophotometric studies were carried out. The levels of serum neopterin, interferon-gamma (IFN- y), indoleamine-2,3 dioxygenase (IDO), and -glutathione S transferase (a-GST) were assessed using an enzyme-linked immunosorbent test. The activity of glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) in erythrocytes was measured using spectrophotometry.*
Result and Discussion: *"Neopterin, IDO, and G6PD levels were significantly higher in the recipient group than in the donor group. The differences in gender and blood groups were statistically insignificant. The rejection reaction developed in 25% of patients and none survived. These findings may facilitate the identification of novel predictive biomarkers for the diagnosis of*

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Submitted / Gönderilme : 05.02.2023
Accepted / Kabul : 18.08.2023
Published / Yayınlanma : 20.09.2023

acute rejection reactions after LDLT. The clinical use of novel non-invasive biomarkers may provide time and cost advantages.
Keywords: *α-GST liver transplantation, G6PD, IFN-γ, indoleamine-2,3-dioxygenase*

ÖZ

Amaç: *Canlı donör karaciğer nakli (CDKN) şu anda dünya çapında en yaygın karaciğer nakli yaklaşımıdır. Rejeksiyon reaksiyonuna yol açabilecek erken nakil sonrası komplikasyonları tespit etmek için greft durumunu öngören uygun biyobelirteçler kullanılmalıdır.*
Gereç ve Yöntem: *Çalışmaya toplam 44 karaciğer alıcısı ve 44 karaciğer donörü dahil edildi, bunlardan ameliyat öncesi kan örnekleri toplandı, immünoassay ve spektrofotometrik analizler yapıldı. Serum neopterin, interferon gama (IFN-y), indolamin-2,3-dioksijenaz (IDO) ve alfa-Glutatyon S- transferaz (a-GST) seviyeleri, enzime bağlı bir immünosorbent deneyi kullanılarak ölçüldü. Eritrositlerdeki glutatyon redüktaz (GR) ve glukoz-6-fosfat dehidrogenaz (G6PD) aktivitesi spektrofotometri ile ölçüldü.*
Sonuç ve Tartışma: *Neopterin, IDO ve G6PD düzeyleri alıcı grupta donör grubuna göre anlamlı olarak yüksekti. Cinsiyet ve kan gruplarındaki farklılıklar istatistiksel olarak anlamsızdı. Hastaların %25'inde reddetme reaksiyonu gelişti ve hiçbiri hayatta kalmadı. Bu bulgular, CDKN sonrası akut rejeksiyon reaksiyonlarının teşhisi için yeni prediktif biyobelirteçlerin tanımlanmasını kolaylaştırabilir. Yeni invaziv olmayan biyobelirteçlerin klinik kullanımı, zaman ve maliyet avantajları sağlayabilir.*
Anahtar Kelimeler: *α-GST karaciğer nakli, G6PD, IFN-γ, indolamin-2,3-dioksijenaz*

INTRODUCTION

Liver transplantation (LT) is currently the just treatment method recommended for patients with developed liver disease, which is life-threatening and cannot be treated by other methods. Patients may develop post-transplant complications associated with immunosuppressive therapy, such as rejection, infection, hypertension, and malignancy [1]. Liver transplantation involves the replacement of diseased liver tissue with a portion of liver tissue with normal functions harvested from a brain-dead or healthy living person. One of the most common complications after liver transplantation is acute rejection, the most common form of which is cell-mediated rejection, which occurs through the recognition of recipient T lymphocytes and the presentation to donor alloantigens by antigen-presenting cells. Acute rejection must be identified as soon as feasible in order to use an effective anti-rejection treatment and to retain the graft's functionality and integrity [2,3]. Liver transplant process can be assessmented by biochemical tests, such as total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (ALP), leukocytosis and eosinophilia, although usual laboratory tests are non-specific and are unsuitable for the effective and timely diagnosis of acute rejection. Liver biopsy, an invasive procedure, can be performed in the event of suspected acute rejection, although the procedure is associated with severe complications. Non-invasive biomarkers include cytokines in saliva, urine, peripheral blood, or other body fluids, or cell surface proteins of various immune cells. As the assessment of such diagnostic biomarkers is not invasive, they have been investigated by researchers for many years as a possible replacement for liver biopsy [4,5].

Neopterin is a chemical of the pyteridine group that is activated by interferon-gamma (IFN-γ), which is made by monocytes, macrophages, dendritic cells, and endothelial cells and released by functional protected T cells. It's been employed as a marker to evaluate cellular consumption [6,7]. Indoleamine 2,3 dioxygenase (IDO) is a cytosolic, heme-containing enzyme involved in the first step of tryptophan catabolism, and has been associated with multiple pathophysiological conditions, including autoimmune disorders, fetomaternal tolerance, cancer, and infectious diseases. It inhibits the proliferation of both T lymphocytes and pathogens by exhausting tryptophan, an essential amino acid, and is expressed by antigen-presenting cells induced by proinflammatory cytokines such as interferon-γ, and its main function is immunosuppressive activity. Interferon-gamma is a cytokine with antiviral, antitumor and immunomodulatory effects, which is crucial for regulations of both congenital and adaptive immune responses [6-9].

IFN-γ is also the strongest inducer of IDO [8,9]. Due to its lower molecular weight and shorter half-life, -glutathione S transferase (α-GST) is a more sensitive biomarker of liver function than common liver tests AST and ALT. α-GST levels were found to be fairly high in patients with acute liver failure and significantly greater in liver transplant recipients who experienced moderate to severe rejection compared to those who did not [10,11]. Glutathione reductase (GR) the main enzyme of the glutathione metabolism converts oxidized glutathione (GSSG), a product of the reactions catalyzed by glutathione S-transferase and glutathione peroxidase to reduced glutathione (GSH). It has an antioxidant effect and is a highly specific marker of liver injury [12,13]. Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that is critical to glucose metabolism and that protects red blood cells from oxidative stress. Nevertheless, researchers define G6PD as a contraindication for liver donation [14].

This study's objective is to contrast numerous protein biomarkers with normal biochemical tests in order to assess their effectiveness as indicators of acute rejection in living donor liver transplants.

MATERIAL AND METHOD

Participants

This prospective cross-sectional study was conducted by the Turgut Özal Medical Center Liver Transplant Institute between January 2018 and January 2020 (Figure 1). The study groups included 44 liver tissue recipients admitted to the transplant institute and 44 tissue donors who donated tissue for these patients. Patients in the liver tissue recipient group were 30 male and 14 female, whereas those in the liver tissue donor group were 27 male and 17 females. The ethics committee of İnönü University Malatya gave its approval for the study, which was carried out in compliance with the Declaration of Helsinki's tenets (Approval No: 2018/144). All participants gave their official approval for the use of their medical data for research. Peripheral venous blood samples from each participant in the trial were taken and put in typical biochemistry tubes for evaluation. The separated sera were put in tubes and kept at -80°C until analysis after the blood samples were centrifuged at 3,500 rpm for 15 minutes at room temperature. The evaluated parameters included neopterin, IFN-γ, IDO, G6PD, GR, a model for end-stage liver disease (MELD) score, total bilirubin, direct bilirubin, hemoglobin, white blood cells (WBCs), platelets, C-reactive protein (CRP), international normalized ratio (INR), creatinine, albumin, sodium, recipient's age, gender and blood group, rejection rate, operation outcome, donor's age, gender and blood group, diagnosis, and serum levels of ALT, AST, gamma-glutamyl transferase (GGT), ALP and α-GST.

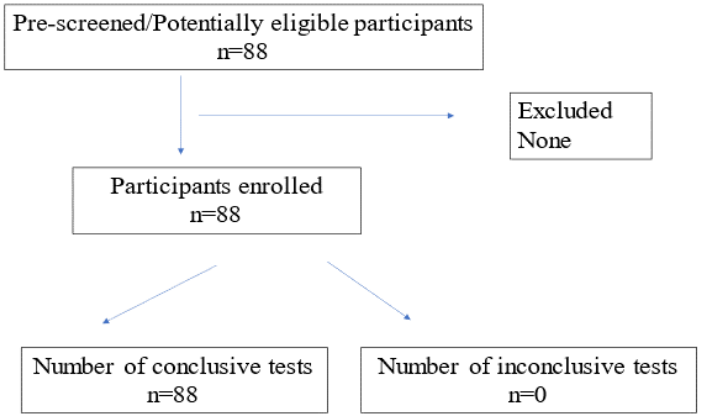

Figure 1. Participant flow diagram

Measurement of Glucose 6-phosphate Dehydrogenase Activity in Erythrocytes

The Beutler method, which relies on the reduction of $NADP^+$ by G6PD in the presence of glucose 6-phosphate, was used to quantify the activity of G6PD in a spectrophotometer at 37°C. The rate of

NADPH synthesis, which is dependent on G6PD activity, was calculated using the rise in absorbance at 340 nm [15].

Measurement of Glutathione Reductase Activity in Erythrocytes

Glutathione reductase activity was measured based on the principle of maximum absorbance of reacting NADPH at 340 nm. The reaction catalyzed by GR results in a decrease in NADPH, and this decrease was monitored spectrophotometrically at 340 nm to determine the enzyme activity [16].

Measurement of Serum Neopterin, IFN-γ, IDO, and α-GST Levels

The serum concentrations of neopterin, IFN-γ, IDO, and α-GST were measured using commercial enzyme-linked immunosorbent assay kits (E3155Hu, ELH-IFNg-1, E0796Hu, and EK1625; Bioassay Technology Laboratory, Ltd.). Serum neopterin, IFN-γ, IDO, and α-GST test measurement ranges were 0.1-38.0 nmol/l, 15-15000 pg/ml, 0.3-90 ng/ml, and 156-10000 pg/ml, respectively. The intra- and inter-assay accuracy coefficients of variation for all ELISA kits were less than 10% and less than 8%, respectively.

Clinical Significance

In the current study, we investigate the non-invasive diagnostic biomarkers for allograft rejection in liver transplant recipients and evaluate the accuracy of these biomarkers in predicting acute rejection by contrasting them with standard biochemical assays used in clinical practice. This is the first study that, to the best of our knowledge, compares the effectiveness of novel protein biomarkers with the outcomes of standard tests. The study's prospective cohort design is another asset. The recipients' serum concentrations of neopterin, IDO, and G6PD were found to be significantly elevated. The creation of novel LDLT diagnostic biomarkers might be made simpler as a result of our findings.

Statistical Analysis

According to power calculations, each group would require at least 21 participants if the biggest neopterin difference between them was 1.4 nmol/l (standard deviation, 0.7 nmol/l), the type I error was 0.05, and the type II error was 0.20. The median (interval) or mean SD is used to present data. The Shapiro-Wilk test was utilized to assess the data's normality. The analysis made use of the independent two-sample t test, Mann-Whitney U test, Pearson 2 test, Yates corrected 2 test, and Fisher exact 2 test where needed. In order to assess the diagnostic performance and the best cutoff values for the variables of interest, a receiver operating characteristic (ROC) curve analysis was carried out. The Biostatistics Department of Inonu University created the web-based DTROC program. The web-based DTROC application, developed by the Biostatistics and Medical Informatics Department of İnonu University Faculty of Medicine, was utilized for the ROC analysis. The correlations between the variables were assessed using the Spearman rank correlation coefficient of 11. P values under 0.05 were regarded as significant. The analyses were done using SPSS version 25 (IBM Corp., Armonk, NY, USA). To calculate odds ratios (ORs), multivariate logistic regression analysis was used.

RESULT AND DISCUSSION

The 44 individuals in the control group had an average age of 32.82 + 8.12 years. Males made up the majority of the contributors. An assessment of blood-type distribution revealed that O(+) was the most common, followed by A(+) in the control group (Table 1). The patient group's 44 participants had a mean age that was higher than that of the control group (46.55 18.82 years). Most tissue recipients were male, similar to the donor group. An assessment of blood-type distribution revealed A(+) to be the most common, followed by O(+) in the patient group (Table 2).

The difference between the two groups median ages was statistically significant ($p<0.001$). While CRP levels were greater in the donor group ($p=0.005$), levels of neopterin, direct bilirubin, AST, ALT, GGT, and total bilirubin were also considerably higher in the patient group ($p<0.001$ for all). Additionally, the donor groups showed significantly higher IDO and G6PD activity ($p=0.028$ for both). Both study groups' GR activity was comparable ($p=0.006$). The patient group's hemoglobin, platelet,

and albumin levels were all lower than those of the control group ($p<0.001$, for all three). Differences in blood type and gender had no impact on the variables under investigation (Table 3). The predictive significance of biomarkers for liver transplants is demonstrated by ROC analysis (Table 4).

Table 1. Demographic and clinical characteristics of the control group

Characteristics		**Liver tissue donors (control group, n=44)**
Age, years		32 (21-50)
Gender	**Female**	17 (38.6)
	Male	27 (61.4)
Blood group	**O (+)**	19 (43.2)
	A (+)	16 (36.4)
	B (+)	8 (18.2)
	AB (+)	1 (2.3)

Values are given as median (minimum-maximum), and number (percentage).

Table 2. Demographic and clinical characteristics of the study group

Characteristics		**Liver tissue recipient (study group, n=44)**
Age, years		50.0 (3.0-72.0)
Gender	**Female**	14 (31.8)
	Male	30 (68.2)
Blood group	**O (+)**	12 (27.3)
	A (+)	22 (50.0)
	B (+)	8 (18.2)
	AB (+)	2 (4.5)
MELD score		23.02±5.08
Diagnosis	**Hepatitis**	14 (31.8)
	Cirrhosis	14 (31.8)
	Liver failure	16 (36.4)
Rejection	**Positive**	11 (25)
	Negative	33 (75)
Operation result	**Live**	33 (75)
	Death	11 (25)

Values are given as median (minimum-maximum), mean ± SD, and number (percentage)

Table 3. Comparison of patient characteristics, and protein biomarker levels in the control and study groups (n=88)

	Group		**P-value[a]**
	Liver tissue donors (control group, n=44)	**Liver tissue recipients (study group, n=44)**	
	Median (Min-Max)	**Median (Min-Max)**	
Age (year)	32 (21-50)	50 (3-72)	**<0.001**
Total Bilirubin (mg/dl)	1.45 (0.42-3.6)	5.45 (1.57-18.07)	**<0.001**
Direct Bilirubin (mg/dl)	0.59 (0.15-1.83)	2.6 (0.77-11.32)	**<0.001**
INR	1.05 (0.85-2.08)	1.95 (1.05-9.86)	**<0.001**
Creatinine (mg/dl)	0.8 (0.5-1.23)	0.83 (0.39-2.05)	0.646
AST (U/L)	170 (14-392)	461.5 (41-3133)	**<0.001**
ALT (U/L)	188 (15-522)	370 (28-2434)	**<0.001**
GGT (U/L)	20 (6-76)	54.5 (15-837)	**<0.001**

Table 3 (*continue*). Comparison of patient characteristics, and protein biomarker levels in the control and study groups (n=88)

		Group		P-value[a]
		Liver tissue donors (control group, n=44)	Liver tissue recipients (study group, n=44)	
		Median (Min-Max)	Median (Min-Max)	
ALP (U/L)		61.5 (30-200)	70 (25-655)	0.317
Hemoglobin (g/dl)		13.9 (10-18.6)	10.55 (5.2-18.1)	**<0.001**
WBC (/L)		19.3 (6.97-30.2)	16 (3.13-65.9)	0.185
Platelets (/L)		244.5 (14-425)	113 (25-1294)	**<0.001**
CRP (mg/l)		0.32 (0.3-6.35)	0.53 (0.3-6.82)	**0.005**
α-GST (µg/l)		0.22 (0.13-2.99)	0.19 (0.05-2.89)	0.324
Neopterin (nmol/l)		0.36 (0.27-2.91)	3.14 (0.31-8654)	**<0.001**
IFN-γ (pg/ml)		0.12 (0.02-2.81)	0.16 (0.02-2.78)	0.126
IDO (ng/ml)		0.4 (0.17-1.76)	0.47 (0.05-1231)	**0.028**
G6PD (units/g/Hgb)		0.02 (0.02-0.03)	0.03 (0.02-0.03)	**0.028**
GR (EU/ml)		0.05 (0.04-0.06)	0.05 (0.04-0.05)	**0.006**
		Mean ± SD	**Mean ± SD**	
Sodium (mEq/l)		136.77±1.93	135.80±3.27	0.058
Albumin (g/dl)		3.32±0.42	2.41±0.65	**<0.001**
		Number (Percent)	**Number (Percent)**	
Gender	**Male**	17 (38.6)	14 (31.8)	0.656
	Female	27 (61.4)	30 (68.2)	
Blood group	**O (+)**	19 (43.2)	12 (27.3)	0.414
	A (+)	16 (36.4)	22 (50.0)	
	B (+)	8 (18.2)	8 (18.2)	
	AB (+)	1 (2.3)	2 (4.5)	

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; G6PD, glucose-6-phosphate dehydrogenase; GGT, gamma-glutamyl transferase; GR, glutathione reductase; GST, glutathione-S-transferase; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-gamma; INR, international normalized ratio; WBC, white blood cells. [a]Values are given as median (minimum-maximum), mean ± SD, or number (percentage). a: Bold values show P <0.05

Table 4. ROC analysis shows the predictive value of biomarkers for liver transplants

Variables	Cutoff	Sensitivity	Specificity	LR+	LR−	PPV	NPV	AUC (95% CI)	*P* value[a]
Age (year)	39.5	72.7 (0.56-0.84)	79.5 (0.30-0.93)	3.56	0.34	78.0	74.5	0.79 (0.68-0.89)	<0.001
Total bilirubin (mg/dl)	2.77	0.95 (0.84-1.00)	0.89 (0.51-0.98)	8.40	0.051	89.4	95.1	0.98 (0.95-1.00)	<0.001
Direct bilirubin (mg/dl)	1.19	0.93 (0.73-1.00)	0.98 (0.85-1.00)	41.00	0.070	97.6	93.5	0.98 (0.97-1.00)	<0.001
INR	1.28	0.91 (0.78-0.98)	0.91 (0.78-0.98)	10.00	0.10	90.9	90.9	0.96 (0.90-0.99)	<0.001
Sodium (mEq/l)	134.5	0.37 (0.23-0.52)	0.89 (0.75-0.96)	3.20	0.72	76.2	58.2	0.62 (0.51-0.72)	0.058
Creatinine (mg/dl)	0.95	0.30 (0.17-0.45)	0.84 (0.70-0.93)	1.86	0.84	65.0	54.4	0.53 (0.41-0.65)	0.652
AST (U/L)	289.5	0.80 (0.65-0.90)	0.93 (0.81-0.99)	11.33	0.24	91.9	80.4	0.88 (0.79-0.94)	<0.001
ALT (U/L)	250.0	0.82 (0.67-0.92)	0.77 (0.62-0.88)	3.60	0.24	78.3	81.0	0.84 (0.74-0.91)	<0.001
GGT (U/L)	41.5	0.64 (0.48-0.78)	0.89 (0.75-0.96)	5.60	0.41	84.8	70.9	0.81 (0.71-0.89)	<0.001

Table 4 (*continue*). ROC analysis shows the predictive value of biomarkers for liver transplants

Variables	Cutoff	Sensitivity	Specificity	LR+	LR−	PPV	NPV	AUC (95% CI)	*P* value[a]
ALP (U/L)	77.5	0.48 (0.33-0.63)	0.80 (0.65-0.90)	2.33	0.66	0.0	60.3	0.56 (0.45-0.69)	0.336
Albumin (g/dl)	2.75	0.68 (0.52-0.81)	0.95 (0.85-0.99)	15.00	0.33	93.8	75.0	0.88 (0.79-0.94)	<0.001
Hemoglobin (g/dl)	11.85	0.66 (0.50-0.80)	0.93 (0.81-0.99)	9.67	0.37	90.6	73.2	0.81 (0.71-0.88)	<0.001
WBC (/L)	14.75	0.48 (0.33-0.63)	0.82 (0.67-0.92)	2.63	0.64	72.4	61.0	0.58 (0.47-0.69)	0.197
Platelets (/L)	185.0	0.84 (0.70-0.93)	0.91 (0.78-0.97)	9.25	0.18	90.2	85.1	0.85 (0.76-0.92)	<0.001
CRP (mg/l)	0.372	0.61 (0.45-0.76)	0.68 (0.52-0.81)	1.93	0.57	65.9	63.8	0.67 (0.56-0.77)	0.003

AUC, area under the curve; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; INR, international normalized ratio; LR−, negative likelihood ratio; LR+, positive likelihood result; NPV, negative predictive value; PPV, positive predictive value; WBC, white blood cells. a: Bold values show P < 0.05

Following LT, the recipient's liver receives the donor's intrinsic bilirubin metabolism, which includes heme oxygenation and bilirubin glucuronidation [17]. In the present study, both the total bilirubin and direct bilirubin levels of the donors were below the cut-off points of 2.77 mg/dl and 1.19 mg/dL, respectively. Liver biochemical tests such as GGT, ALT, AST, ALP, and INR are major and sensitive predictors of acute rejection episodes. A previous study identified liver activity tests such as AST/ALT, GGT, ALT, and AST as sensitive predictors of acute rejection in a group of patients receiving liver transplants from living donors, although these enzymes were not associated with the severity of acute rejection [18]. Our study found INR, AST, ALT, GGT, and ALP levels in the donors to be below the cut-off points. A prevalent ailment among individuals who suffer from liver disease is anemia. Despite the high prevalence of anemia among kidney, heart, and lung transplant recipients, little is known regarding the occurrence, progression of anemia after LT, and available treatments. Depending on the criteria used to define anemia, the reported incidence of anemia following LT ranges from 4.3% to 28.2% [19]. In the present study, the hemoglobin levels of the donors were above the cut-off point, whereas the hemoglobin levels of the recipients were both below the cut-off point and below the levels of the donors. Generally, the cause of anemia is not identified and a multifactorial mechanism has been suggested. The most common cause of unexplained anemia may be immune-suppressive medication-induced bone marrow suppression. Experimental and clinical studies have identified a dual role of platelets in liver transplant patients, causing both beneficial and harmful effects. Recent studies have shown that, despite the fact that a low platelet count is usually thought to be a risk factor for perioperative bleeding, platelet aggregation in patients with cirrhosis may not be as poor as previously thought [20]. In the current study, thrombocytopenia was found in a few of the recipients, and we discovered that the recipient group's platelet count was 53.8% lower than the donor group's, which may be due to hemodilution, immune responses, or platelet sequestration in the liver graft after reperfusion. Inflammatory markers are crucial for predicting the prognosis of several disorders, such as cirrhosis and hepatocellular cancer, as well as the mortality rate following liver transplantation and the effectiveness of the procedure. A important indicator of post-transplant mortality is CRP, which is assessed prior to liver transplantation [21]. In the current investigation, we founded that the receivers' CRP level was 65.6% greater than the donors.

Two crucial plasma proteins, albumin and fibrinogen levels, are measured for the monitoring of liver function following liver transplantation. Albumin levels were 12.6% below the cut-off limit in the current investigation [22].

After a significant correlation between neopterin levels and organ rejection following organ transplants, such as kidney, liver, heart, and lung, neopterin was regarded as an important marker in organ transplant patients [23]. A study involving kidney transplant patients found neopterin levels to be elevated in patients with irreversible organ rejection and reached such high levels as 500–1000 nmol/l

in six patients [24]. Another study investigated serum neopterin levels in heart transplant patients, and found low neopterin levels in patients with stable organ functions, while those with organ rejection had significantly increased levels of neopterin [25]. Neopterin serum levels were found to be considerably higher 10 days after transplantation in patients with bacteremia in a study looking at the relationship between postoperative serum neopterin levels and post-transplant septicaemia and death in liver tissue recipients [26].

In a study, it was discovered that blood IFN- γ levels dramatically increased 3 days after transplantation, notably in allograft recipients, and that graft life was also markedly prolonged. It is therefore believed that an important link exists between the IFN-γ producing natural killer (NK) cells and the innate and adaptive immune response immediately after transplantation. IFN- γ is produced immediately after transplantation and its serum levels peak 3 days after transplantation. In the same study, host-produced NK cells, and to a much lesser extent, donor NK cells, have been shown to be the source of most of the IFN- γ produced in the early post-transplantation period. It has further been shown that serum IFN- γ levels are significantly reduced and graft survival is significantly prolonged in the absence of NK cells [27]. Karahanova et al. reported that IFN-γ levels could be used as a significant parameter in the earliest preoperative period in patients undergoing liver transplantation [28]. The control of the immunological response depends on IDO [29]. Recent studies suggest that IDO may have a substantial immunomodulatory role in a range of events, including allergies, tumor immunology, autoimmunity, HIV infection, and transplant immunity [30]. Serum IDO activity was evaluated 30 days after heart transplantation as a part of a study looking into the impact of IDO on rejection. It was discovered that individuals with acute rejection had considerably higher IDO activity than patients without acute rejection [31]. One group of researchers found that kidney transplant recipients had significantly greater IDO levels than the control group [32]. Following renal transplantation, Kaden et al. investigated at kynurenine levels in relation to IDO activity and discovered elevated kynurenine levels in patients who were experiencing acute rejection [33]. The authors thus concluded these levels could serve as a reliable diagnostic tool in the early period. In the present study, a comparison of the IDO levels of the liver transplant recipients and the control group revealed higher IDO levels in the recipients than in the donors.

As GSTs are cytosolic enzymes found inside of cells, serum GST levels are a stronger indicator of cell damage than protein expression. Due to the short half-life of GST (90 min), variations in GST levels are strongly correlated with ongoing liver cell death. Particularly in cases of acute liver failure, α-GST is highly elevated. Furthermore, compared to patients who experience mild or no rejection following liver transplantation, patients who experience moderate to severe post-LT rejection also have considerably higher α-GST levels. As an additional finding, α-GST is a strong correlation with ALT,

AST, and bilirubin. Despite reports that α -GST is a hallmark of acute cellular rejection and a sensitive indicator of liver damage, it has been proposed that α -GST is useless and unspecific as a marker for the diagnosis and treatment of tissue rejection [34]. When our study results were evaluated, neopterin and IDO levels increased in liver tissue recipients due to the activation of the cellular immune system. This immune activation underlying rejection reactions in tissue recipients is expected. Due to the stimulation of the manufacture of this enzyme, which shields red blood cells from oxidative stress, G6PD levels are higher in tissue recipients compared to donors. As a result, while the cellular immune system is activated as a result of liver transplantation, an increase in the level of protective enzymes has been observed with a feedback mechanism against liver damage.

ACKNOWLEDGEMENTS

The Department of Scientific Research Projects at İnönü University provided funding for the present study (Project number: TDK-2019-1641).

AUTHOR CONTRIBUTIONS

Concept: T.T.Ş., H.Y., S.Y., T.Ç., S.Ü.; Design: Ö.F.Ç., S.Ü.; Control: T.T.Ş., Ş.Y., S.Ü.; Sources: T.T.Ş., S.Y., S.Ü.; Materials: T.T.Ş., H.Y., S.Y., N.B.T., S.Ü.; Data Collection and/or

Processing: Ö.F.Ç., Ş.Y., S.Ü.; Analysis and/or Interpretation: H.Y., N.B.T., Ş.Y., S.Ü.; Literature Review: S.Y., T.Ç., S.Ü.; Manuscript Writing: Ö.F.Ç., S.Ü.; Critical Review: S.Y., T.Ç., S.Ü.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

This study was approved by the İnönü University Malatya Clinical Research Ethics Committee (Approval No: 2018/144).

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 47(3): 967-977, 2023
Doi: 10.33483/jfpau.1274764

ÖZGÜN MAKALE / ORIGINAL ARTICLE

KİMYASALLARIN OLUŞTURDUĞU ALERJİK TEMAS DERMATİT POTANSİYELİNİN DOĞRUDAN PEPTİT REAKTİVİTE YÖNTEMİ İLE DEĞERLENDİRİLMESİ

ASSESSMENT OF THE POTENTIAL OF ALLERGIC CONTACT DERMATITIS BY CHEMICALS BY DIRECT PEPTIDE REACTIVITY METHOD

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ÖZ

Amaç: *Kozmetik ürün, farklı kimyasal bileşiklerin bir kombinasyonu olup genellikle cilt koşullarını veya insan vücudunun kokusunu temizlemek ve iyileştirmek için kullanılır, güzelliği arttırır. AB'de, bitmiş kozmetik ürünler üzerinde hayvan testlerinin kullanılmasına ilişkin bir test yasağı 2004'ten beri yürürlükteyken, 2013'te deri hassasiyet testi de dahil olmak üzere her türlü hayvan toksisitesi testi için bir pazarlama yasağı yürürlüğe girmiştir.*

Gereç ve Yöntem: *Deri hassasiyet testi için ECVAM tarafından üç farklı in-vitro ve bir kimyasal yöntem geliştirilmiş ve onaylanmıştır. Doğrudan Peptit Reaktivite Deneyi (DPRA) bir in-chemico yöntemidir. Testin amacı, kimyasalların deri hassasiyet potansiyelinin değerlendirilmesine katkıda bulunmaktır. Düşük moleküler ağırlıklı maddelerin (haptenler) cildteki proteinlere kovalent bağlanması olan haptenizasyon, alerjide önemli bir mekanizma olarak kabul edilmektedir. Bu nedenle, DPRA gibi peptit reaktivite deneylerinden elde edilen bilgiler, kimyasalların deri hassasiyet potansiyelinin değerlendirilmesi hakkında bilgi sağlar. Çalışmamızda hassasiyet potansiyeli bilinen kimyasalları uygulanarak DPRA'yı kılavuza göre kurmak amaçlanmıştır.*

Sonuç ve Tartışma: *Kozmetik bileşenlerin deri hassasiyet sınıflandırmasına yönelik DPRA çalışmamızın sonuçları, bu kimyasallar ile yapılan önceki in-vivo ve ex-vivo çalışmaların sonuçlarıyla uyumlu bulunmuştur. Çalışmaya eklenen ve kuvvetli irritasyon özelliğine sahip sodyum dodesil sülfat (SDS) uygulaması, DPRA yönteminin irritasyon ve alerji ayrımını yapabileceğini göstermesi açısından önem taşımaktadır. Daha güvenilir bir deri hassasiyet değerlendirmesi sağlamak için iki veya daha fazla yaklaşımdan elde edilen en iyi sonuçların nasıl birleştirileceği*

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Gönderilme / Submitted : 03.04.2023
Kabul / Accepted : 21.08.2023
Yayınlanma / Published : 20.09.2023

değerlendirilmelidir. Alternatif yöntemlerden elde edilen veriler, tek başına bir yöntem olarak kullanılmamalı, kanıt yaklaşımının ağırlıklı bir parçası olarak diğer bilgilerle birlikte değerlendirilmelidir. Bu nedenle, duyarlaştırıcı ve duyarlaştırıcı olmayan maddeler arasında ayrım yapmak mümkündür.
Anahtar Kelimeler: *Alerjik temas dermatit potansiyeli, DPRA, hapten, in-chemico*

ABSTRACT

Objective: *Cosmetics are composed of a variety of chemical compounds and are typically applied to the skin for purpose of cleaning, improvement of skin conditions and also body odor, as well as enhancing beauty. Since 2004, there has been an animal testing restriction on completed cosmetic goods in the EU, and in 2013, a marketing ban for all animal toxicity experiments, including skin sensitization tests, went into effect. ECVAM has created and verified three different in vitro and one in-chemico method for skin sensitization tests.*
Material and Method: *The In-chemico method known as Direct Peptide Reactivity Assay (DPRA) is used. The test's objective is to aid in the evaluation of chemicals' potential to cause skin sensitivity. An essential process in allergies is thought to be haptenization, in which a low molecular weight substance called haptens creates a covalent bond with proteins in the skin. Therefore, the evaluation of the skin sensitization potential of chemicals is based on data from peptide reactivity tests, such as DPRA. Our study's goal was to build up the DPRA in accordance with the guidelines by using compounds known for their sensitization potency.*
Result and Discussion: *Our DPRA study's susceptibility classification of cosmetic compounds produced results that agreed with those of earlier in-vivo and ex-vivo tests on these substances. In order to demonstrate that, the DPRA approach can distinguish between irritation and allergy, sodium dodecyl sulfate (SDS) was used in this study, which has a strong irritation feature. A more accurate estimate of the sensitization potential can be achieved by integrating the best results from two or more methods. The weight-of-evidence technique should be used in conjunction with information from various sources rather than using it as a stand-alone strategy. As a result, a differentiation between sensitizing and non-sensitizing chemicals can be understood.*
Keywords: *Allergic contact dermatitis potency, DPRA, hapten, in-chemico*

GİRİŞ

Kozmetik ürün güvenliği için kullanılan toksisite testlerinde deney hayvanı kullanımı Mart 2013 yılında tamamen yasaklanmıştır [1,2]. İnsan sağlığının ve çevrenin korunmasını amaçlayan Avrupa yönetmeliklerinde belirtildiği üzere, kimyasalların deri hassasiyeti potansiyellerinin ölçümü için hayvan kullanımını tamamen ortadan kaldırmak için hayvan deneylerine alternatif yöntemlerin geliştirilmesine ihtiyaç duyulmaktadır. Deri hassasiyeti, yasal düzenlemelerde, insanlarda alerjik temas dermatiti (ACD)'ni ifade etmek için kullanılan bir terimdir; deri hassasiyeti potansiyeli, kimyasalların güvenlik ve risk değerlendirmelerinde dikkate alınan önemli bir sağlık son noktasıdır.

Deri hassasiyeti değerlendirilmesi için mekanik temelli ve hayvanlarda uygulanmayan test yöntemlerinin özellikle kozmetikler için geliştirilmesi büyük önem taşımaktadır. Deri hassasiyeti için mevcut olan bazı testler, hayvanların kullanımına dayandığı için günümüzde kozmetik güvenliği açısından önemini yitirmiştir. Bunlar arasında geleneksel kobay testleri (Buehler Testi ve Kobay Maksimizasyon Testi [3] ve Lokal Lenf Düğümü Testi (LLNA) [4,5], radyo-izotopik olmayan varyantlar bulunmaktadır [6]. LLNA, geleneksel kobay testlerine göre bir iyileştirme yöntemi olarak kabul edilmesine ve insan maruziyetinde güvenli seviyeleri belirlemek için tam risk değerlendirmesinde gerekli olan deri hassasiyetinin anlaşılmasında büyük bir öneme sahip olmasına rağmen, deney hayvanlarının kozmetik ürün testlerinde kullanımının yasaklanmasından sonra yerine geçebilecek alternatif (*in vitro* ve *in chemico*) yöntemler araştırılmaktadır. REACH'e göre bu son nokta için hayvansal olmayan, alternatif yöntemlerin geliştirlmesi ve uygulanmasına daha fazla ihtiyaç vardır [7,8].

Deri hassasiyeti için farklı alternatif yöntemler geliştirilmiştir. Bu yöntemlerden biri olan Doğrudan Peptit Reaktivite Yönteminin (Direct Peptide Reactivity Asssay-DPRA) aynı zamanda *in chemico* bir yöntemdir. Alerjik temas dermatiti mekanizmasında rol oynayan haptenizasyon

olayı temel alınarak geliştirilmiştir. DPRA, potansiyel sensitizanların, lizin veya sistein (Ac-RFAACAA-COOH ve AcRFAAKAA-COOH) içeren sentetik peptitlerle reaktivitesini ölçerek, deri sensitizasyonu advers etki yolağını gösterir. UV dedektörlü yüksek basınçlı sıvı kromotografisinde (UV-YPSK) elde edilen piklerin değerlendirildiği ECVAM (European Center for Validation of Alternative Methods) tarafından da 2013 yılında valisayonu tamamlanan bir yöntemdir [9]. Validasyon, yöntemin tekrarlanabilirliği (laboratuvarlar içinde ve laboratuvarlar arasında) hakkında sonuçların tutarlılığına göre yapılmaktadır [10], 2015 yılında OECD test kılavuzuna (TG) girmiştir ve özellikle kozmetik firmaları tarafından yaygın olarak kullanılmaktadır [11,12].

Alerjik temas dermatit (alerjik kontakt dermatit), çok önemli sosyoekonomik etkileri olan ve sık görülen deri hastalıklarından biri olan Tip IV (Gecikmiş tip hipersensitivite)'ün bir alt tipidir. [13]. Alerjik temas dermatiti, kimyasal maddelere maruziyette en sık görülen advers etki olması açısından büyük önem taşımaktadır [14]. Bir kimyasal maddenin piyasaya sürülmesinden önce mutlaka biyouyumluluk ve toksisite testlerinden biri olan alerjik kontakt dermatit (deri sensitizasyon) testinin yapılması zorunludur [15]. Kozmetik ürün içeriğinde birçok farklı kimyasal madde bulunmaktadır ve listelenen 30.000 kimyasal madde vardır [16]. Bu 30.000 kimyasalın ortalama %86'sının toksisitesinin, insan ve çevre üzerinde etkilerine ilişkin veriler halen yetersizdir. Avrupa Birliği'ndeki en son kimyasal güvenlik uygulaması olan REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals-Kimyasalların Kaydı, Değerlendirilmesi, Yetkilendirilmesi ve Sınırlandırılması) sisteminde değişiklikler mevcuttur. Bu değişim ile birlikte, üreticinin ürün başvuru dosyalarında toksisite verilerini sunması zorunlu olup nihai ürünün insan sağlığı üzerindeki güvenliği üreticinin sorumluluğuna verilmektedir [17,18].

Alerjik temas dermatiti, düşük moleküler ağırlıklı reaktif kimyasalların neden olduğu gecikmiş tipte bir aşırı duyarlılık reaksiyonudur [19]. Alerjik temas dermatit (ACD) deri hassasiyetine neden olma yeteneğine sahip maddelerle ilişkili olarak immün sistem aracılığıyla ortaya çıkan bir toksisite reaksiyonudur [14]. Deri hassasiyet potansiyelinin değerlendirilmesi, yeni ve mevcut maddelerin güvenlik değerlendirmesinin önemli bir bileşenini temsil etmektedir.

Alerjik temas dermatite yol açan cilt hassaslaşması insidansı, sanayileşme ile yoğun olarak kullanılan potansiyel hassaslaştırıcılar nedeniyle artmaktadır. Bu kimyasalları tespit etmek için gerekli testler insan testleri, hayvan testleri ve *in vitro* testler olarak ayrılabilir. Çalışmamız için yama testi sonuçlarına göre insan testlerinde en sık kullanılan testlerden biri olan parafenilendiamin (PPD), Peru balzamı ve koku karışımı seçilmiştir [20]. Bu makalenin amacı, yaygın olarak kullanılan bu maddelerin alerjik temas dermatit potansiyelini saptamak ve değerlendirmek için alternatif bir yöntem olan DPRA'yı kullanmaktır.

GEREÇ VE YÖNTEM

DPRA

OECD Test Klavuzu'nda No 442C: "Deri Hassasiyetinde Doğrudan Peptid Reaktivite Testi (DPRA)" olarak belirtilmektedir. TG 442C, göre deride hassasiyet oluşturanlar ve hassasiyet oluşturmayanlar arasındaki farkı desteklemek için önerilen bir *in chemico* prosedür olarak kabul edilmektedir.

Araştırmamızda kullanılan yöntem detayları, Doğrudan Peptid Reaktivite Testinde EURL - ECVAM doğrulama çalışmasında kullanılan Standart Çalışma Prosedürüne (SOP) dayanmaktadır [11,21].

Kullanılan Peptidler ve Çözeltiler

Sistein veya lizin içeren sentetik heptapeptitler, (sistein peptit - Ac-RFAACAA COOH; lizin peptidi - Ac-RFAAKAA-COOH) kullanılmıştır (JPT Peptide Technologies GmbH, Berlin, Almanya). Sistein ve lizin çözeltileri sırasıyla 0,501 mg/ml ve 0,518 mg/ml konsantrasyonlarda hazırlanmıştır.

Sistein peptiti için; 100 mM sodyum fosfat tampon (pH: 7.5), lizin peptiti için; 100 mM amonyum asetat tampon (pH: 10.5) hazırlanmıştır.

Standart Peptit Çözeltilerinin Hazırlanması

8 ml tampon çözetisini (Sistein peptidi için pH: 7.5 fosfat tamponu, lizin peptidi için pH: 10.2 amonyum asetat tamponu) 2 ml asetonitril ile karıştırarak yaklaşık 10 ml seyreltme tamponu (dilüsyon tamponu) hazırlanmıştır. 1600 µl peptit stok solüsyonunu (0.667 mM'de) 400 µl asetonitril ile seyrelterek 0.534 mM'de başlangıç standardı "STD-1" hazırlanmıştır. Seri seyreltme yaparak 0.534 mM - 0.0167 mM aralığını kapsayan peptit stok çözeltisinin standartları hazırlanmış ve Tablo 1'de gösterilmiştir.

Tablo 1. Standart peptit çözeltileri

	STD-1	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7 (dilüsyon tampon çözeltisi)**
mM peptit (Sistein, Lizin)	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

Test Kimyasallarının ve Pozitif Kontrol Çözeltisinin Hazırlanması

Çalışmamızda test kimyasalı olarak PPD (parafenilendiamin), geraniol, izoöjenol ve SDS (sodyum dodesil sülfat) kullanılmıştır. Pozitif kontrol olarak sinnamik aldehit kullanılmıştır. Test kimyasalları ve pozitif kontrol çözeltileri her iki peptit için de (lizin ve sistein) protokol doğrultusunda tartılarak asetonitril'de çözülmüştür. SDS (sodyum dodesil sülfat)'nin asetonitril içerisindeki çözünürlüğü az olduğu için distile su içerisinde çözülerek test kimyasal çözeltisi hazırlanmıştır.

Numunelerin Hazırlanması ve Ko-elüsyon (Co-elution) Kontrolü

Test kimyasallarının ve pozitif kontrolün her iki peptit ile üçlü numuneleri hazırlanmıştır. Aşağıda her bir numunenin 220 nm'de absorbsiyonunu ve bir peptit ile benzer bir retansiyon süresine sahip olup olmadığını ve veri analizine müdahale edip edemeyeceğini doğrulamak için peptit olmadan bir numune hazırlanmıştır. Bu durum ko-elüsyon (co-elution) kontrol olarak adlandırılmaktadır. Test kimyasallarının inkübasyonunda kullanılan peptitlerin hazırlanışı Tablo 2'de gösterilmiştir.

Tablo 2. Test kimyasallarının inkübasyonunda kullanılan peptitlerin hazırlanışı

1:10 oranı Sistein Peptit	**1:50 oranı Lizin Peptit**
750 mikrolitre sistein stok çözeltisi (Ko-elüsyon kontrolü için pH: 7.5 olan fosfat tampon çözeltisi) + 250 mikrolitre test kimyasal çözeltisi (Referans kontrol-A için uygun bir solvent-asetonitril)	750 mikrolitre lizin stok çözeltisi (Ko-elüsyon kontrolü için pH: 10.2 olan amonyum asetat tampon çözeltisi) + 250 mikrolitre test kimyasal çözeltisi (Referans kontrol-A için uygun bir solvent-asetonitril)

Test kimyasalı ve peptit çözeltisi, belirtilen miktarlarda küçük şişelere eklendikten sonra vortekslenip karanlıkta 24 saat oda sıcaklığında (25°C'de) bekletilerek oluşan numune serisinin YPSK analizi gerçekleştirilmiştir.

Referans Kontrol Hazırlanması

Referans kontrol, test kimyasalını çözmek için kullanılan çözücü ile peptit inkübasyonuna dayanır. Referans kontrol A, asetonitril ile farklı peptitlerin inkübasyonu ile hazırlanmıştır. Amaç çözücüden kaynaklı bir protein eksilmesinin olup olmadığını tespit etmektir.

Her standart, test kimyasal ve kontrol numunelerinden eşit hacimlerde sisteme enjekte edilmiştir.

Yöntem Koşulları

Agilent 1100 serisi YPSK-PDA (ABD) sistemi kullanılmıştır. Zorbax SB-C18 2.1 mm x 100

mm x 3.5 µ kolon üzerinde ayırım gerçekleştirilmiştir.

Mobil faz A, %0.1 h/h TFA içeren su; 1000 ml distile su içerisine 1 ml TFA (trifloroasetik asit) eklenerek hazırlanmıştır.

Mobil faz B, %0.085 h/h TFA içeren ACN; 1000 ml asetonitril içerisine 0.85 ml TFA eklenerek hazırlanmıştır. YPSK koşulları Tablo 3'de gösterilmiştir.

Tablo 3. YPSK Koşulları

Kolon	Zorbax SB-C18 2.1 mm x 100 mm x 3.5 µ			
Kolon sıcaklığı	30ºC			
Numune sıcaklığı	25ºC			
Dedektör	Miktar tayini için 220 nm sinyalli Sabit Dalgaboyu absorbans UV dedektörü			
Enjeksiyon hacmi	7 µl			
Çalışma süresi	20 dakika			
Akış hızları	**Zaman**	**Akış**	**%A**	**%B**
	0 dk	0.35 ml/dk	90	10
	10 dk	0.35 ml/dk	75	25
	11 dk	0.35 ml/dk	10	90
	13 dk	0.35 ml/dk	10	90
	13.5 dk	0.35 ml/dk	90	10
	20 dk	0.35 ml/dk		

Veri Analizi

Her test kimyasalı için peptit konsantrasyonu, 220 nm dalga boyunda belirlenmiştir. YPSK'dan elde edilen uygun piklerin pik alanı ölçümü yapılmış ve standart serisinden (STD-1 ile STD-7) elde edilen doğrusal kalibrasyon eğrileri kullanılarak peptit konsantrasyonu hesaplanmıştır. Her test kimyasalının peptitin tükenme yüzdesi, aşağıdaki formülle belirlenmiştir.

$$\%\text{Peptit Tükenmesi} = \left[1 - \left(\frac{\text{Peptit Pik Alanı}}{\text{Referans Kontrol Ortalama Pik Alanı}}\right)\right] \times 100$$

Elde edilen pik alanları ve standartların konsantrayonlarına dayalı olarak doğrusal bir kalibrasyon eğrisi oluşturulmuştur.

Yöntemin Geçerlilik Kriterleri

Elde edilen ölçümler sonucunda kalibrasyon doğrusallığı $r^2 > 0.990$ olarak bulunmalıdır**.** Referans kontrol-A'nın ortalama peptit konsantrasyonu 0.50 +/- 0.05 mM olmalıdır [11]. OECD klavuzunda yer alan ve pozitif kontrol için ortalama Peptit Tükenme Yüzde değerleri Tablo 4'de gösterilmiştir.

Tablo 4. Sinnamik aldehit (pozitif kontrol) için üç enjeksiyonun ortalama Peptit Tükenme Yüzde değerleri

	Yüzde peptit tükenmesi (Sistein için)		**Yüzde peptit tükenmesi (Lizin için)**	
Pozitif kontrol (Sinnamik aldehit)	Alt sınır	Üst sınır	Alt sınır	Üst sınır
	60.8	100.0	40.2	69.4

Pozitif kontrol tekrarları için maksimum standart sapmalar; Yüzde sistein tükenmesi için standart sapma < %14.9. Yüzde lizin tükenmesi için standart sapma < %11.6 olmalıdır [11].

Değerlendirme Modeli

OECD klavuzunda yer aldığı üzere, kimyasal maddelerin değerlendirme modelinde, ölçüt olarak sistein ve lizin proteinlerinin % tükenme ortalama değerleri ve sadece sistein protein yüzde tükenmesi değerleri kullanılmış ve Tablo 5'de gösterildiği gibi sınıflandırılma yapılmıştır.

Tablo 5. Sistein 1:10 / Lizin 1:50 Tahmin Modeli

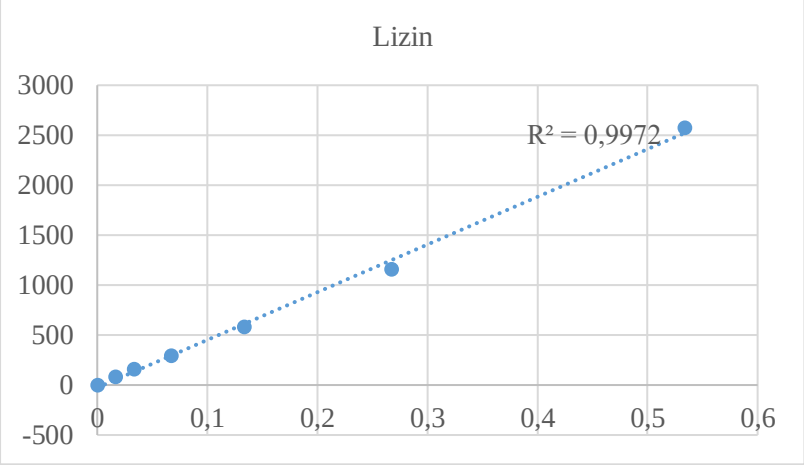
Sistein ve Lizin % Tükenme Ortalaması	Reaktivite Sınıfı	DPRA Tahmini[2]
%0 < ortalama % tükenme < %6.38	Reaktivite yok veya minimum düzeyde	Negatif
%6.38 < ortalama % tükenme < %22.62	Düşük reaktivite	Pozitif
%22.62 < ortalama % tükenme < %42.47	Orta reaktivite	
%42.47 < ortalama % tükenme < %100	Yüksek reaktivite	

Tablo 6. Sistein 1:10 Tahmin Modeli

Sistein (Cys) % Tükenmesi	Reaktivite Sınıfı	DPRA Tahmini[2]
%0 < Cys % tükenme < %13.89	Reaktivite yok veya minimum düzeyde	Negatif
%13.89 < Cys % tükenme < %23.09	Düşük reaktivite	Pozitif
%23.09 < Cys % tükenme < %98.24	Ortalama reaktivite	
%98.24 < Cys % tükenme < %100	Yüksek reaktivite	

SONUÇ VE TARTIŞMA

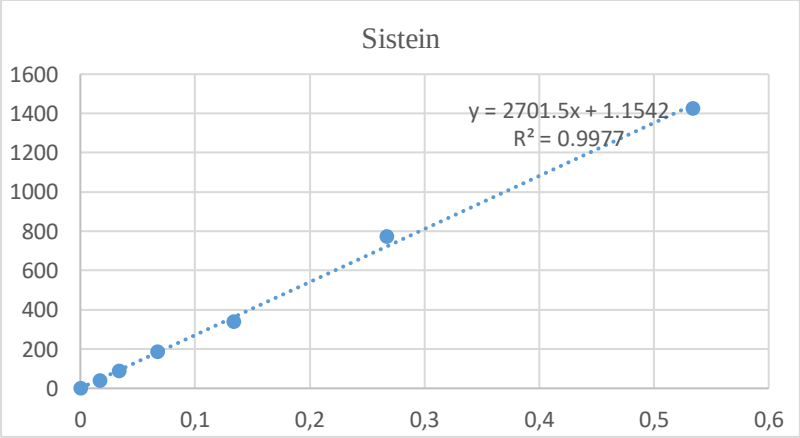
Standartlardan (STD-1 ile STD-7) türetilen doğrusal kalibrasyon doğrusu kullanılarak test kimyasalları ve pozitif kontrol ile hazırlanan numunelerin lizin ve sistein konsantrasyonu hesaplanmıştır (Şekil 1, Şekil 2). Protokol doğrultusunda hazırlanan lizin ve sistein çözeltileri 24 saat inkübe edildikten sonra her numunede peptit konsantrasyonu 220 nm'de verdiği pik alanından hareketle belirlenmiş ve Tablo 7 ve Tablo 8'de gösterilmiştir.



Şekil 1. Lizin için kalibrasyon standart doğru grafiği. Grafikte x konsantrasyon; y ise pik alanını ifade etmektedir. (r^2= 0.9972)

Tablo 7. Test kimyasallarının, pozitif kontrolün lizin ile inkübasyonu sonucu hazırlanan numunelerin pik alanları

Kimyasal	Pik alanı	Ortalama ± standart sapma (SS)
Parafenilendiamin (PPD	61	71 ± 10
	81	
	71	
Geraniol	1865	1853.3 ± 11.50
	1842	
	1853	
İzoöjenol	1823	1820.3 ± 2.51
	1818	
	1820	
Sodyum dodesil sülfat (SDS) (TC-4)	14.89	16.63 ± 1.74
	18.37	
	16.63	
Pozitif kontrol (sinnamik aldehit)	179	183.5 ± 4.5
	188	
	183.5	
Referans kontrol-A	1948	1973.3 ± 25.501
	1999	
	1973	



Şekil 2. Sistein için kalibrasyon standart doğru grafiği. Grafikte x konsantrasyon; y ise pik alanını ifade etmektedir. (r2= 0.9977)

Tablo 8. Test kimyasallarının, pozitif kontrolün sistein ile inkübasyonu sonucu hazırlanan numunelerin pik alanları

Kimyasal	Pik alanı	Ortalama ± standart sapma (SS)
Parafenilendiamin (PPD	11	10.6 ± 0.57
	10	
	11	
Geraniol	848	850.3 ± 2.51
	853	
	850	
İzoöjenol	83	80.4 ± 2.50
	78	
	80.2	

Tablo 8 (*devamı*). Test kimyasallarının, pozitif kontrolün sistein ile inkübasyonu sonucu hazırlanan numunelerin pik alanları

Kimyasal	Pik alanı	Ortalama ± standart sapma (SS)
Sodyum dodesil sülfat (SDS)	1084	1083.6 ± 1.52
	1085	
	1082	
Pozitif kontrol (sinnamik aldehit)	112	111.45 ± 1.17
	110.1	
	112.25	
Referans kontrol-A	1264	1261.6 ± 2.51
	1262	
	1259	

Tablo 9'da gösterilen lizin, sistein ve ışığında, çalışmamızda kullandığımız *in chemico* bir test yöntemi olan DPRA ile parafenilendiamin, geraniol, izoöjenol, SDS'nin allerjik kontakt dermatit oluşturma potansiyallerine dair veriler elde edilmiştir.

Tablo 9. Test kimyasallarının, pozitif kontrolün sistein, lizin, toplam peptit tükenme yüzdeleri

	% Sistein Tükenmesi	% Lizin Tükenmesi	% Peptit Tükenmesi
Pozitif Kontrol (sinnamik aldehit)	91.16	90.58	Yüksek
PPD (parafenilendiamin)	99.11	96.2	Yüksek
Geraniol	32.71	6.08	Düşük
İzoöjenol	93.63	6.59	Yüksek
SDS (sodyum dodesil sülfat)	14.16	99.15	Yüksek

DPRA ile haptenizasyon olarak kabul edilen, düşük moleküler ağırlıklı maddelerin (haptenlerin) deri proteinlerine kovalent bağlanmasıyla temsil edilen cilt hassaslaşmasının başlatılması süreci incelenmiştir. Kullanılan test maddeleri aktivitelerine göre çok düşük, düşük, orta veya yüksek olarak sınıflandırılmıştır.

Kozmetik bileşenlerin hassasiyet sınıflandırmasına yönelik DPRA çalışmamızın sonuçları, tahriş edici kimyasal SDS dışında önceki *in-vivo* ve *ex-vivo* çalışmaların sonuçlarıyla uyumlu bulunmuştur. lizin tükenme yüzdesi yanlış pozitif sonuç vermis olup sistein tükenmesi daha güvenilir olarak saptanmıştır. Sonuçlarımıza göre, lizin ve sistein kombinasyonunun kılavuzlara göre önerildiği bu yöntemde sadece sistein tükenmesi sonuçlarının da kullanılabileceği önerilmiştir. Çalışmamızın kapsamında, DPRA ile kimyasalların hassasiyet potansiyelini araştırmak için doğrulanmış bir yöntem oluşturulmuştur. Yöntemin özellikle irritasyon ve alerji ayrımını yapabilme yeteneğinin tespiti için SDS'nin (sodyum dodesil sülfat) de test kimyasalı olarak araştırılması yönteme önemli bir katkı sağlamıştır.

Alerjik kontakt dermatit, önemli bir mesleki ve çevresel sağlık sorunu olarak karşımıza çıkmaktadır. Hassasiyet potansiyelinin ölçümü, kişisel bakım ürünlerinde ve kozmetik ürünler içerisinde bulunan bileşenlerin geliştirilmesi, risk değerlendirmesinde temel bir unsurdur.

Kullanılan kimyasal maddenin alerjik kontakt dermatite neden olma potansiyelini yapılan test sonuçları göstermektedir [22]. Alternatif yöntemler, test başına hayvan sayısını azaltırken, hayvan refahını da göz ardı etmeden kullanılan yöntemlerin dışında ve/veya yer değiştirme ile sonuçlanan yeni teknikleri içermektedir, bu da toksikolojik bir son noktayı belirlemektedir. Konu, amaç ve tekniğe uygunsa *in vivo* yöntemler tercih edilerek veya entegre şekilde kullanılabilmektedir. Alternatif yöntemlerin entegre edilmesi, *in vivo* yöntemlere olan ihtiyacı azaltmaktadır. Günümüzde kullanılacak kimyasal madde sayısının sürekli arttığı düşünüldüğünde, alternatif yöntemlerin kullanılması hassasiyet

ölçümünün optimizasyonuna yarar sağlayacaktır [15]. Düşük moleküler ağırlıklı kimyasalların deri hassasiyet reaksiyonlarını tetiklediği ve ortaya çıkardığı süreç oldukça karmaşıktır ve kimyasalın cilde nüfuz etme, protein ile reaksiyona girme ve hücre aracılı bağışıklık tepkisini tetikleme yeteneği ile ilgili birçok faktöre bağlıdır. Bu nedenle, deri hassasiyet ölçümünde tek bir yöntem kullanılması önerilmemektedir.

DPRA kullanılarak yapılan çalışmalarda farklı kimyasal maddelerin deri hassasiyet potansiyelleri değerlendirilirken oksidasyon ile bozulma sonucunda çıkan ürünlerin peptit tüketimine etkisinin araştırıldığı çalışmalara da rastlanmıştır [2,23,24].

DPRA, deri hassasiyet potansiyelinin belirlenmesinde hızlı, ucuz ve uygulanabilir yöntemlerden biri olması nedeniyle oldukça avantajlıdır. Ancak DPRA yöntemi, bilinmeyen veya değişken kompozisyondaki madde ve karışımların, metal bileşiklerin, kompleks reaksiyon ürünlerinin, biyolojik materyallerin ve prohaptenlerin tespitinde kullanılamaz [2]. Yanlış negatif sonuçlara neden olmamak için sistein veya lizin dışındaki aminoasistlere karşı yapılan tercihli reaktifliğe sahip kimyasallar test edilmemelidir.

Hayvan deneylerine alternatif yöntemler, dünya çapında giderek daha önemli hale gelmektedir. ancak kabul edilen alternatif yöntemlerin, test maddelerinin deri hassasiyet potansiyelini belirlemede hayvan modellerine kıyasla yeterli olmaları gerekmektedir.

Alternatif yöntemlerin entegrasyonu *in vivo* yöntemlere olan ihtiyacı azaltsa da, kullanılacak olan test maddesinin duyarlaştırıcı potansiyelini belirlemek için hayvan modellerine kıyasla kabul edilen alternatif yöntemler doğru ve güvenilir sonuç vermelidir. Çalışmamızda kullandığımız alternatif bir yöntem olan DPRA'nın, hayvan modelleri ile elde edilen sonuçlarla benzer sonuçlar vermesi yöntemin geçerliliğini doğrulamaktadır, ancak daha fazla çalışmaya ihtiyaç vardır.

Özetle, hayvan modellerine alternatif yöntemlerin uygulama alanını genişletmek ve gelişimlerini tamamlamak için düzenleyici otoriteler, bilim ve endüstri arasındaki işbirliğinin devam etmesi ve yeni veri tabanlarının oluşturulması yeni sentezlenen kimyasal maddelerin duyarlaştıcıcı potansiyellerini tahmin etmekte büyük önem taşımaktadır.

TEŞEKKÜR

Ankara Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi tarafından desteklenmiştir (Proje No: 19L0237003).

YAZAR KATKILARI

Kavram: Ö.Ü., A.G., N.C., P.K.; Tasarım: Ö.Ü., A.G., N.C., P.K.; Denetim: Ö.Ü., A.G., N.C., P.K.; Kaynaklar: Ö.Ü., A.G., N.C., P.K.; Malzemeler: Ö.Ü., A.G., N.C., P.K.; Veri Toplama ve/veya İşleme: Ö.Ü., A.G., N.C., P.K.; Analiz ve/veya Yorumlama: Ö.Ü., A.G., N.C., P.K.; Literatür Taraması: Ö.Ü., A.G., N.C., P.K.; Makalenin Yazılması: Ö.Ü., N.C.; Kritik İnceleme: Ö.Ü., A.G., N.C., P.K.; 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.

KAYNAKLAR

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 978-986, 2023
Doi: 10.33483/jfpau.1311496

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IN SILICO EVALUATION OF SARS-COV-2 PAPAIN-LIKE PROTEASE INHIBITORY ACTIVITY OF SOME FDA-APPROVED DRUGS

FDA ONAYLI BAZI İLAÇLARIN SARS-COV-2 PAPAİN-LİKE PROTEAZ İNHİBİTÖR AKTİVİTESİNİN İN SİLİKO DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *In this study, it was aimed to perform in silico studies on the papain-like protease structure of SARS-CoV-2 (PDB: 7JIT) of 1300 FDA-approved drugs downloaded from the ZINC database.*
Material and Method: *A molecular docking study was performed with PLpro (PDB ID: 7JIT) using four different molecular docking programs for a total of 1300 FDA-approved drugs obtained from the ZINC database. Conivaptan and amphotericin B were obtained in docking analysis with AutoDock Vina and Sybyl-X, respectively. Docking analysis with Glide SP and Glide XP resulted in fludarabine and panobinostat, respectively. Molecular dynamics simulations were performed for a period of 120 ns to check the stability of these four drugs.*
Result and Discussion: *The reliability of the results obtained using four different molecular docking programs on the SARS-CoV-2 papain-like protease of 1300 drug molecules was checked by reinserting the co-crystal ligand. Protein-ligand interactions between fludarabine, conivaptan, amphotericin-B, panobinostat, and PLpro were given. In the molecular dynamics study, RMSD, RMSF, Rg, and SASA analyses were performed for four systems. It was observed that RMSD remained constant for all 120 ns for all four systems except for amphotericin B, which deviated slightly towards the end of 120 ns. No significant fluctuation was noticed in the RMSF graphics for all four systems.*
Keywords: *Molecular docking, molecular dynamics, SARS-CoV-2, ZINC*

ÖZ

Amaç: *Bu çalışmada ZINC veri tabanından indirilen 1300 adet FDA onaylı ilacın SARS-CoV-2'nin papain-like proteaz yapısı üzerinde (PDB:7JIT) in siliko çalışmalarının yapılması amaçlanmıştır.*

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Submitted / Gönderilme : 08.06.2023
Accepted / Kabul : 21.08.2023
Published / Yayınlanma : 20.09.2023

Gereç ve Yöntem: *ZINC veri tabanından elde edilen toplam 1300 FDA onaylı ilaç, dört ayrı moleküler doking programı kullanılarak PLpro (PDB ID: 7JIT) ile moleküler doking çalışması gerçekleştirildi. AutoDock Vina ve Sybyl-X ile doking analizinde, sırasıyla conivaptan ve amfoterisin B elde edildi. Glide SP ve Glide XP ile doking analizi sırasıyla fludarabin ve panobinostat ile sonuçlandı. Bu dört ilacın stabilitelerini kontrol etmek için 120 ns'lik bir süre boyunca moleküler dinamik simülasyonları gerçekleştirildi.*
Sonuç ve Tartışma: *1300 ilaç molekülünün SARS-CoV-2 papain benzeri proteazı üzerinde dört farklı moleküler doking programı kullanılarak elde edilen sonuçların güvenilirliği, ko-kristal ligandın yeniden yerleştirilmesiyle kontrol edildi. Fludarabin, conivaptan, amphotericin-B, panobinostat ve PLpro arasındaki protein-ligand etkileşimleri verildi. Moleküler dinamik çalışmasında dört sistem için RMSD, RMSF, Rg ve SASA analizleri yapıldı. 120 ns'nin sonlarına doğru hafifçe sapan amfoterisin B hariç, RMSD'nin dört sistemde de 120 ns'nin tamamında sabit kaldığı gözlendi. Dört sistemin tümü için RMSF grafiklerinde önemli bir dalgalanma fark edilmedi.*
Anahtar Kelimeler: *Moleküler dinamik, moleküler doking, SARS-CoV-2, ZINC*

INTRODUCTION

Coronaviruses (CoVs) are enveloped RNA viruses that have been responsible for three life-threatening viral epidemics in the last 20 years. They feature a 30 kb non-segmented positively sensitive RNA genome that has been known since the mid-1960s [1,2]. According to the World Health Organization, there were 8096 confirmed cases of SARS (severe acute respiratory syndrome) in 2002-2003 (mortality = 9.6%) and 2494 confirmed cases of MERS (Middle East respiratory disease) between 2012 and 2016. As of August 16, 2023, there were 769,774,646 confirmed COVID-19 cases, with 6,955,141 deaths reported to the WHO. The actual number is believed to be significantly higher. The invisible part of the iceberg brings along uncertainties. As a result, COVID-19 is the most serious epidemic to threaten humanity, both physically and financially, since the Spanish flu of 1918-1920. One of the most crucial areas being researched is the disease's spread patterns. The virus is known to spread in the foreground through droplets produced by coughing, sneezing, or talking. Fever, cough, shortness of breath, nasal discharge, nasal congestion, sneezing, sore throat, and smell and taste problems are the most prevalent symptoms. The binding of distinct cellular receptors and various structural characteristics of S-proteins explain these clinical symptom discrepancies. These variables all contributed to COVID-19's rapid dissemination [3,4].

The target cell membrane is bound by the S protein homotrimer, which creates projections on the virus surface. The virion is shaped by the M protein. The E protein is involved in virus recovery and release. The N protein is involved in virion packing and virus integrity versus intracellular defense mechanisms [5]. Although the roles of the majority of non-structural proteins (NSPs) in viral replication have been determined, the roles of a few remain unknown [6]. Viral proteases are an appealing target for therapeutic development since they are essential for viral replication. It is unique to each virus, allowing for targeted therapies with the minimum of hazardous side effects. Antagonizing ubiquitin and ubiquitin-like changes is a frequent strategy by which viral proteases influence innate immune pathways [7]. SARS-CoV-2 encodes two functioning proteases: papain-like protease (PLpro, NSP3) and 3-chymotrypsin-like cysteine protease (Mpro or 3CLpro, NSP5) [8]. PLpro produces NSP1, NSP2, and NSP3, and 3CLpro generates the remaining 13 non-structural proteins [7]. Mpro's primary job as a positive RNA virus is to degrade viral polyproteins that are required for virus development, replication, and invasion [9]. Inhibition of PLpro affects virus replication through inadequate viral protein processing and may also affect distant PLpro activities including deubiquitination, de-ISGylation, and innate anti-host immune reactions [10]. SARS-CoV PLpro is a cysteine protease with several major functions, including the processing of the viral polyprotein chain for viral protein maturation, irregular host inflammation responses by deubiquitylation, and disrupting host type I interferon antiviral immune responses by removing interferon-induced gene 15 [11]. Thus, inhibition of PLpro activity can halt viral replication and impair its role in host immune response evasion, making it an excellent anti-viral drug target.

In this study, a molecular docking study of 1300 FDA-approved drug molecules obtained from

the ZINC database (https://zinc.docking.org/) was performed on the crystal structure of Papain-Like Protease (PDB: 7JIT) of SARS CoV-2 using four separate programs. Molecular docking studies of all compounds were performed on AutoDock Vina, Glide SP, Glide XP, and Sybyl-X programs. The results of the studies were evaluated separately and a compound with the best binding energy (kcal/mol) was selected in each program. Conivaptan was obtained in docking analysis with AutoDock Vina, amphotericin B was obtained in docking analysis with Sybyl-X, fludarabine was obtained in docking analysis with Glide SP, and panobinostat was obtained in docking analysis with Glide XP (Figure 1). The reliability of all programs was checked by re-docking of the co-crystal ligand. The RMSD values and protein-ligand interactions of these drugs were given and presented with visuals. In addition, molecular dynamics simulations were performed using Gromacs Version 2020.4 for a period of 120 ns to check the stability of these four drugs. RMSD, RMSF, Rg, SASA values, and intermolecular hydrogen bond numbers for all systems were presented with graphics.

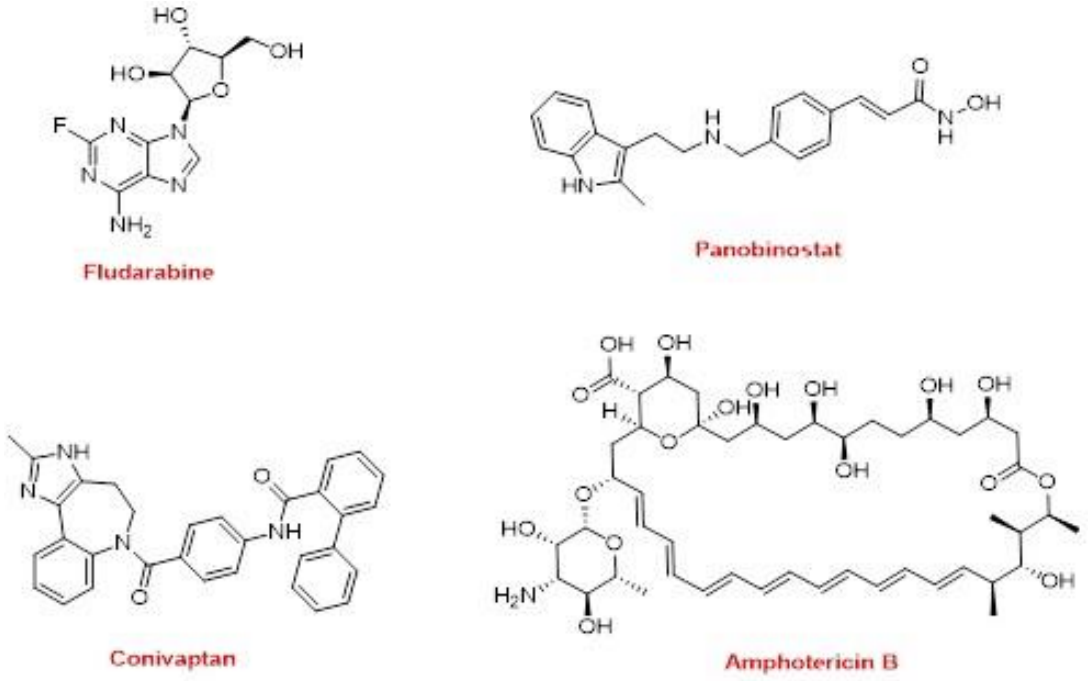

Figure 1. Chemical structures of fludarabine, panobinostat, conivaptan, and amphotericin-B

MATERIAL AND METHOD

Molecular Docking

Molecular docking studies were performed using Schrödinger Glide SP (Standard Precision) [12] and Glide XP (Extra Precision) [13], Autodock Vina v1.1.2 [14], and Sybyl-X v2.1.1 software [15]. The 3D crystal structure of the papain-like protease was downloaded from the protein databank (PDB:7JIT) [16]. Protein preparation was performed using the 'Protein Preparation Wizard' module of the Schrödinger suite (release 2022-3). 1300 FDA-approved drug molecules downloaded from the ZINC database [17,18] were downloaded in 3D SDF file format. The ligands were prepared using the 'LigPrep' module of the Schrödinger suite. The results of the studies performed in AutoDock Vina, Glide SP, Glide XP, and Sybyl-X programs were evaluated. 2D and 3D interactions of ligand and protein were determined and exhibited via BIOVIA Discovery Studio Visualizer v21.1 and UCSF Chimera v1.17.1.

Molecular Dynamics Simulation

Molecular dynamics simulations were performed using Gromacs Version 2020.4 [19]. The

procedure was carried out using the method given in the literature [20-22]. Root mean square deviation (RMSD) and root mean square fluctuation (RMSF), the radius of gyration (Rg), and solvent accessible surface area (SASA) analyses were performed in a standard molecular dynamics simulation of 120 ns duration. Molecular dynamics simulation trajectories were monitored with VMD-Visual Molecular Dynamics v1.9.4 and graphs were generated with the QtGrace Tool v0.2.6.

RESULT AND DISCUSSION

Molecular Docking

Traditional ways of discovering novel medicinal medications are costly and time-consuming. For this reason, many experimental and high-throughput simulation methods have been used in drug design in recent years. The "Molecular Docking Method" is one of these methods. Molecular docking studies are critical in identifying whether or not the millions of molecules thus produced are useful therapeutic ingredients. It is impossible to analyze each of the millions of chemical substances *in vitro*; thus, molecular docking studies play a critical role in determining the most effective molecules [23-25].

A total of 1300 FDA-approved drugs obtained from the ZINC database were subjected to molecular insertion with PLpro (PDB ID: 7JIT) using four molecular docking software programs. All programs were checked for their reliability by re-docking the co-crystal ligand, resulting in acceptable RMSD values of 1.002 Å (AutoDock Vina), 0.824 Å (Glide SP), 0.772 Å (Glide XP) and 0.826 (Sybyl-X) (Figure 2). After this validation, the docking of FDA-approved drugs was carried out using the same parameters for each software. Docking analysis with AutoDock Vina and Sybyl-X resulted in the acquisition of conivaptan and amphotericin B, respectively. Docking with the Glide program resulted in fludarabine and panobinostat from SP and XP docking, respectively. The four compounds acquired also showed molecular interactions with various residues of PLpro at the active site (Figure 3). In Table 1, protein-ligand interactions between fludarabine, conivaptan, amphotericin-B, panobinostat, and papain-like protease (PDB ID: 7JIT) were given. Molecular dynamics simulations were also performed to check the stability of these four drugs.

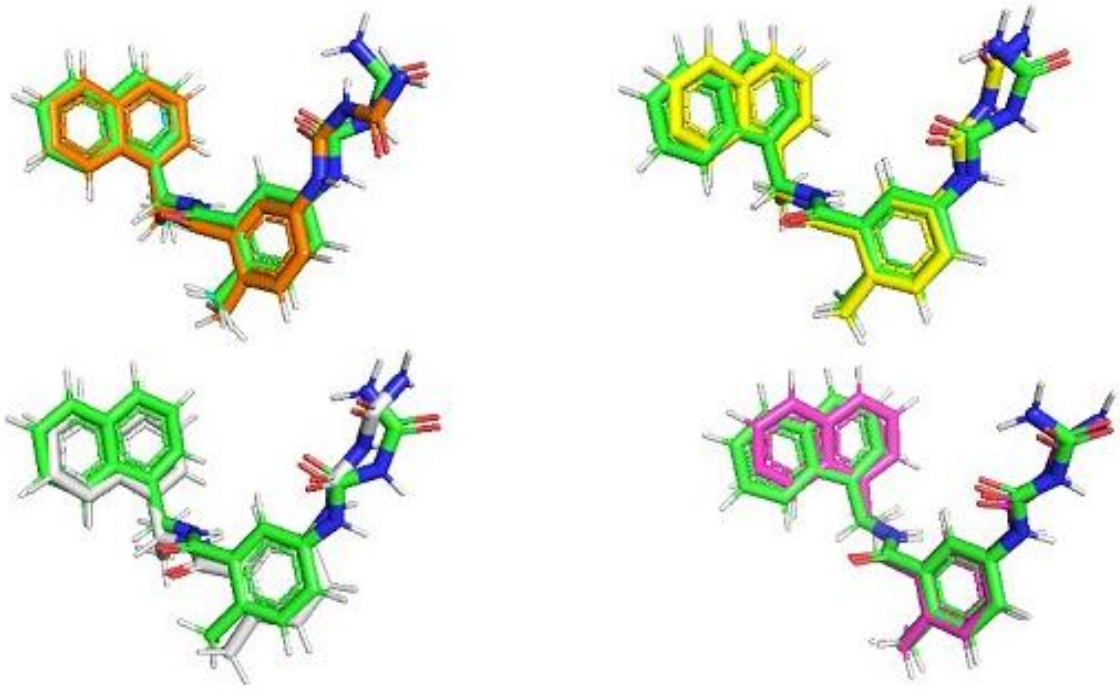

Figure 2. Superimposition of docking poses obtained with AutoDock Vina (orange), Glide SP (yellow), Glide XP (magenta), and Sybyl-X (gray) with the natural conformation of Y95. RMSD values were measured as 1.002 with AutoDock Vina, Glide SP 0.824, Glide XP 0.772, and Sybyl-X 0.826, respectively

Table 1. Protein-ligand interactions between fludarabine, conivaptan, amphotericin-B, panobinostat, and papain-like protease (PDB ID: 7JIT)

Compound	Protein-ligand Interactions
Fludarabine	LEU A:162, TYR A:268, GLN A:269, GLY A:163, ASP A:164, ARG A:166
Panobinostat	GLY A:163, GLY A:271, LEU A:162, GLN A:269, ASP A:164, MET A:208, GLUA:167, PRO A:247
Conivaptan	TYR A:263, PRO A:248, ASP A:164, TYR A:264, PRO A:247, TYR A:268, LYS A:157
Amphotericin B	ASP A:164, ARG A:166, GLU A:167, GLN A:269, LEU A:162, TYR A:263, TYR A:268, TYR A:264, PRO A:248

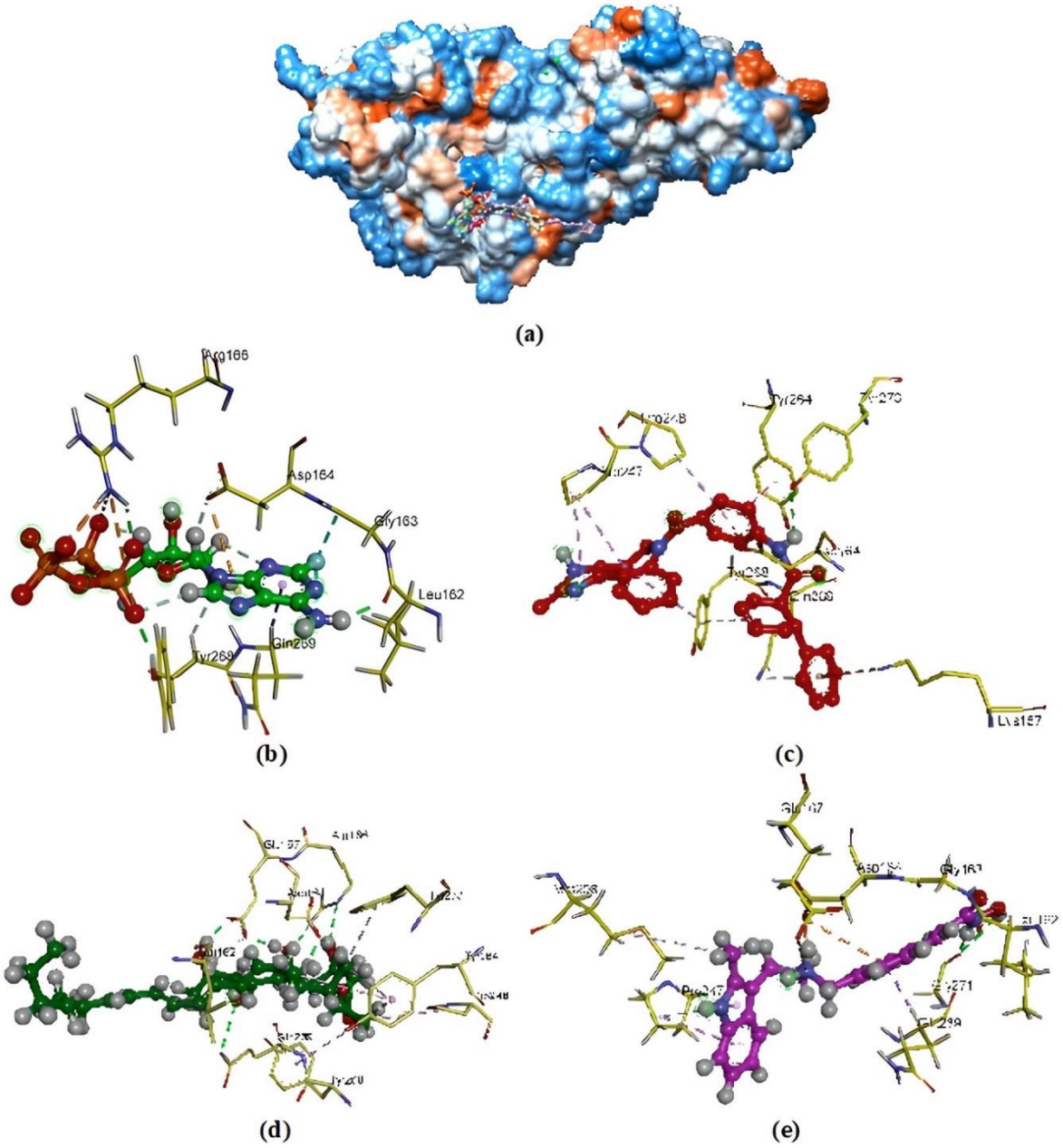

Figure 3. Protein-ligand interactions of superimposition (a), fludarabine (b), conivaptan (c), amphotericin B (d), and panobinostat (e) at the papain-like protease active site

Molecular Dynamics Simulations

Molecular dynamics (MD) simulations create an interface between experiment and theory by being used to predict the dynamic properties of complex systems that cannot be calculated analytically as the equivalent of experiments [26]. MD simulation systems are multi-particle systems that use numerical integration of Newton's Law of Motion's classical theory to describe the motion of atoms and molecules to construct a dynamic trajectory ranging from nanometer to micrometer scale. Such simulations help to answer significant unanswered questions in biology and chemistry. It also contributes continuously to the drug development process [27,28].

The docked complexes of the fludarabine, conivaptan, amphotericin-B, and panobinostat with PLpro were used as the beginning coordinates for molecular dynamics simulations, and their stabilities were tested for 120 ns. All systems were tested for stability by graphing their RMSD, RMSF, radius of gyration, and solvent-accessible surface areas (Figure 4).

The RMSD analysis indicates how much the atoms in the protein structure have shifted away from their normal positions before and during the simulations. RMSD analysis, in other words, enables the tracking of dynamic changes in protein structure. When an inhibitor is present in the active site of the target-containing residue in perfect protein-ligand MD simulations, it fluctuates less and interacts with the ligand [29]. The RMSD for all four systems was found to be steady for the whole 120 ns, with the exception of amphotericin B, which showed a minor departure near the end of the 120 ns.

The average deviation of a particle (for example, a protein residue) from a reference position (usually the particle's time-averaged position) over time is measured by RMSF [30]. As a result, the RMSF examines the parts of structures that deviate the most (or least) from their average structure.

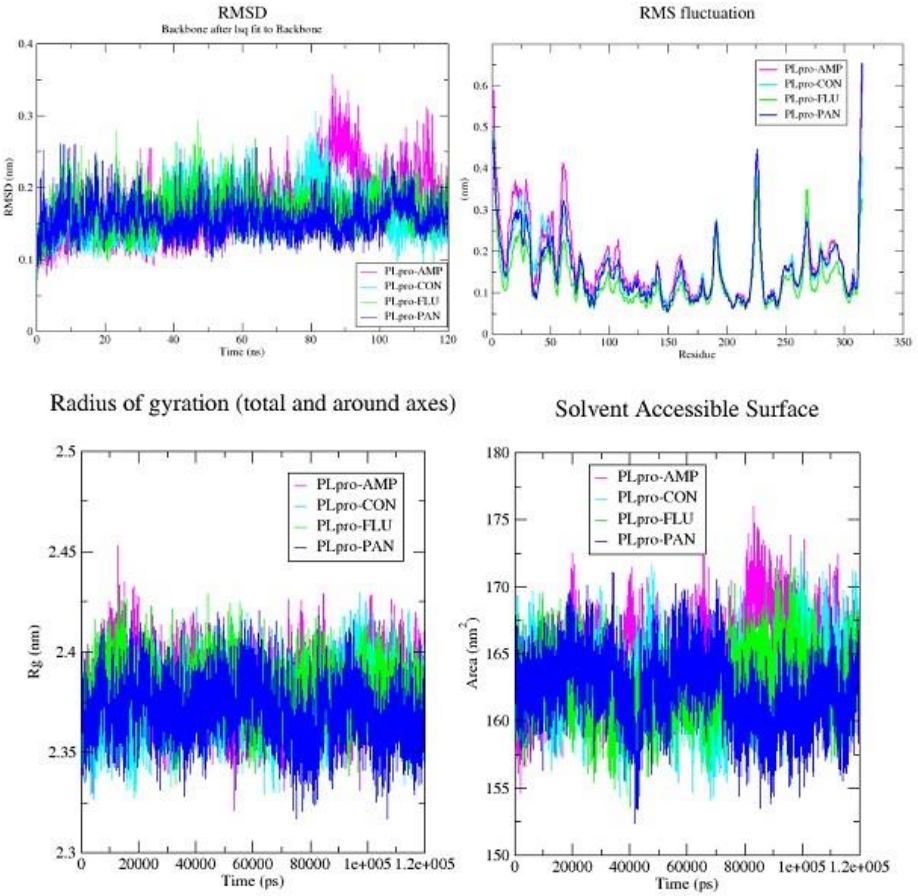

Figure 4. Molecular dynamics simulation of amphotericin B, conivaptan, fludarabine, and panobinostat with the papain-like protease active site. (a) RMSD of apo- and ligand-bound PLpro, (b) RMS fluctuation, (c) Rg, and (d) SASA values during the period of simulation

The Rg value, which is another analysis used to measure system stability, was also computed for each simulation group. A protein's radius of rotation (Rg) is a measure of its compactness. If a protein folds stably, its Rg value will most likely remain constant. If a protein unfolds rather than folding, its radius of rotation (Rg) changes over time [31]. As a result, the compactness of the four complexes was compared. It was measured with modest fluctuation values ranging from 2.32 to 2.45 nm. Over time, PLpro-CON, PLpro-FLU, and PLpro-PAN showed a more consistent trend.

The surface area of a biomolecular structure accessible by a solvent is defined as SASA (solvent accessible surface area) analysis [32]. To begin the modeling investigations, the SASA values of the four protein structures were determined and are shown in Figure 4. SASA measurements of fludarabine, conivaptan, amphotericin B, and panobinostat were done after binding to the active site of papain-like protease to determine the value of the solvent reaching the protein surfaces. The SASA value resulting from the interaction of amphotericin B with PLpro was larger than the others.

In protein-ligand or DNA-ligand interactions, the presence and amount of hydrogen bonds may signal that the ligand will interact more with the macromolecule and create a more stable complex [33]. As a result, the time-dependent number and fluctuation of hydrogen bonds were investigated. During the 120 ns simulation, as shown in Figure 5, amphotericin B typically has 1 to 4 hydrogen bonds, conivaptan has 1 to 5 hydrogen bonds, fludarabine has 1 to 8 hydrogen bonds, and panobinostat 1 to 7 hydrogen bonds.

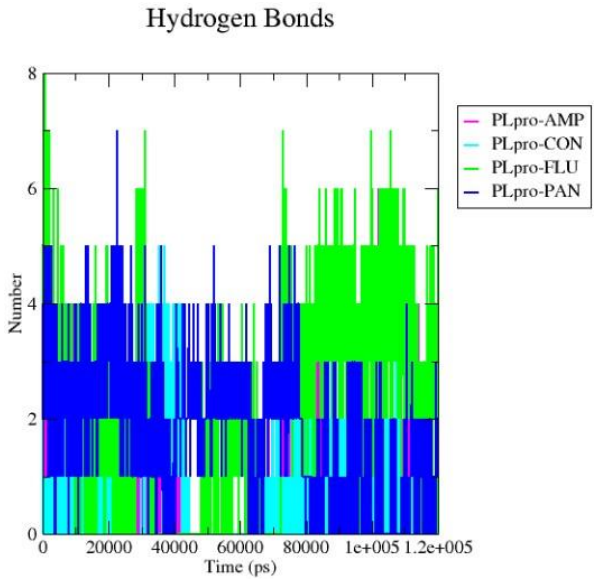

Figure 5. Intermolecular H bond number between papain-like protease active sites and amphotericin B, conivaptan, fludarabine, and panobinostat for 120 ns (PDB ID: 7JIT)

As a result, 1300 FDA-approved drugs collected from the ZINC database were molecular docked with PLpro (PDB ID: 7JIT) using four distinct molecular docking tools in this study. Protein-ligand interactions of drugs acquired by each program were demonstrated. In addition, molecular dynamics simulations were run for 120 ns to test the stability of these four drugs. All systems were subjected to RMSD, RMSF, Rg, and SASA evaluations, and all graphs were displayed. This study, which is supported by *in silico* research, will be valuable in identifying therapeutic compounds that are thought to be effective against SARS-CoV-2 PLpro.

ACKNOWLEDGEMENTS

All molecular dynamics simulations reported were performed utilizing TÜBİTAK (The Scientific and Technological Research Council of Turkey) ULAKBİM (Turkish Academic Network and Information Centre), High Performance and Grid Computing Centre (TRUBA resources).

AUTHOR CONTRIBUTIONS

Concept: M.E.; Design: M.E.; Control: M.E.; Sources: M.E.; Materials: M.E.; Data Collection and/or Processing: M.E.; Analysis and/or Interpretation: M.E.; Literature Review: M.E.; Manuscript Writing: M.E.; Critical Review: M.E.; 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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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 47(3): 987-993, 2023
Doi: 10.33483/jfpau.1337313

ÖZGÜN MAKALE / ORIGINAL ARTICLE

FLOROKİNOLONLARIN HPLC-DAD İLE ANALİZİ İÇİN YENİ YÖNTEM GELİŞTİRİLMESİ

THE NEW METHOD DEVELOPMENT FOR THE DETERMINATION OF FLUOROQUINOLONES BY HPLC-DAD

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ÖZ

Amaç: *Bu çalışmanın amacı, farklı nesil florokinolonlardan; siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasinin ayrılması ve eş zamanlı analizine olanak sağlayan yeni, kolay, hızlı ve hassas bir HPLC-DAD yöntemi geliştirmektir.*
Gereç ve Yöntem: *Literatürlerde, etken maddede florokinolonların tek başına veya ikili karışımlarının ayrılmaları, analizi ve miktar tayinleri ile ilgili çeşitli yöntemler mevcuttur. Bu çalışmada siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasin için en etkili ayırımı sağlayacak yeni bir HPLC-DAD yönteminin oluşturulması hedeflenmiştir. Farklı asidik ve bazik hareketli fazlar, tampon çözeltiler ve ayırım tipleri denenmiştir. En etkili ve seçici yöntemin XTerra, C18 (100 x 4.6 mm, tanecik boyutu 3.5 µm) analitik kolon ve metanol:borat tamponu (pH=9.1, 100 mM) içeren hareketli faz ile gradient elüsyonla 0.6 ml/dak akış hızında gerçekleştirilmiştir. Genel olarak florokinolonların kromatografik tekniklerle analizinde floresan dedektör kullanıldığı gözlenmiştir. Yaptığımız çalışmada ise ayırım 280 nm'de DAD dedektörü kullanılarak başarılmış ve siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasinin eş zamanlı tayinleri gerçekleştirilmiştir. Kalibrasyon eğrileri çalışılan florokinolonların herbiri için 0.5-10 µg/ml konsantrasyon aralığında doğrusaldır. Geliştirilen yöntem için validasyon çalışmaları da yapılmıştır.*
Sonuç ve Tartışma: *Siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasinin eş zamanlı tayinine izin veren basit, hızlı, hassas ve valide bir HPLC-DAD yöntemi geliştirilmiştir.*
Anahtar Kelimeler: *Enrofloksasin, HPLC-DAD, levofloksasin, moksifloksasin, siprofloksasin*

ABSTRACT

Objective: *The aim of this study was to develop a new, simple, rapid and sensitive HPLC-DAD*

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Gönderilme / Submitted : 03.08.2023
Kabul / Accepted : 22.08.2023
Yayınlanma / Published : 20.09.2023

method for the separation and simultaneous analysis of ciprofloxacin, levofloxacin, enrofloxacin and moxifloxacin from different generation of fluoroquinolones.

Material and Method: *In the literature, there are various methods for the separation, analysis and quantification of fluoroquinolones alone or in binary mixtures in active substance. In this study, it was aimed to develop a new HPLC-DAD method that would provide the most effective separation for ciprofloxacin, levofloxacin, enrofloxacin and moxifloxacin. Different acidic and basic mobile phases, buffer solutions and separation types were tested. The most efficient and selective method XTerra, C18 (100 x 4.6 mm, particle size 3.5 µm) analytical column and mobile phase containing methanol:borate buffer (pH=9.1, 100 mM) were used for gradient elution at a flow rate of 0.6 ml/min. In general, fluorescent detector was used in the analysis of fluoroquinolones by chromatographic techniques. In our study, separation was achieved by using DAD detector at 280 nm and simultaneous determinations of ciprofloxacin, levofloxacin, enrofloxacin and moxifloxacin were performed. The calibration curves were linear in the concentration range of 0.5-10 µg/ml for each of the fluoroquinolones studied. Validation studies were also performed for the developed method.*

Result and Discussion: *A simple, rapid, sensitive and validated HPLC-DAD method allowing simultaneous determination of ciprofloxacin, levofloxacin, enrofloxacin and moxifloxacin was developed.*

Keywords: *Ciprofloxacin, enrofloxacin, HPLC-DAD, levofloxacin, moxifloxacin*

GİRİŞ

Kinonlonlar, sentetik antimikrobiyal ajanlardır ve 1962'de klorokin sentezinin bir yan ürünü olan nalidiksik asit olarak keşfedilmişlerdir. Sonrasında farklı sübstitüentlerin eklenmesi yoluyla kinolon çekirdeğinin modifikasyonu ile farklı florokinolonlar keşfedilmiştir. Norfloksasin, 6. karbon pozisyonuna bir flor eklenmesi sonrası elde edilen ilk "florokinolondur" (Şekil 1 Owens ve Ambrose [3]). Diğer ikinci nesil kinolonlar ise; siprofloksasin, ofloksasin, levofloksasin, enrofloksasin, enoksasin, fleroksasin, lomefloksasin, pefloksasin ve rufloksasindir. 1980'lerden beri bakteriyel enfeksiyonları tedavi etmek için kullanılan bir antibiyotik sınıfıdır. Bakteriyel DNA replikasyon sürecinde topoizomeraz II ve IV'ü inhibe ederek bakterilerin ölümüne yol açarlar. Florokinolonlar Gram-pozitif, Gram-negatif ve atipik bakterilere karşı kullanılan başlıca antibakteriyel ilaçlardır. Dört gruba ayrılırlar. Antibakteriyel ajanların konsantrasyonlarının izlenmesi etkili tedavi sağlar ve antibiyotiklere karşı bakteriyel direncin artmasını önler. Ayrıca, olası toksikolojik etkilere karşı koruyucu tedavi yaklaşımına katkı sağlamaktadır. Florokinolonlar idrar yolu enfeksiyonları, solunum yolu enfeksiyonları, deri ve yumuşak doku enfeksiyonları dahil olmak üzere çok çeşitli bakteriyel enfeksiyonların tedavisinde yaygın olarak kullanılmaktadır. Ayrıca bazı gastrointestinal ve cinsel yolla bulaşan enfeksiyonların tedavisinde de kullanılmaktadırlar [1-4]. İlerleyen çalışmalar ile daha geniş spektruma, daha yüksek etkinliğe sahip çeşitli yeni nesil florokinolonlar (üçüncü ve dördüncü nesil) üretilmiştir [5]. Sparfloksasin ve levofloksasin üçüncü, moksifloksasin dördüncü nesil bileşiklerdir ve sıklıkla tedavide tercih edilen kinolonlar arasında yer alırlar [6].

Siprofloksasin, levofloksasin, moksifloksasin ve enrofloksasin kimyasal yapıları ve etkili oldukları bakteri türlerinin spektrumu bakımından farklılık gösterirler. Örneğin, siprofloksasin ve levofloksasin hem Gram-negatif hem de Gram-pozitif bakterilere karşı etkiliyken, moksifloksasin ve ofloksasin Gram-pozitif bakterilere karşı daha etkilidir. Enrofloksasin domuz, kedi ve köpeklerde Gram-negatif ve bazı Gram-pozitif bakterilere karşı kullanılmaktadır [6-7].

Farklı matrislerde florokinolonların analizi için birçok enstrümantal teknik kullanılmıştır. Literatürde genellikle yüksek performanslı sıvı kromatografisi (HPLC) ve floresans detektör kullanılarak yapılan çalışmalar ile bazı yeni çalışmalarda voltametrik analiz uygulamaları, fluoresans spektroskopisi, kütle spektrometrisi uygulamaları da mevcuttur [8-13]. HPLC ve diyod sıralı dedektör (DAD) ile florokinolonların analizine yönelik ise kısıtlı sayıda çalışma mevcuttur [14-15]. Bu çalışmada siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasinin HPLC-DAD sistemi ile eş zamanlı olarak analizine olanak sağlayacak, basit, seçici ve valide bir analitik yöntem oluşturulması amaçlanmıştır.

GEREÇ VE YÖNTEM

Cihaz

Shimadzu sıvı kromatografi sistemi (LC-2030C), pompa (LC-10AT VP), yazılım (Class-VP 5.03), kontrol sistemi (SCL-10A VP), otomatik örnekleyici (SIL-10AD VP), ve diyod sıralı dedektör (DAD, SPD-10A VP)'den oluşmaktadır. Ayırım XTerra, C18 (100 x 4.6 mm, tanecik boyutu 3,5 µm) analitik kolon (Waters, Milford, MA, ABD) ile analiz sıcaklığı (30°C), 0.6 ml/dak akış hızında metanol-borat tamponu içeren (100 mM, pH 9.1) hareketli faz ile gradient elüsyonda yapılmıştır. DAD dedektör 280 nm dalga boyuna ayarlanmıştır. pH 0.1 M formik asit kullanılarak ayarlanmıştır. Enjeksiyon hacmi 10 µl olarak seçilmiştir. Tüm çözeltilerde tip 1 su kullanılmıştır (Simplicity 185 Water System, Millipore Corp., Bedford, MA, ABD). Analiz sonunda analitik kolon, hacminin yaklaşık 20 katı HPLC sınıfı su ve ardından metanol ile yıkanmıştır. Son olarak, kolon saf metanol içinde saklanmıştır. Bu prosedür her analiz sonrasında uygulanmıştır.

Kimyasallar

Bu çalışmada siprofloksasin (SF) ve levofloksasin (LF) analitik standartları Drogsan İlaçları San. ve Tic. A.Ş.'den temin edilmiştir, moksifloksasin (MF), enrofloksasin (EF) ve kafein (iç standart, IS) analitik standartları Sigma-Aldrich (ABD)'den satın alınmıştır. Metanol, asetonitril (HPLC saflıkta), borik asit ve formik asit Sigma-Aldrich (ABD)'den satın alınmıştır. SF, LF, MF ve EF ile kafeinin stok çözeltileri 1 mg/ml olacak şekilde hareketli fazda hazırlanmış ve analizde kullanılacak çözeltiler, istenilen konsantrasyon değerlerine stok çözeltilerin hareketli faz ile seyreltilmesiyle günlük olarak hazırlanmıştır. Stok çözeltiler -20°C'da 1 hafta süresince saklanmıştır.

Kromatografik Çalışmalar

Çalışmada optimum kromatografik ayırma koşullarının belirlenmesi amacıyla çeşitli hareketli faz bileşimlerinde ve farklı sabit fazlarda denemeler yapılmıştır. Bu amaçla hareketli faz organik düzenleyici seçimi için metanol ve asetonitrilin florokinolonların analizine etkisi değişik konsantrasyonlarda çalışılarak incelenmiştir. Asetonitril kullanılarak hazırlanan hareketli faz ile iyi bir ayırım elde edilememiş, florokinolon piklerinde çakışmalar gözlemlenmiştir. En iyi ayırım için metanol tercih edilmiştir. Ek olarak en iyi ayırımın elde edilmesi amacıyla çeşitli sabit faz denemelerinin yapıldığı çalışmada da C8 ve C18 özelliğinde dolgu maddeleri içeren analitik kolonların 100-250 mm uzunluğunda olan sabit fazlar denenmiş ve XTerra, C18 (100 x 4.6 mm, tanecik boyutu 3.5 µm) en iyi ayırımın elde edildiği sabit faz olarak seçilmiştir. Yine hareketli faz bileşimi optimizasyon çalışmalarında değişik tampon çözeltiler ve pH denemeleri yapılmış bu amaçla fosfat tamponu ve borat tamponu ile yapılan çalışmalarda pH değerinin 3 ila 9.1 aralığında ayırım için kapasite faktörü ve pik simetri oranları ile olan etkileri incelenmiştir. En iyi ayırım hedeflenen dört florokinolon ve kafein (iç standart) için hareketli faz bileşiminin metanol (A) ve borat tamponu (B) (100 mM, pH 9.1) ile gradient elüsyonda elde edilmiştir. İç standart olarak kromatogramda diğer etken madde pikleri ile çakışmaması ve iyi bir ayırım elde edilmesi sebebiyle kafein tercih edilmiştir. Uygulanan gradient ayırım program Tablo 1'de verilmiştir. Hareketli faz 0.45 µm gözenek çaplı membran filtreden süzülmüş ve ultrasonik banyoda 15 dk bekletilerek çözünen gazlar uzaklaştırılmıştır.

Tablo 1. Analizde uygulanan gradient ayırım programı

Zaman (dk)	Metanol (A) (%)	Borat Tamponu (B) (%)
0-2	35	65
3-15	50	50
16	35	65

Validasyon Çalışmaları

Validasyon çalışmaları ICH gereklilikleri kapsamında incelenmiştir [16-17]. Bu kapsamda; elde edilen verilere göre her bir ilaca ait alıkonma zamanı, kapasite faktörü, kuyruklanma faktörü ve teorik

tabaka sayısı hesaplanmıştır. Sistem uygunluk testi 1 µg/ml konsantrasyondaki standart karışım çözeltisinin altı tekrarlı enjeksiyonu ile gerçekleştirilmiştir. Doğrusallık çalışmalarında kromatogramlardan elde edilen pik eğri altı alan değerleri iç standarda (1 µg/ml) ait pik eğri altı alan değerlerine oranlanlanmış ve her bir florokinolonun konsantrasyon değerine karşı grafiğe geçirilerek kalibrasyon eğrileri elde edilmiştir. Elde edilen eğrilerden korelasyon katsayısı (r) değerleri incelenerek yöntem doğrusallığı değerlendirilmiştir. Ayrıca duyarlılık, seçicilik, doğruluk ve kesinlik çalışmaları üç farklı konsantrasyon değerinde (0.5, 1 ve 10 µg/ml, n=6) yapılmış, gün-içi ve günler-arası tekrarlı analiz sonuçları değerlendirilmiştir. İki farklı analizci tarafından yapılan tekrarlı analiz sonuçlarının karşılaştırılması ile tutarlılık analizleri de yapılmıştır. Sonuçlar t testi ve standart hata hesaplamaları yapılarak değerlendirilmiştir. Gözlenebilme sınırı (LOD) değeri; tekrarlanan (n=6) farklı standart çözelti analiz için sinyal/gürültü (S/G) değerinin 3'e eşit olduğu değer olarak alınmıştır. Alt tayin sınırı (LOQ) değeri ise tekrarlanan (n=6) farklı çözelti analiz için BSS değerinin %5'e eşit ve küçük olduğu değer olarak alınmıştır [18-19]. Analiz sıcaklığının ve hareketli faz akış hızının ayırıma etkisinin araştırılması çalışmasında 24 ila 35°C aralığında ve 0.4 ml/dk, 0.6 ml/dk, 0.8 ml/dk değerlerinde çalışmalar yapılmıştır.

SONUÇ VE TARTIŞMA

Bu çalışmada siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasinin HPLC-DAD sistemi ile eş zamanlı olarak analizine olanak sağlayacak yeni, basit ve seçici bir analiz yönteminin oluşturulması amaçlanmıştır. Yapılan çalışmalarda en iyi ayırım koşullarının ve en uygun organik düzenleyici konsantrasyonunun seçimi için bir dizi deneysel çalışmalar yapılmış aynı zamanda geliştirilen yöntem için validasyon parametreleri de incelenmiştir. Yöntem, siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasin için 0.5-10 µg/ml aralığında doğrusal olarak bulunmuştur. Elde edilen eğri denklemlerinden r^2 değerleri 1'e çok yakın olarak hesaplanmıştır (Tablo 2). En iyi ayırım koşullarının belirlenmesi çalışmaları kapsamında elde edilen verilere göre her bir etken maddeye ait alıkonma zamanı, kapasite faktörü, kuyruklanma faktörü ve hesaplanan teorik tabaka sayısı değerleri Tablo 3'de verilmiştir.

Tablo 2. Yöntem için elde edilen doğrusal aralık ve hesaplanan değerler (n=6)

Florokinolon	Doğrusal Aralık (µg/ml)	Eğim	Kesişim	r	r^2	Eğim SH*	Kesişim SH*	LOD (µg/ml)
SF	0.5-10	0.807	0.012	0.9998	0.9996	0.064	0.014	0.15
LF	0.5-10	0.620	-0.161	0.9998	0.9996	0.059	0.021	0.15
EF	0.5-10	0.651	0.234	0.9999	0.9998	0.078	0.019	0.15
MF	0.5-10	1.677	0.082	0.9999	0.9999	0.098	0.017	0.15

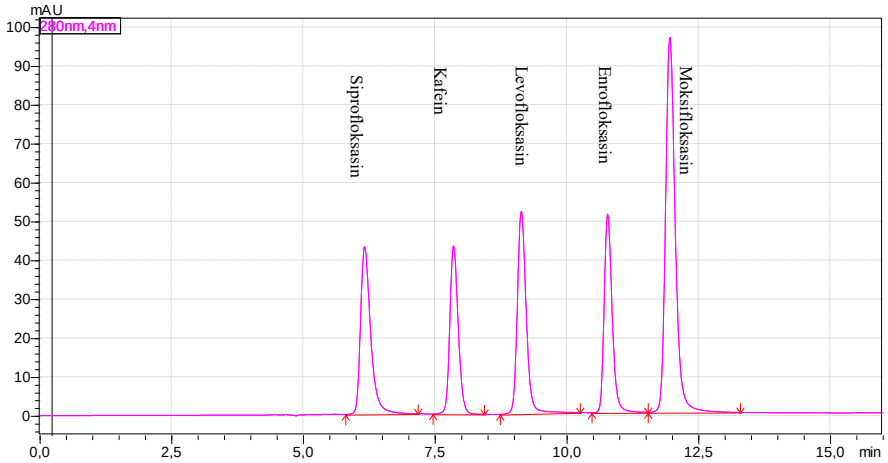
SH*: Eğim ve kesişimin standart hatası

Tablo 3. Sistem uygunluk parametrelerinin değerlendirilmesi (n=6)

Florokinolon	Alıkonma Zamanı (dk)	Kapasite Faktörü	Kuyruklanma Faktörü	Teorik Tabaka Sayısı
SF	6.20±0.01	2.11	1.31±0.01	5860.11
LF	9.14±0.02	3.58	1.12±0.01	17286.53
EF	10.73±0.01	4.37	1.08±0.00	29950.78
MF	12.05±0.01	4.94	1.20±0,01	23600.80

Çalışma kapsamında incelenen dört faklı florokinolon için iyi bir ayırım elde edilmiştir (Şekil 1). Validasyon çalışmaları kapsamında yapılan duyarlılık, seçicilik, doğruluk ve kesinlik parametreleri için üç farklı konsantrasyon değerinde (0.5, 1 ve 10 µg/ml, n=6) deneyler yapılmış, gün-içi ve günler-arası tekrarlı analiz sonuçları bağıl hata (BH), bağıl standart sapma (BSS) ve bağıl standart hata (BH) değerleri hesaplanarak değerlendirilmiştir (Tablo 4). Yöntemin seçiciliğinin belirlenmesi amacıyla

yapılan çalışmalar sonucunda SF, LF, EF, MF ve IS piklerinin alıkonma zamanlarında hareketli fazın gradient sisteminden kaynaklanan herhangi bir girişimin olmadığı tespit edilmiştir.



Şekil 1. SF, LF, EF, MF ve kafeine ait kromatogram (1 µg/ml)

Tablo 4. Gün içi ve günler arası kesinlik ve doğruluk çalışmaları (n=6)

Konsan. (µg/ml)		SF		LF		EF		MF	
		Gün İçi	Günler Arası	Gün İçi	Günler Arası	Gün İçi	Günler Arası	Gün İçi	Günler Arası
0.5	$\bar{x}$	0.49	0.49	0.50	0.50	0.49	0.49	0.48	0.49
	SH	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.01
	BSS	2.23	2.01	1.23	0.50	2.01	1.95	0.16	4.13
	BH	1.06	-1.47	-0.83	0.13	-1.47	-1.47	-3.89	6.35
1.0	$\bar{x}$	0.99	1.02	0.97	1.01	0.99	1.02	1.04	1.01
	SH	0.05	0.03	0.01	0.03	0.02	0.03	0.02	0.03
	BSS	1.57	3.41	0.89	3.29	1.57	3.41	0.63	3.29
	BH	0.65	1.62	3.20	0.77	0.65	1.62	1.98	0.77
10.0	$\bar{x}$	9.99	9.99	9.94	9.95	9.99	9.99	10.00	9.99
	SH	0.10	0.02	0.01	0.03	0.02	0.02	0.02	0.02
	BSS	0.15	0.15	0.11	0.30	0.15	0.15	0.16	0.16
	BH	0,.14	0.12	0.57	0.46	0.14	0.12	0.05	0.06

$\bar{x}$: Ortalama, SH: Standart hata, BSS: Bağıl standart sapma, BH: Bağıl standart hata

Geliştirilen yöntem için HPLC-DAD sistem uygunluğu alıkonma zamanı, enjeksiyon tekrarlanabilirliği, kapasite faktörü, kuyruklanma faktörü ve teorik tabaka sayısı esas alınarak değerlendirilmiştir. İncelenen parametreler için 6 tekrarlı yapılan deneyler sonucunda elde edilen veriler kullanılmıştır. Elde edilen değerler (Tablo 3) kabul edilen şartları sağlamaktadır kapasite faktörü>2, kuyruklanma faktörü≤1.5 ve teorik tabaka sayısı>2000] içinde bulunmuş olup sistem hedeflenen ilaçların analizi için uygun olarak bulunmuştur [16-17].

Geliştirilen yöntem için en iyi ayırım; XTerra, C18 (100 x 4.6 mm, tanecik boyutu 3,5 µm) analitik kolon kullanılarak metanol:borat tamponu (pH=9.1, 100 mM) içeren hareketli faz ile 0.6 ml/dak akış hızında gradient elüsyon ile gerçekleştirilmiştir. Analiz sıcaklığının ayırıma etkisi üç farklı sıcaklık değerinde çalışılarak incelenmiş ve analiz sıcaklık değeri 30 °C olarak yine akış hızının ayırıma etkisinin

araştırılması çalışmasında 0.4 - 0.8 ml/dk aralığında çalışmalar yapılmış ve 0.6 ml/dk olarak en iyi ayırım koşulu belirlenmiştir. LOD ve LOQ değerleri her bir florokinolon için sırasıyla 0.15 ve 0.5 µg/ml olarak saptanmıştır. Tutarlılık çalışmalarında iki farklı analizci tarafından elde edilen değerler karşılaştırıldığında anlamlı bir farklılık bulunmamıştır ($p<0.05$) (Tablo 5).

Tablo 5. Geliştirilen yöntemin tutarlılık verileri (n=6)

Florokinolon	1 µg/ml standart madde için eğri altı alan/iç standart eğri altı alan		
	Analizci 1	Analizci 2	P<0.05
	$\bar{x}$± SH	$\bar{x}$± SH	
SF	0.827±0.007	0.819±0.008	0.046
LF	0.457±0.005	0.454±0.006	0.014
EF	0.902±0.006	0.893±0.001	0.045
MF	1.797±0.013	1.713±0.011	0.033

$\bar{x}$: Ortalama, SH: Standart hata, p: Kesişimin olasılık değeri

Bu çalışmada, farklı nesil florokinolonlardan olan siprofloksasin, levofloksasin, enrofloksasin ve moksifloksasinin HPLC-DAD sisteminde eş zamanlı analizine olanak sağlayan yeni kolay, basit, seçici ve valide bir analiz yöntemi geliştirilmiştir. Geliştirilen yöntem literatürde yer alan HPLC-DAD yöntemlerinden farklı ve kısa sürede ayırım gerçekleştirebilmesine olanak sağlaması açısından bir alternatif sunmaktadır. Geliştirilen analiz yöntemi ilaç düzeyi takibinde ve değişik uygulamalarda yeni ve basit bir ayırım yöntemi olarak uygulanabilir.

YAZAR KATKILARI

Kavram: A.D., E.D.G.T., F.O.; Tasarım: A.D., E.D.G.T., F.O.; Denetim: A.D., E.D.G.T., F.O.; Kaynaklar: A.D., E.D.G.T., F.O.; Malzemeler: A.D., E.D.G.T., F.O.; Veri Toplama ve/veya İşleme: A.D., E.D.G.T., F.O.; Analiz ve/veya Yorumlama: A.D., E.D.G.T., F.O.; Literatür Taraması: A.D., E.D.G.T., F.O.; Makalenin Yazılması: A.D., E.D.G.T., F.O.; Kritik İnceleme: A.D., E.D.G.T., F.O.; 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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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 994-1002, 2023
Doi: 10.33483/jfpau.1344356

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ASSESSMENT OF AWARENESS AND BEHAVIORAL HABITS TO REDUCE DIETARY EXPOSURE TO MYCOTOXINS

MİKOTOKSİNLERE DİYETLE MARUZİYETİ AZALTMAK İÇİN FARKINDALIK DÜZEYİ VE DAVRANIŞSAL ALIŞKANLIKLARININ DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *Mycotoxins are known as secondary fungal metabolites that cause biochemical, physiological, and/or pathological changes in many species, including animals and plants. According to the Food and Agriculture Organization (FAO), these substances contaminate about 25% of all food in the world. Mycotoxins are responsible for many different disorders affecting the gastrointestinal, urogenital, vascular, renal, and nervous systems, as well as cancers. Key strategies for preventing and controlling exposure to mycotoxins include controlling toxin formation, implementing surveillance and monitoring programs to prevent human exposure, detoxifying the mycotoxins through biological, chemical, and physical means, and promoting dietary diversification. Although exposure cannot be avoided entirely, implementing monitoring programs is crucial to minimize it. In general, prevention of exposure to mycotoxins should receive greater emphasis. This study aims to provide information about mycotoxins and raise awareness about mycotoxin exposure.*

Material and Method: *This cross-sectional survey study was conducted with voluntary participants in Turkey between January 25, 2023-July 25, 2023, via electronic questionnaire. The questionnaire included socio-demographic data, income status, general dietary habits, frequency of consumption of mycotoxin-rich foods, knowledge about mycotoxins, and implementation of measures to prevent mycotoxin exposure.*

Result and Discussion: *A total of 796 participants (52.6% female, 46.7% male, and 0.6% other) were included in the study. This study concludes that the participants considered both the price and quality of food to be important factors. The study indicates that a high percentage of participants are unaware of the concept of mycotoxin and the associated health risk. A statistically significant change was found between whether the participants were knowledgeable about nutrition and whether they knew the concept of mycotoxin ($p<0.05$). There was a statistically significant difference*

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Submitted / Gönderilme : 17.08.2023
Accepted / Kabul : 27.08.2023
Published / Yayınlanma : 20.09.2023

in participants' knowledge of healthy nutrition and knowledge of the health hazards of mycotoxins. ($p<0.05$). The results indicate a requirement for comprehensive management against mycotoxins and mycotoxin exposure, which can cause several health problems in humans.
Keywords: *Moldy foods, mycotoxins, mycotoxin exposure*

ÖZ

Amaç: *Mikotoksinler, hayvanlar ve bitkiler de dahil olmak üzere birçok türde biyokimyasal, fizyolojik ve/veya patolojik değişikliklere neden olan ikincil mantar metabolitleri olarak bilinmektedir. Gıda ve Tarım Örgütü'ne (FAO) göre, bu maddeler dünyadaki tüm gıdaların yaklaşık %25'ini kirletmektedir. Mikotoksinler gastrointestinal, ürogenital, vasküler, renal ve sinir sistemlerini etkileyen birçok farklı rahatsızlığın yanı sıra kanserlerden de sorumludur. Mikotoksinlere maruz kalmanın önlenmesi ve kontrol altına alınmasına yönelik temel stratejiler arasında toksin oluşumunun kontrol altına alınması, insanların maruz kalmasını önlemek için gözetim ve izleme programlarının uygulanması, mikotoksinlerin biyolojik, kimyasal ve fiziksel yollarla detoksifiye edilmesi ve diyet çeşitliliğinin teşvik edilmesi yer almaktadır. Maruziyet tamamen önlenemese de, izleme programlarının uygulanması maruziyeti en aza indirmek için çok önemlidir. Genel olarak, mikotoksinlere maruz kalmanın önlenmesine daha fazla önem verilmelidir. Bu çalışma, mikotoksinler hakkında bilgi vermeyi ve mikotoksin maruziyeti konusunda farkındalık yaratmayı amaçlamaktadır.*
Gereç ve Yöntem: *Bu kesitsel anket çalışması, 25 Ocak 2023-25 Temmuz 2023 tarihleri arasında Türkiye'deki gönüllü katılımcılarla elektronik anket yoluyla gerçekleştirilmiştir. Anket sosyo-demografik veriler, gelir durumu, genel beslenme alışkanlıkları, mikotoksin bakımından zengin gıdaların tüketim sıklığı, mikotoksinler hakkında bilgi ve mikotoksin maruziyetini önlemeye yönelik tedbirlerin uygulanmasını içermektedir.*
Sonuç ve Tartışma: *796 katılımcı (%52,6 kadın, %46,7 erkek ve %0,6 diğer) çalışmaya dahil edilmiştir. Bu çalışma, katılımcıların gıdanın hem fiyatını hem de kalitesini önemli faktörler olarak gördükleri sonucuna varmıştır. Çalışma, katılımcıların yüksek bir yüzdesinin mikotoksin kavramından ve buna bağlı sağlık riskinden habersiz olduğunu göstermektedir. Katılımcıların beslenme konusunda bilgili olup olmamaları ile mikotoksin kavramını bilip bilmemeleri arasında istatistiksel olarak anlamlı bir değişim bulunmuştur ($p<0.05$). Katılımcıların sağlıklı beslenme bilgisi ile mikotoksinlerin sağlığa zararları bilgisi arasında istatistiksel olarak anlamlı bir fark bulunmuştur. ($p<0.05$). Sonuçlar, insanlarda çeşitli sağlık sorunlarına neden olabilen mikotoksinlere ve mikotoksin maruziyetine karşı kapsamlı bir yönetimin gerekliliğine işaret etmektedir.*
Anahtar Kelimeler: *Küflü gıdalar, mikotoksinler, mikotoksin maruziyeti*

INTRODUCTION

Filamentous fungi synthesize mycotoxins as secondary metabolites. Mycotoxins are chemically diverse and can cause a variety of toxic effects in humans [1]. The term "mycotoxicosis" was first employed in 1952 in a study on animal diseases. The discovery of aflatoxins in the UK in 1960, following the death of 100,000 turkey poults, marked the beginning of modern mycotoxin research [2]. The term mycotoxin typically refers to the secondary metabolites of fungi, such as Aspergillus, Penicillium, Fusarium, and Clariceps, which parasitize or feed on our food crops or livestock feed [3].

These are molecules with variable structures, ranging from small heterocyclic rings that weigh up to 50 Da to larger structures, consisting of irregularly arranged 6-8 heterocyclic rings with a combined molecular weight exceeding 500 Da. [4]. Mycotoxins are found in a variety of products ranging from raw agricultural products such as corn, barley, oats, fruits, and grasses to commercial products such as aquaculture, beverages, fruit and vegetable-derived products [5].

Toxicity typically arises following ingestion, although it can also result from exposure through dermal contact or inhalation, which may lead to adverse effects. The sources and origins of fungi that produce mycotoxins are varied and, in most cases, still under investigation [6]. Mycotoxins' effects on human health depend on various factors, including their type, concentration, conjugation forms, duration of exposure, pharmacokinetics, and accumulation, ams well as the age, gender, immune system, and health status of the individual exposed to them [7]. Mycotoxins are responsible for many different

disorders affecting the gastrointestinal, urogenital, vascular, renal, and nervous systems as well as cancers [8]. Some mycotoxins weaken immunity and are therefore thought to reduce resistance to infectious diseases [9]. Exposure to mycotoxins can occur directly through the consumption of infected food and feed; humans can also be indirectly affected by consuming animals fed infected feed [10]. These toxic substances can be present in various food sources and have diverse chemical structures and various molecular mechanisms of action that impact multiple organs and systems [11]. As some fungi can generate different mycotoxins and crops can be contaminated with multiple fungal species simultaneously, several mycotoxins can often co-occur in food products. Mycotoxins that contaminate food products together can exhibit synergistic or antagonistic activities [12]. Several critical strategies for preventing and controlling exposure to mycotoxins include controlling toxin formation, implementing surveillance and monitoring programs to avoid human exposure, detoxifying through biological, chemical, and physical processes, and promoting dietary diversification.

MATERIAL AND METHOD

Questionnaire

This study was conducted between January to June of 2023 using a web-based data collection form to collect data after obtaining written informed consent from people in Turkey. Approval for the study was obtained from the Non-Interventional Clinical Research Ethics Committee of Çukurova University Non-Interventional Clinical Studies Ethics Committee (Decision Number: 68 dated 6th January 2023).

In this study, a questionnaire was created to assess the level of knowledge of mycotoxins and their exposure among participants. The questionnaire started with a section for voluntary information before moving on to the questions. The survey included questions on socio-demographic data, income status, general dietary habits, frequency of consumption of mycotoxin-rich foods, knowledge about mycotoxins, and measures taken to prevent mycotoxin exposure.

Statistical Analysis

Data evaluation was conducted using SPSS 20.0. Descriptive analysis involved the use of frequency, percentage, mean, and standard deviation values. We analyzed the data for normality and then used t-tests for independent groups in comparisons of two groups, and One-Way ANOVA for comparisons of more than two groups. We also conducted cross-tabulations and chi-square statistics. We considered *p*-values of 0.05 or less as statistically significant in all tests.

RESULT AND DISCUSSION

Demographic Data

The sample of the study consisted of 796 volunteer participants. Table 1 displays the sociodemographic characteristics of the participants.

The average age of the individuals who participated in the study was 28.51±2.67. 52.6% of the participants were female and 46.7% were male. A large number of the individuals participating in the survey (18.7%) stated that their economic income was between 10 thousand and 15 thousand TL. The educational status of the participants was distributed as primary school (1.8%) secondary school (2.4%) and high school (16.5%) university (70.5%) master's degree (6.5%) doctorate (2%).

Descriptive Statistical Analysis of Participants' Dietary Habits

When the diet types of the participants were analyzed, it was observed that the rate of omnivorous was (95%) (Table 2).

The places where participants prefer to eat were questioned. It is observed that the majority of individuals (85.9%) prefer to eat at home (Table 3).

Participants were asked whether they considered themselves to have sufficient knowledge about healthy nutrition. 57.4% of the participants stated that they did not have enough knowledge about nutrition and 33.7% stated that they had enough knowledge.

The participants' opinions on the quality and price of food were evaluated. It can be concluded that for the majority of participants (58.3%), the quality of food (45.2%) and the price of food (45.2%) are very important.

Table 1. Demographic data

		Frequency (n)	Percentage (%)
Gender			
	Female	419	52.6
	Male	372	46.7
	Participants who do not specify gender	5	.6
Education Status			
	No formal education	3	.4
	Primary School	14	1.8
	Middle School	19	2.4
	High School	131	16.5
	University	561	70.5
	Master's Degree	52	6.5
	PhD	16	2.0
Monthly income	No monthly income	191	24.0
	0 - 2.000 TL	99	12.4
	2.000-5.500 TL	93	11.7
	5.500 - 10.000 TL	120	15.1
	10.000 - 15.000 TL	149	18.7
	15.000-20.000 TL	58	7.3
	20.000- 25.000 TL	19	2.4
	25.000 - 30.000 TL	12	1.5
	30.000-35.000 TL	2	.3
	35.000TL and above	18	2.3
	Not want to specify	35	4.4

Table 2. Diet types

What type of diet do you prefer?				
	Frequency	**Percent**	**Percent**	**Cumulative Percent**
Vegan	20	2.5	2.5	2.5
Vegetarian	17	2.1	2.1	4.6
Omnivorous	759	95.4	95.4	100.0

Table 3. Dining venue preferences

		Frequency (n)	Percentage (%)
Dining venue preferences	Home	684	85.9
	Cafe/Restaurant	112	14.1

The frequency with which participants consume different foods was analyzed. Spices (57.9%) and coffee (46.5%) have the highest daily consumption rates. These were followed by bakery products, fruit, and dairy products (Figure 1).

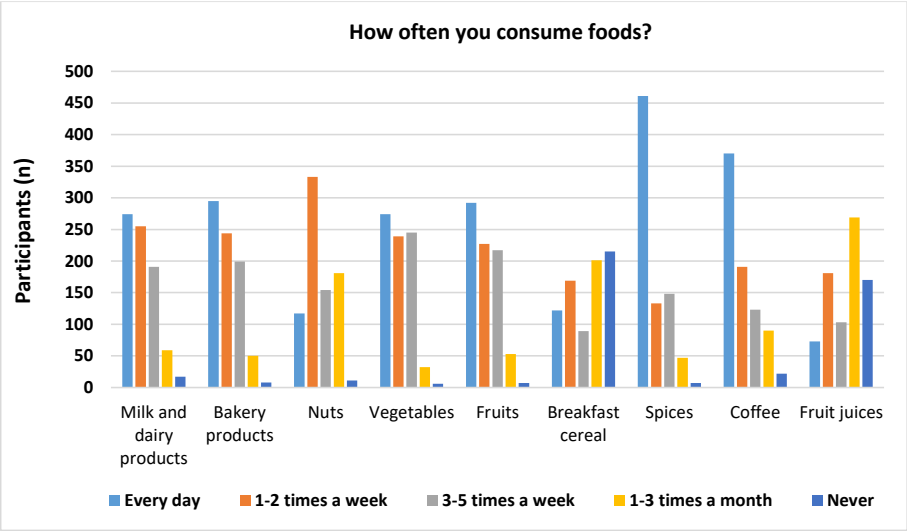

Figure 1. Daily consumption rates of foods

Descriptive Statistical Analysis of Participants' Mycotoxin Awareness and Healthy Consumption of Foods

In this study, we aimed to measure the mycotoxin awareness of the participants and the questions they were asked and the response patterns are given in Table 4.

Table 4. Descriptive statistics

		Frequency (n)	Percentage (%)
I have heard of mycotoxin before	Yes	210	26.4
	No	586	73.6
I know the health hazards of mycotoxins	Yes	135	17.0
	No	541	68.0
	Partily	120	15.1
I buy food as fresh as possible and consume it in a short time	Strongly disagree	45	5.7
	Disagree	61	77
	Partially agree	65	8.2
	I agree	312	39.2
	Completely agree	313	39.3
I store food in a clean, dry, and cool place	Strongly disagree	37	4.6
	Disagree	64	8.0
	Partially agree	42	5.3
	I agree	315	39.6
	Completely agree	338	42.5
I clean areas such as the refrigerator and pantry once a week	Strongly disagree	53	6.7
	Disagree	122	15.3
	Partially agree	189	23.7
	I agree	249	31.3
	Completely agree	183	23.0
I throw away moldy food immediately	Strongly disagree	37	4.6
	Disagree	72	9.0
	Partially agree	60	7.5
	I agree	188	23.6
	Completely agree	439	55.2

Table 4 (*continue*). Descriptive statistics

		Frequency (n)	Percentage (%)
I do not consume spoiled milk and dairy products	Strongly disagree	44	5.5
	Disagree	56	7.0
	Partially agree	21	2.6
	I agree	155	19.5
	Completely agree	520	65.3
I store cereals and flour in a dry place	Strongly disagree	44	5.5
	Disagree	55	6.9
	Partially agree	38	4.8
	I agree	225	28.3
	Completely agree	434	54.5
I buy fruit and vegetables that are as intact as possible, not injured or dented	Strongly disagree	38	4.8
	Disagree	70	8.8
	Partially agree	38	4.8
	I agree	221	27.8
	completely agree	429	53.9
I don't eat rotting fruit, I don't make compotes and jams	Strongly disagree	51	6.4
	Disagree	86	10.8
	Partially agree	79	9.9
	I agree	201	25.3
	Completely agree	379	47.6
Even if there is mold on a piece of bread, I throw it away whole	Strongly disagree	74	9.3
	Disagree	109	13.7
	Partially agree	97	12.2
	I agree	196	24.6
	Completely agree	320	40.2
I throw away meat or sausage when it gets moldy	Strongly disagree	39	4.9
	Disagree	68	8.5
	Partially agree	32	4.0
	I agree	177	22.2
	Completely agree	480	60.3
I don't consume moldy nuts, I throw them away when they are moldy	Strongly disagree	38	4.8
	Disagree	63	7.9
	Partially agree	31	3.9
	I agree	184	23.1
	Completely agree	480	60.3
I buy spices in small quantities and consume them quickly	Strongly disagree	73	9.2
	Disagree	114	14.3
	Partially agree	145	18.2
	I agree	230	28.9
	Completely agree	234	29.4
I don't give moldy food to animals	Strongly disagree	68	8.5
	Disagree	84	10.6
	Partially agree	62	7.8
	I agree	171	21.5
	Completely agree	411	51.6

A comparison of the responses of the participants to the questions in Table 4 with all of the responses. There is a statistically significant difference ($p<0.05$) in whether or not participants consume

moldy foods depending on their gender. Female participants use moldy foods 372 (46.7%) less than male individuals 419 (52.6%).

The analysis was performed on the consumption of moldy foods according to the diet types of the participants. There was a statistically significant difference ($p<0.05$) in the consumption of moldy foods according to the participants' diet types. Individuals with vegetarian and vegan diets consumed significantly less moldy foods than those with omnivorous diets. A statistically significant difference was found between whether the participants were knowledgeable about healthy eating and whether they knew the concept of mycotoxins ($p<0.05$). Participants who were knowledgeable about healthy eating had a higher rate of having heard of the concept of mycotoxins.

A statistically significant difference ($p<0.05$) has been found between the answers of the participants regarding the consumption of moldy food and their knowledge about healthy nutrition. 29.9% of the participants who think that they are knowledgeable about nutrition do not consume food that has started to turn moldy. Among the participants who did not think they were knowledgeable about nutrition, 50.3% did not consume food that had started to turn moldy.

The answers of the participants to the question "I do not eat rotten fruits and do not make jam from them" have a statistically significant change according to their monthly income level ($p<0.05$). While 45.1% of the participants with an income level of 20 thousand TL and below answered that they completely agree with the question, 2.5% of the participants with an income level of 20 thousand TL and above answered that they completely agree with the question. There is a statistically significant difference ($p<0.05$) between whether or not participants are aware that moldy food can pose a risk to their health, depending on how much importance participants attach to food quality. It can be seen that participants who are aware of the risk also attach importance to quality. A comparative analysis of the income level of the participants and their knowledge of the health hazards of mycotoxins was performed. No statistically significant change was found ($p>0.05$).

An estimated 500 million people, mostly living in rural areas, are exposed to precarious levels of mycotoxins, according to the World Health Organization. Several factors promote frequent contamination of food crops by mycotoxigenic fungi, leading to the alarming statistic mentioned above. These factors include climatic conditions, poor agricultural practices, poverty, inadequate knowledge about mycotoxins among primary food producers, and a lack of mycotoxin regulations [13].

The study conducted in two north-central Nigerian states reports a comprehensive overview of multiple mycotoxin contamination of various foods consumed by households, including cereals, nuts, and legumes. At least 80% of the respondents in each state (Nasarawa: 84%; Niger: 80%) indicated the ability to identify molds in foods and stored grains by discoloration of food items. However, only 43% and 15% of the respondents from Nasarawa and Niger states, respectively, were aware of what mycotoxins are, and only 26% and 11% of respondents, respectively, were aware of possible food handling practices to reduce mycotoxin contamination in food [14]. In our study, the frequency of those who consumed foods that started to mold was 2.8% and the frequency of those who had heard of mycotoxins was 26.4%. In addition, the frequency of people who were aware of the health hazards of mycotoxins was determined as 68%.

A study conducted in southwestern Nigeria assessed the perceived attitudes, practices, and knowledge of fermented food vendors regarding fungal colonization of foodstuffs, a precursor to mycotoxins. The result shows a wide knowledge gap among those surveyed ($n = 86$), as 98% were unable to link fungi to mycotoxin contamination and perceived associated health risks. A significant number of people in both developed and developing countries are poorly informed about contaminants in food. The majority of participants (93%) were women. Few participants had no formal education (11%), while most of those who did have a primary education (61%). Mycotoxins are at the forefront of chronic food toxicants, usually occurring below levels that cause acute health effects, but such levels can cause long-term health effects among humans and animals. It can therefore be difficult to link various health complications to mycotoxin exposure, which strongly supports the poor perception of respondents in the Nigerian study on the issue. That study provided insights into the safety of fermented foods produced in Nigeria and likewise raised vendors' awareness of fungal and mycotoxin contamination and associated health risks. It was observed that there was a wide knowledge gap among the respondents regarding this aspect of food safety [15]. In our study, there was a wide knowledge gap

among the respondents, as 26.4% had heard of mycotoxins, 46.7% of the respondents in our study were male and 52.6% were female. Very few of the participants in our study had no formal education (0.4), whereas most of those who had education had a university education (70.5%). Our study provided us with information about mycotoxin exposure and awareness among people living in Turkey.

A study conducted in Belgium showed that around 70% of the participants believed mycotoxins have the potential to cause toxicity in humans or animals. Consumers, in general, lack knowledge about mycotoxins as biohazards [16].

In a study conducted in Germany, an online questionnaire was administered to a cohort of university students (n=186) to investigate knowledge about mycotoxins and adherence to behavioral practices or habits that may influence the risk of mycotoxin exposure. The results of the study indicate an overall rather low level of knowledge about mycotoxins in the studied cohort and a poor perception of the risks associated with them compared to similar studies; about half of the group was not familiar with the term "mycotoxin". In the German study, 54% of respondents indicated familiarity with mold toxins and 48% of all respondents answered yes to the question "Have you ever heard the term mycotoxin?". In general, the health risks of mycotoxins ranged from "quite risky" to "extremely risky". Similarly, 55% of respondents indicated that they were "concerned" or "very concerned" about the presence of mycotoxins in food. Following an intervention text, 64% of respondents answered that they were already aware of the health hazards caused by mold toxins in food. Consequently, mycotoxin prevention strategies should not stop at the retail level; in particular, disclosure and information on health risks from mycotoxins are recommended to reduce the risk of exposure in private households or informal trade markets [17]. In our study, an electronic questionnaire was administered to 796 volunteers. As a result of the survey, the prevalence of those who did not consume moldy foods was 87.1%. The frequency of people who thought they were knowledgeable about nutrition was 33.7%. There was a statistically significant difference between the participants' knowledge of nutrition and their knowledge of mycotoxin ($p=0.000$). As a result of our study, it is concluded that people have not heard of the concept of mycotoxin before and do not know the health hazards of mycotoxins.

The variation in consumer food safety knowledge and practices across demographic categories and possibly socioeconomic, educational, and cultural levels [18] could account for the differences in knowledge and awareness levels observed between our study group and previously published studies.

In conclusion, as a result of this study, it is concluded that volunteer individuals have not heard of the concept of mycotoxin before and do not know the health hazards of mycotoxins sufficiently.

It is an undeniable fact that individuals attach importance to both the quality and price of food.

According to the gender of the participants, whether they consume moldy foods or not varies statistically significantly ($p<0.05$).

A statistically significant change was found between whether the participants were knowledgeable about nutrition and whether they knew the concept of mycotoxin ($p<0.05$). According to these results, there is a need for comprehensive management against mycotoxins and mycotoxin exposure, which pose many problems for human health. Therefore, necessary plans should be made by the relevant organizations to raise awareness about mycotoxin hazards and exposure.

AUTHOR CONTRIBUTIONS

Concept: G.D.; Design: G.D.; Control: G.D.; Sources: G.D., N.N.D.; Materials: G.D., N.N.D.; Data Collection and/or Processing: G.D., N.N.D.; Analysis and/or Interpretation: G.D., N.N.D.; Literature Review: N.N.D.; Manuscript Writing: G.D.; Critical Review: G.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

All procedure was approved by the Non-Interventional Clinical Research Ethics Committee of Çukurova University Non-Interventional Clinical Studies Ethics Committee (Decision Number: 68

dated 6th January 2023).

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 1003-1010, 2023
Doi: 10.33483/jfpau.1344014

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ELECTROCHEMICAL INVESTIGATION OF OTILONIUM BROMIDE USING BORON-DOPED DIAMOND AND GLASSY CARBON ELECTRODES

CAMSI KARBON VE BOR KATKILI ELMAS ELEKTROTLAR KULLANARAK OTİLONYUM BROMÜRÜN ELEKTROKİMYASAL DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *Using cyclic (CV) and differential pulse (DPV) voltammetric techniques, the electrochemical research of otilonium bromide (OTB) was carried out over a wide pH range (0.3–12) at glassy carbon electrodes (GCE) and boron-doped diamond electrodes (BDDE). The typical electrochemical behavior of OTB was identified as being dependent on the type of working electrode and pH. This research aims to provide a brand-new electroanalytical technique for measuring OTB in buffer solutions.*

Material and Method: *All experiments employed the typical three-electrode cell of 10 ml capacity in conjunction with a platinum wire counter electrode, a BDDE and GCE working electrode, and an Ag/AgCl reference electrode. NOVA 1.8 software and an AUTOLAB 204 potentiostat/galvanostat were used for electrochemical measurements.*

Result and Discussion: *The electrochemical behavior of OTB, which belongs to a class of drugs called 'antispasmodics' (spasm and cramps reliever), primarily used to treat irritable bowel syndrome (IBS), and other gastrointestinal conditions characterized by motility problems, painful bowel spasms and distension (swelling and bloating in the belly area), was examined in 0.1 M H_2SO_4*

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Submitted / Gönderilme : 15.08.2023
Accepted / Kabul : 28.08.2023
Published / Yayınlanma : 20.09.2023

at BDDE and GCE. The electrooxidation mechanism was also investigated by conducting CV investigations at various pH levels throughout a broad pH range (pH 0.3-12.0). Understanding the mechanism was aided by scan rate investigations, which revealed that diffusion was controlled for both electrodes. The proposed technique was successfully used to determine OTB under optimal conditions.
Keywords: *Boron-doped diamond electrode, differential pulse voltammetry, drug assay, glassy carbon electrode, Otilonium bromide*

ÖZ

Amaç: *Döngüsel (CV) ve diferansiyel darbe (DPV) voltametrik teknikler kullanılarak, otilonyum bromürün (OTB) elektrokimyasal araştırması, bor katkılı elmas elektrotlarda (BDDE) ve camsı karbon elektrotlarda (GCE) geniş bir pH aralığında (0.3–12) gerçekleştirildi. OTB'nin tipik elektrokimyasal davranışının, çalışan elektrot tipine ve pH'a bağlı olduğu tespit edildi. Bu araştırmanın amacı, tampon çözeltilerde OTB'yi ölçmek için yepyeni bir elektroanalitik teknik sağlamaktır.*
Gereç ve Yöntem: *10 ml kapasiteli tipik tek bölmeli üç elektrotlu hücre, tüm çalışmalarda bir BDDE ve GCE çalışma elektrotu, bir platin tel karşıt elektrot ve bir Ag/AgCl referans elektrotu ile birlikte kullanıldı. Elektrokimyasal ölçümleri gerçekleştirmek için NOVA 1.8 yazılımı ve bir AUTOLAB 204 potansiyostat/galvanostat kullanıldı.*
Sonuç ve Tartışma: *"Antispazmodikler" (spazm ve kramp giderici) adı verilen bir ilaç sınıfına ait olan OTB'nin elektrokimyasal davranışı, öncelikle hassas bağırsak sendromunu (IBS) ve hareketlilik sorunları, ağrılı bağırsak spazmları ve şişkinliği (şişme) ile karakterize edilen diğer gastrointestinal rahatsızlıkları tedavi etmek için kullanılır. ve göbek bölgesinde şişkinlik), GCE ve BDDE'de 0.1 M H_2SO_4'te incelenmiştir. Geniş bir pH aralığı (pH 0.3-12.0) boyunca çeşitli pH seviyelerinde CV araştırmaları yapılarak elektrooksidasyon mekanizması da araştırıldı. Mekanizmanın anlaşılması, difüzyonun her iki elektrot için de kontrol edildiğini ortaya koyan tarama hızı araştırmalarıyla desteklendi. Önerilen yöntem, optimal koşullar altında OTB'yi belirlemek için başarıyla kullanılmıştır.*
Anahtar Kelimeler: *Bor katkılı elmas elektrot, camsı karbon elektrot, diferansiyel puls voltammetrisi, ilaç analizi, Otilonyum bromür*

INTRODUCTION

Otilonium bromide (OTB; Figure 1) is an antispasmodic medicament used to treat Irritable Bowel Syndrome symptoms. The smooth muscles of the stomach and intestine contractions are relieved with OTB. The medicament relieves painful muscle spasms by relaxing the muscles in the intestine's walls. As a result, it lessens bloating, discomfort, and pain in the stomach caused by spasms, cramps, and gas development [1-4].

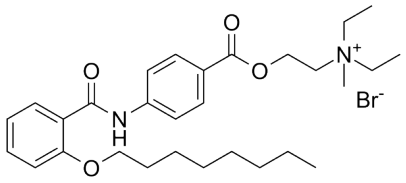

Figure 1. Structure of Otilonium Bromide (OTB)

In the 19th century, two main branches of natural sciences - chemistry and electrical science - merged to form electrochemical science. Examining pharmaceuticals can be done using an intriguing approach called electrochemical sensing. Electrochemical sensing techniques can be used for various purposes, including the quick and simultaneous examination of numerous samples with high sensitivity and low detection limits. All these investigations require extremely affordable devices. Thus, we have chosen electrochemical techniques over others because of their low cost. Other approaches include the use of many organic solvents and labor-intensive steps. In drug analysis, electrochemical techniques are

frequently employed. Voltammetric methods are popular because of their rapidness, sensitivity, and the fact that they only require simple equipment. This study employed differential pulse voltammetry (DPV), a method that is often utilized [5-9].

The glassy carbon electrode (GCE) is commonly considered for voltammetry and numerous applications in drug analysis [10]. The boron-doped diamond electrode (BDDE) has been of great interest as the electrode for different electroanalytical applications. It has advantages over conventionally used electrodes [11,12], such as its low background currents, large potential window (up to 3 V), thermal shock resistance, corrosion resistance towards aggressive media, and extreme electrochemical stability [5-9].

This study aimed to create novel pulse voltammetric methods for directly determining OTB using contemporary drug assay methods. Using CV and DPV, this work also aims to investigate the precise voltammetric oxidation mechanism of the OTB on BDDE and GCE.

MATERIAL AND METHOD

Apparatus

NOVA 1.8 software and an AUTOLAB 204 (Eco Chemie, Utrecht, and The Netherlands) potentiostat/galvanostat were used to conduct the electrochemical measurements. The typical three-electrode cell of 10 ml capacity was employed in conjunction with an Ag/AgCl reference electrode, a BDDE and GCE working electrode, a Pt wire counter electrode, and in all studies. Before each measurement, the BDDE or GCE was manually polished on a smooth polishing pad with slurries prepared from 0.01 m aluminum oxide, and it was then thoroughly cleaned with double-distilled water.

Chemicals

The following chemicals were purchased from Sigma-Aldrich: sodium phosphate monobasic, sulfuric acid, acetic acid, methanol, phosphoric acid, sodium hydroxide, sodium dihydrogen phosphate dihydrate, sodium acetate trihydrate, sodium phosphate, and acetonitrile. All the reagents were of analytical grade and utilized. All experiments were performed at room temperature; to prevent deterioration, all solutions were shielded from light and employed within 24 hours. The OTB utilized in the electrochemical studies was given by DEVA Holding AS. Istanbul/Turkey Analytical grade chemicals were employed to make solutions at all stages of the study, and they weren't further purified before use.

Electrochemical Study

For drawing the calibration graph of OTB using 0.1 M H_2SO_4 solutions (pH 0.3), the DPV technique was applied with the optimum parameters of scan rate 0.010071 mVs^{-1}, interval time 0.5 s, step potential of 0.005 V, modulation amplitude of 0.05 V, modulation time 0.05 s. CV was performed at a scan rate of 0.05 Vs^{-1}. All the electrochemical studies were applied at room temperature.

Preparation of Standard and Calibration Solutions

For electrochemical experiments, various supporting electrolytes, including buffers of phosphate (pH 2.0 – 8.0), acetate (pH 3.7 – 5.7), H_2SO_4 solutions (0.1 and 0.5 M), and Britton Robinson buffers (pH 2.0 – 12.0), were created. Recordings of DPV voltammograms were made after each aliquot was added. Working solutions of OTB for the voltammetric studies were made from the stock solution (1 x 10^{-2} M) in bidistilled water by diluting it with the chosen supporting electrolyte.

RESULT AND DISCUSSION

At the GCE and BDDE, the CV approach was used to learn more about the extensive electrochemical analysis of OTB. The analysis of OTB for both GCE and BDDE used the DPV approach. To the best of our knowledge, there hasn't been any published research on OTB's electrochemical determination in the literature.

In our investigation, bare GCE and BDDE were used to conduct the first electrochemical

examination and OTB determination. The influence of pH values and supporting electrolytes on the electrochemical current peaks of OTB were studied using DPV and CV in the pH range of 0.30 to 12.00 utilizing GCE and BDDE in order to determine the most appropriate experimental settings.
On GCE (Figure 2A) and BDDE (Figure 2B), the maximum voltammetric peak for 1 x 10^{-3} M OTB was obtained using 0.1 M H_2SO_4 as the supporting electrolyte. Between pH 0.30 and 12.00, it was oxidized on both electrodes, yielding two distinct, irreversible oxidation peaks for GCE and BDDE. For GCE and BDDE, respectively, main peak potential values of roughly +1.00 V and +1.50 V were noted. This distinction clearly showed that OTB is simply oxidized on the GCE in comparison with the BDDE. The absence of a cathodic reaction at either electrode during reverse scanning revealed that the electrooxidation of OTB is irreversible.

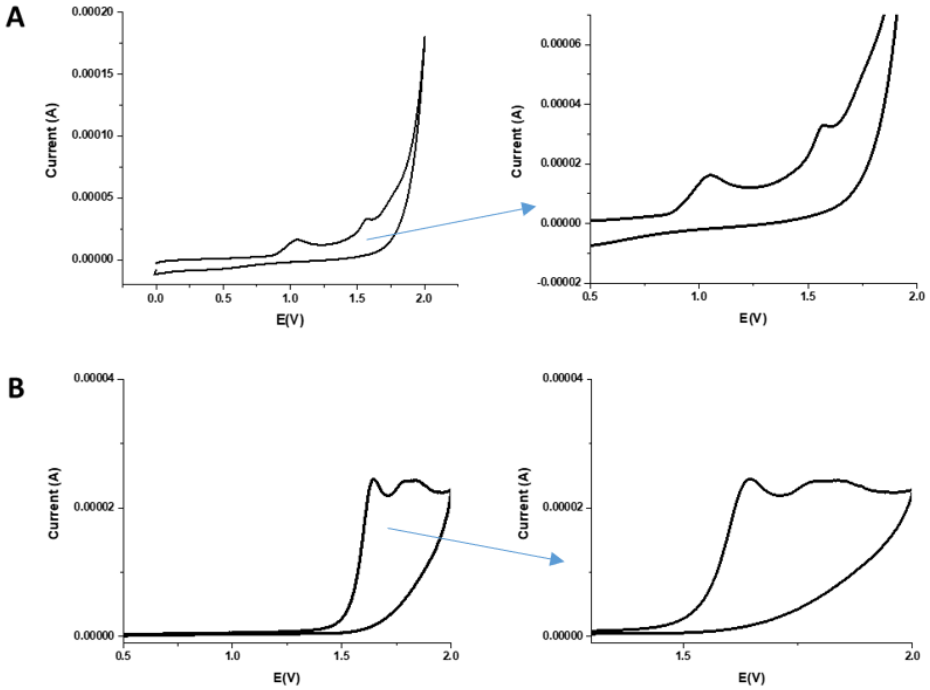

Figure 2. Cyclic voltammograms of 1x10^{-3} M OTB with GCE (A) and BDDE (B) in 0.1M H_2SO_4 at scan rate 0.5 V/s

To obtain distinctive information on the voltammetric behavior of OTB, the DPV method was applied by GCE and BDDE. It was observed that the Ep values of OTB for both electrodes shifted to less positive potential values. A higher peak value was observed for OTB using the BDDE compared to the GCE.

The effect of supporting electrolyte and pH value on the peak current and potential for 1 x 10^{-3} M OTB was examined for GCE and BDDE. The shifting of Ep values of OTB showed the presence of protons in the electrochemical oxidation procedure for GCE and BDDE. Using the DPV technique, the highest peak current was observed in the 0.1 M H_2SO_4 medium for both electrodes. The following equation indicates the influence of pH on the peak potential. For both electrodes, the dependency between Ep and pH can be stated with the following equations:

$$Ep\ (mV) = 986.27 - 19.721\ pH;\ r = 0.992 \text{ for GCE (pH 0.3 – 8.0)}$$

$$Ep\ (mV) = 1646.4 - 28.703\ pH;\ r = 0.964 \text{ for BDDE (pH 0.3 – 4.0)}$$

The slope values obtained from the above equation show that the number of electrons equals the

number of protons in the electrochemical oxidation process.

Scan Rate Study

The influence of scan rate works was performed to provide important knowledge on the electrochemical redox mechanism procedure and define whether the electrochemical process happened under the adsorption or diffusion control mechanism. The effect of the scan rate using CV between 0.01 and 1 V/s on the peak potential and current was examined in selected supporting electrolytes, where the maximum peak current was acquired in pH experiments with a GCE and BDDE.

The effect of scan rate (ν) on the Ip values of 1 x 10^{-3} M OTB has been investigated in 0.1 M H_2SO_4 for BDDE and GCE.

As illustrated in the following equations, the Ip was linear to the square root of scan rate ($\nu^{1/2}$) for BDDE and GCE in the between of 0.01 - 1.00 Vs^{-1}:

$$Ip\ (\mu A) = 0.5193\ \nu^{1/2}\ (Vs^{-1}) + 0.7304\ (r = 0.9656;\ n=10)\ \text{at GCE in 0.1 M } H_2SO_4$$

$$Ip\ (\mu A) = 2.5415\ \nu^{1/2}\ (Vs^{-1}) - 6.1322\ (r = 0.9763;\ n=10)\ \text{at BDDE in 0.1 M } H_2SO_4$$

According to the linear dependency of the Ip – $\nu^{1/2}$, the electrooxidation process of OTB is a diffusion-controlled process on GCE and BDDE.

The following equations showed a linear relationship between the logarithms of Ip (log Ip) and scan rate (log ν):

$$\log Ip\ (\mu A) = 0.562\ \log \nu\ \ (Vs^{-1}) - 0.3721\ (r = 0.9931;\ n = 10)\ \text{at GCE in 0.1 M } H_2SO_4$$

$$\log Ip\ (\mu A) = 0.501\ \log \nu\ (Vs^{-1}) + 0.1145\ (r = 0.9953;\ n = 10)\ \text{at BDDE in 0.1 M } H_2SO_4$$

The slope value of the linear relationship of the log Ip and the log ν was calculated as 0.562 and 0.501 for both electrodes in 0.1 M H_2SO_4, which is near the theoretical value of 0.50, obviously approving the diffusion-controlled mechanism.

For Ep, in 0.1 M H_2SO_4, at GCE and BDDE, the linear relationship of the Ep versus log ν was revealed as:

$$Ep\ (mV) = 0.0721\ \log \nu\ (Vs^{-1}) + 1.2474\ (r = 0.9952;\ n = 10)\ \text{at GCE in 0.1 M } H_2SO_4$$

$$Ep\ (mV) = 0.0374\ \log \nu\ (Vs^{-1}) + 1.7055\ (r = 0.9973;\ n = 10)\ \text{at BDDE in 0.1 M } H_2SO_4$$

When the scan rate was increased, the peak potential of the OTB's oxidation mechanism for GCE and BDDE was proven to be irreversible, and the peak potential values were moved to be more positive without the reverse reduction peak. The irreversible electrochemical mechanism shifts the Ep to around 30/n mV with greater positive potentials for a 10-fold improvement in scan rate. "α" is the anodic charge transfer coefficient, and "n" is the electron transfer number, respectively. Ep values were shifted to 72.1 and 37.4 mV in 0.1 M H_2SO_4 at GCE and BDDE, respectively. Since the value is typically regarded to be 0.5, the values of n = 2.40 (2) and 1.25 (1) for GCE and BDDE, respectively, were discovered.

Analytical Applications

The relationship between OTB peak current and concentration was studied to assess the analytical applications of OTB using GCE and BDDE. The electrochemical oxidation process of OTB was monitored as a diffusion control mechanism on both electrodes. The fast and sensitive electrochemical technique, DPV, was applied to determine OTB in 0.1 M H_2SO_4 on BDDE and GCE. Under the optimum conditions, the corresponding characteristics of the linear regression analysis are calculated in Table 1 for both electrodes [13,14]. Both electrodes' performance in various concentrations of OTB is given in Figure 3.

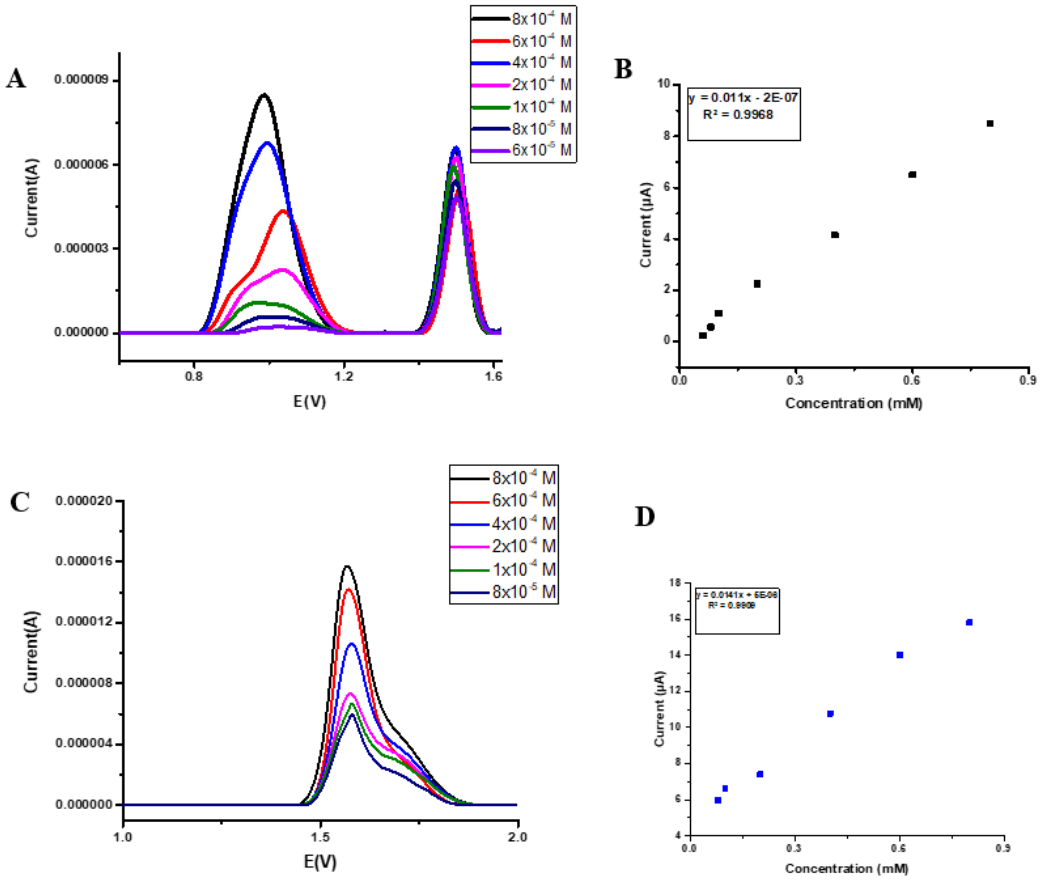

Figure 3. DP voltammograms of the developed sensor in different OTB concentrations in 0.1 M H_2SO_4 using A) GCE and B) BDDE

Based on the results of the experiments, GCE and BDDE were proposed for the detection of OTB using DPV under the optimum experimental conditions (step potential of 0.005 V, modulation amplitude of 0.05 V, modulation time 0.05 s, interval time 0.5 s, scan rate 0.01 mV s^{-1}, in 0.1 M H_2SO_4). As seen in Figure 3A, the anodic currents that appeared at 1022.2 mV were linearly increased in the range of concentrations from 6 x 10^{-5} – 8 x 10^{-4} M with a linear regression equation as follows:

$$Ip\ (\mu A) = 0.011\ C_{OTB}\ (\mu M) - 2 \times 10^{-7} (R^2 = 0.9968)$$ for GCE in 0.1 M H_2SO_4

As seen in Fig. 3B, the anodic currents that appeared at 1576 mV were linearly increased in the range of concentrations from 8x10^{-5} – 8x10^{-4} M with a linear regression equation as follows:

$$Ip\ (\mu A) = 0.0141\ C_{OTB}\ (\mu M) + 5 \times 10^{-6}\ (R^2 = 0.9909)$$ for BDDE in 0.1 M H_2SO_4

The other important validation data, including precision, LOD, LOQ, and linearity range, were figured out and are given in Table 1. The LOD and LOQ were figured out from the equation as follows:

LOD = 3.3 s/m

LOQ = 10 s/m

where "s" is the standard deviation of five experiments and "m" is the slope of the calibration graph. The LOD and LOQ were calculated to be 1.17 x 10^{-5} M and 3.86 x 10^{-6} M, 4.92 x 10^{-6} M, and 1.49x10^{-5} M, for GCE and BDDE, respectively, all cases in 0.1 M H_2SO_4. LOD results approved that the developed DPV technique using GCE is quite similar to the proposed DPV technique using BDDE.

The precision (reproducibility and repeatability) of the technique was assessed in terms of RSD% and connected results are given in Table 1. Repeatability RSD% results of peak current were found to

be 0.86 and 0.62, for GCE and BDDE, respectively, in 0.1 M H_2SO_4.

Table 1. Validation and regression information of the calibration graphs of Otilonium Bromide in 0.1 M H_2SO_4 with GCE and BDDE using DPV

	GCE	BDDE
Measured potential (V)	1.0222	1.5761
Linearity range (M)	$6x10^{-5} – 8x10^{-4}$	$8x10^{-5} – 8x10^{-4}$
Slope (µA M^{-1})	0.011	0.0141
Intercept (µA)	$2x10^{-7}$	$5x10^{-6}$
Correlation coefficient	0.9968	0.9909
LOD (M)	$1.17x10^{-5}$	$4.92x10^{-6}$
LOQ (M)	$3.86x10^{-6}$	$1.49x10^{-5}$
Repeatability of peak current (RSD %)*	0.86	0.62
Reproducibility of peak current (RSD %)*	1.19	0.95

*Each value is an average of five measurements

OTB has been assayed by various advanced analytical techniques such as high-performance liquid chromatography [15-17] , and capillary electrophoresis [18], and two methods based on derivative spectrophotometry are available [19,20], out of which one is estimation in combination with diazepam. In addition to this, an electroanalytical method is also not found in the literature review. Although chromatographic assay techniques allow simultaneous diagnosis of more than one analyte, they have some disadvantages compared to voltammetric techniques. It contains time-consuming sample preparation periods, undefined reaction time, is quite expensive, and less green assay. All these published methods are required highly sophisticated instrumentation. Otherwise, the electroanalytical techniques are low cost, sensitive, rapid analysis times, high accuracy and precision, selective, eco-friendly, practical methods.

The primary objective of this investigation has been to elucidate the electrochemical investigation and analysis of OTB using GCE and BDDE. This study demonstrated the proposed and validated DPV procedure as speedy, simple, accurate, and precise. It was performed directly as standard quality control for a tablet dosage form, eliminating the use of organic solvents or expensive devices. CV and DPV methods were developed to determine OTB with GCE and BDDE. The proposed method offers significant advantages such as low-cost, fast performance, and easy instrumentation is required.

ACKNOWLEDGEMENTS

The pharmaceutical firm DEVA Holding A.S. provided the information, for which the authors are grateful.

AUTHOR CONTRIBUTIONS

Concept: L.K., E.B.A., S.A.Ö.; Control: L.K., E.B.A., S.A.Ö.; Sources: L.K., E.B.A., S.A.Ö.; Data Collection and/or Processing: L.K., E.B.A., S.A.Ö.; Analysis and/or Interpretation: L.K., E.B.A., S.A.Ö.; Literature Review: L.K., E.B.A., S.A.Ö.; Manuscript Writing: L.K., E.B.A., S.A.Ö.; Critical Review: L.K., E.B.A., S.A.Ö.; 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., 47(3): 1011-1019, 2023
Doi: 10.33483/jfpau.1344300

ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARATIVE LEAF ANATOMY OF SOME *ASYNEUMA* GRISEB. & SCHENK TAXA

BAZI ASYNEUMA GRISEB. & SCHENK TAKSONLARININ KARŞILAŞTIRMALI YAPRAK ANATOMİSİ

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ABSTRACT

Objective: *The Asyneuma Griseb. & Schenk is represented by 34 accepted species worldwide and its native range covers a wide area from East Central Europe to Japan and Northern Indo-china. The plants of Asyneuma are characterized by herbaceous, simple or branched inflorescences, purple, violet or blue corolla and capsule fruits. Studies show that the Campanulaceae family contains bioactive compounds phenylethanoid (phenylproponoid), alkaloids, cyanogenetic heteroside, flavonoid, triterpene, anthocyanin, phenolic acid, essential oil, coumarin and polysaccharide. In addition, it is known that plants in the family have antioxidant, wound healing, anti-inflammatory, analgesic, antiobesity, expectorant antihepatotoxic, antitumoral, antiatherosclerotic, neuroprotective, antidepressant, tonic and α-glucosidase inhibitory activities. In this study, the anatomical structures of Asyneuma limonifolium subsp. limonifolium, A. limonifolium subsp. pestalozzae (Boiss.) Damboldt, A. linifolium subsp. linifolium and A. linifolium subsp. nallihanicum Kit Tan & Yıldız leaves were examined.*

Material and Method: *Asyneuma limonifolium subsp. limonifolium, A. limonifolium subsp. pestalozzae (Boiss.) Damboldt, A. linifolium subsp. nallihanicum Kit Tan & Yıldız were collected from Ankara. A. linifolium subsp. linifolium was collected from Antalya. Plant parts preserved in alcohol (70%). Microscopic sections were taken using a razor blade. Tissues were stained with Sartur's reagent and examined with a light microscope. Microphotographs were taken with a camera attached to a light microscope.*

Result and Discussion: *The results showed that, the anatomical structures of the basal and cauline leaves of Asyneuma limonifolium subsp. limonifolium and A. limonifolium subsp. pestalozzae were similar. The leaves are bifacial and the palisade parenchyma 1-2 rows. In addition, the unicellular,*

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Submitted / Gönderilme : 16.08.2023
Accepted / Kabul : 31.08.2023
Published / Yayınlanma : 20.09.2023

non-glandular hairs and anomocytic stomata were observed on both epidermal surfaces. Besides, the leaf of A. linifolium subsp. linifolium is monofacial while that of A. linifolium subsp. nallihanicum is bifacial. Also, unicellular hairs were present on epidermal layer of A. linifolium subsp. linifolium while A. linifolium subsp. nallihanicum were not. The anomocytic stomata were determined on the upper and lower leaf surfaces of both subspecies.
Keywords: *Asyneuma, Campanulaceae, medicinal plants, pharmaceutical botany, plant anatomy*

ÖZ

Amaç: *Asyneuma Griseb. & Schenk, dünya çapında kabul görmüş 34 türle temsil edilmektedir ve doğal yayılışı Doğu Orta Avrupa'dan Japonya ve Kuzey Çin-hindi'ye kadar geniş bir alanı kapsamaktadır. Asyneuma bitkileri, otsu, basit veya dallı çiçekler mor, menekşe veya mavi renkli ve kapsül meyveler ile karakterize edilir. Campanulaceae familyasının feniletanoid (fenilproponoid), alkaloidler, siyanogenetik heterosit, flavonoit, triterpen, antosiyanin, fenolik asitler, uçucu yağ, kumarin ve polisakkarit gibi biyoaktif bileşikler içerdiğini göstermektedir. Ayrıca familya bitkilerinin antioksidan, yara iyileştirici, antienflamatuar, analjezik, antiobezite, balgam söktürücü, antihepatotoksik, antitümöral, antiaterosklerotik, nöroprotektif, antidepresan, tonik ve α-glukosidaz inhibitör aktiviteleri olduğu bilinmektedir. Bu çalışmada Asyneuma limonifolium subsp. limonifolium, A. limonifolium subsp. pestalozzae (Boiss.) Damboldt, A. linifolium subsp. linifolium ve A. linifolium subsp. nallihanicum Kit Tan & Yıldız yapraklarının anatomik yapıları incelenmiştir.*
Gereç ve Yöntem: *Asyneuma limonifolium subsp. limonifolium, A. limonifolium subsp. pestalozzae (Boiss.) Damboldt, A. linifolium subsp. nallihanicum Kit Tan & Yıldız Ankara'dan toplanmıştır. A. linifolium subsp. linifolium Antalya'dan toplanmıştır. Bitki kısımları alkolde (%70) korunmuştur. Jilet kullanılarak mikroskobik kesitler alınmıştır. Dokular Sartur reaktifi ile boyanmıştır ve ışık mikroskobunda incelenmiştir. Işık mikroskobuna bağlı bir kamera ile mikrofotoğrafları çekilmiştir.*
Sonuç ve Tartışma: *Sonuçlar, Asyneuma limonifolium subsp. limonifolium ve A. limonifolium subsp. pestalozzae taban ve gövde yapraklarının benzer olduğunu göstermiştir. Yapraklar bifasiyal ve palizat parenkiması 1-2 sıralıdır. Ayrıca, tek hücreli örtü tüyleri ve anomostik stomalar her iki epidermal yüzeyde de gözlenmiştir. Bunun yanında, A. linifolium subsp. linifolium'un yaprağı monofasiyal, A. linifolium subsp. nallihanicum'un ise bifasiyaldir. Ek olarak, A. linifolium subsp. linifolium'un epidermal tabakasında tek hücreli tüyler bulunurken, A. linifolium subsp. nallihanicum'da ise yoktur. Anomostik stomalar, her iki alt türün yaprak üst ve alt yüzeylerinde tespit edilmiştir.*
Anahtar Kelimeler: *Asyneuma, bitki anatomisi, Campanulaceae, farmasötik botanik, tıbbi bitkiler*

INTRODUCTION

Folk medicine and medicinal plants have a long history in the Anatolia and many traditional practices are still used today. Studies have documented the diversity of plant species used in folk medicine and their traditional uses in the treatment of many diseases in Türkiye [1-7]. Overall, these studies and many others highlight the richness and traditional uses of medicinal plants in Türkiye. More research is needed to better understand the therapeutic potential of these herbs and ensure their sustainable use.

Campanulaceae Juss. family includes 94 accepted genera worldwide [8]. The family has a native range from the tropics to the subtropics and includes herbs, trees and shrubs [9]. Leaves are alternate and exstipulate; the flowers are hermaphrodite, epigynous, actinomorphic; inflorescence 1-many flowered spicate, racemiform or paniculate; the fruit is an erect or nodding capsule; seeds are numerous, small and usually shiny [10].

Studies show that the Campanulaceae family contains bioactive compounds phenylethanoid (phenylproponoid), alkaloids, cyanogenetic heteroside, flavonoid, triterpene, anthocyanin, phenolic acid, essential oil, coumarin and polysaccharide [11-22]. In addition, it is known that family plants have antioxidant, wound healing, anti-inflammatory, analgesic, antiobesity, expectorant, antihepatotoxic, antitumoral, antiatherosclerotic, neuroprotective, antidepressant, tonic and α-glucosidase inhibitory activities [23-35].

The genus *Asyneuma* Griseb. & Schenk is represented by 34 accepted species worldwide and its native range covers a wide area from East Central Europe to Japan and Northern Indo-china [8]. The

plants of *Asyneuma* are characterized by herbaceous, simple or branched inflorescences, purple, violet or blue corolla and capsule fruits [36].

Light microscopy is an effective and practical method that is frequently used in the analysis and diagnosis of herbal drugs. Elucidation of plant tissues is very important to correctly describe and determine of medicinal plants [37-39]. In this study, the anatomical structures of *Asyneuma limonifolium* subsp. *limonifolium*, *A. limonifolium* subsp. *pestalozzae* (Boiss.) Damboldt, *A. linifolium* subsp. *linifolium* and *A. linifolium* subsp. *nallihanicum* Kit Tan & Yıldız leaves were examined.

MATERIAL AND METHOD

Asyneuma limonifolium subsp. *limonifolium* (*mh23012*) (Figure 1A), *A. limonifolium* subsp. *pestalozzae* (Boiss.) Damboldt (*mh23013*) (Figure 1B), *A. linifolium* subsp. *nallihanicum* Kit Tan & Yıldız (*mh23019*) (Figure 1D) were collected from Ankara. *A. linifolium* subsp. *linifolium* (*mh23023*) (Figure 1C) was collected from Antalya. Plant parts were preserved in alcohol (70%). Microscopic sections were taken using a razor blade. Tissues were stained with Sartur reagent and examined with a light microscope. Microphotographs were taken with a camera attached to a light microscope.

Figure 1. A: *Asyneuma limonifolium* subsp. *limonifolium*, B: *A. limonifolium* subsp. *pestalozzae*, C: *A. linifolium* subsp. *linifolium*, D: *A. linifolium* subsp. *nallihanicum* (photo: Şeyda Yayla)

RESULT AND DISCUSSION

Asyneuma limonifolium subsp. *limonifolium*

The basal leaf is bifacial (Figure 2). The midrib is domed outward in both sides. It is more protruding in the abaxial side. The upper and lower epidermis layers of the midrib are composed of square-rectangular cells. The cuticle covering the epidermis layer is thicker at the bottom. Both epidermal layers of the midrib are covered with unicellular non-glandular hairs, it is observed more intensely especially in the lower epidermis of the midrib. In the midrib, the vascular bundle is embedded in thin-walled, round-shaped parenchymatous cells. The xylem is surrounded by the phloem on the

abaxial side. The upper and lower epidermal cells of the leaf lamina are observed as square-rectangular. The palisade parenchyma is in 1-2 rows and located below the upper epidermis. The spongy parenchyma is observed below the palisade parenchyma. The unicellular non-glandular hairs and anomocytic stomata with 3-5 subsidiary cells were seen in the both surface sections of the leaf.

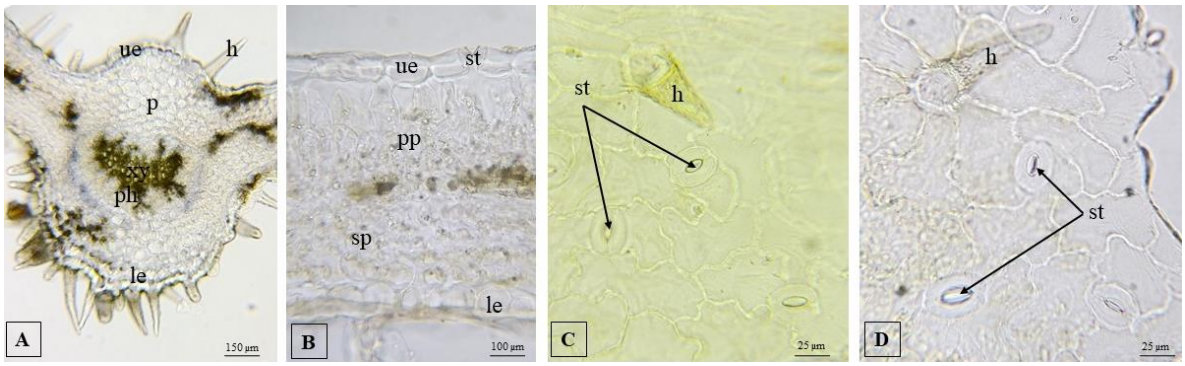

Figure 2. Basal leaf anatomical features of *A. limonifolium* subsp. *limonifolium* (A: Midrib cross section, B: Lamina cross section, C: Lamina upper surface section, D: Lamina lower surface section); h: unicellular hair, le: lower epidermis, p: parenchyma, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem

The cauline leaf is bifacial (Figure 3). The upper and lower epidermis of the midrib consists of square-rectangular cells. The midrib protrudes outward on both surfaces, but it is more on the abaxial surface. The vascular bundle is embedded in thin-walled parenchymatous cells in the midrib, and the phloem is located on the abaxial side of xylem. The mesophyll has 1-2 rows of palisade parenchyma. The unicellular non-glandular hairs and anomocytic stomata with 3-5 subsidiary cells were seen in the both surface sections of the cauline leaf.

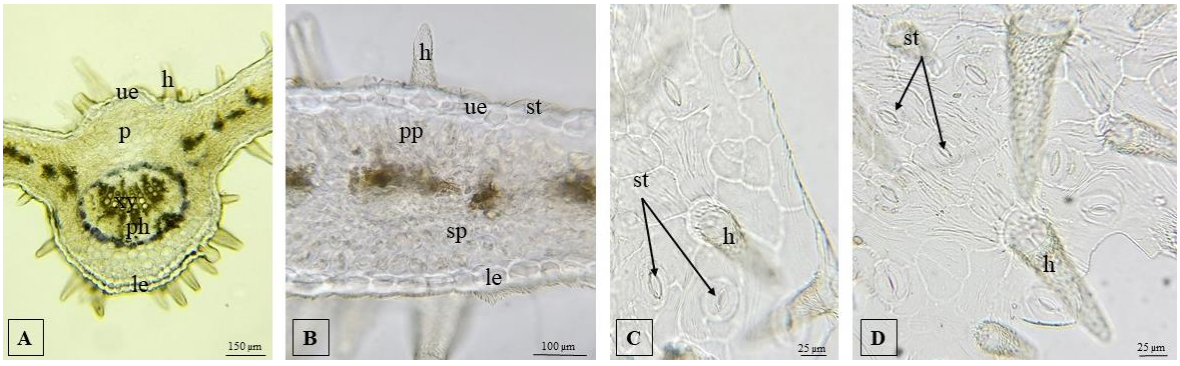

Figure 3. Cauline leaf anatomical features of *A. limonifolium* subsp. *limonifolium* (A: Midrib cross section, B: Lamina cross section, C: Lamina upper surface section, D: Lamina lower surface section); h: unicellular hair, le: lower epidermis, p: parenchyma, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem

Asyneuma limonifolium* subsp. *pestalozzae

The basal leaf is bifacial (Figure 4). The midrib is domed outward in both sides. It is more protruding in the abaxial side. The cuticle is thick on both epidermal surfaces. In the midrib, the phloem is arc-shaped and surrounds the xylem from the abaxial side. The epidermal layer is composed of square-rectangular cells. Palisade parenchyma has 1-2 rows. The epidermis layer contains unicellular non-glandular hairs and stomata. The stomata are anomosytic type and have 3-5 subsidiary cells.

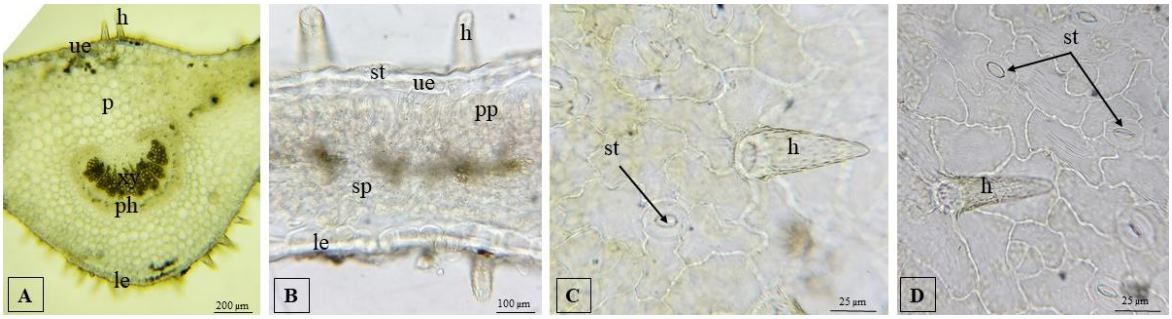

Figure 4. Basal leaf anatomical features of *A. limonifolium* subsp. *pestalozzae* (A: Midrib cross section, B: Lamina cross section, C: Lamina upper surface section, D: Lamina lower surface section); h: unicellular hair, le: lower epidermis, p: parenchyma, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem

The cauline leaf is bifacial (Figure 5). The midrib is slightly domed in the adaxial side and extremely in the abaxial side. The main vein is embedded in thin-walled parenchymatous cells and the phloem is surrounded the abaxial side of xylem. Unicellular, non-glandular hairs are found more densely on the lower surface of the cauline leaf. The anomocytic stomata are located on the both epidermis surfaces, with 3-5 subsidiary cells. The upper epidermal cells of the cauline leaf lamina are similar to the lower epidermal cells, square-rectangular in shape and larger than the lower epidermal cells.

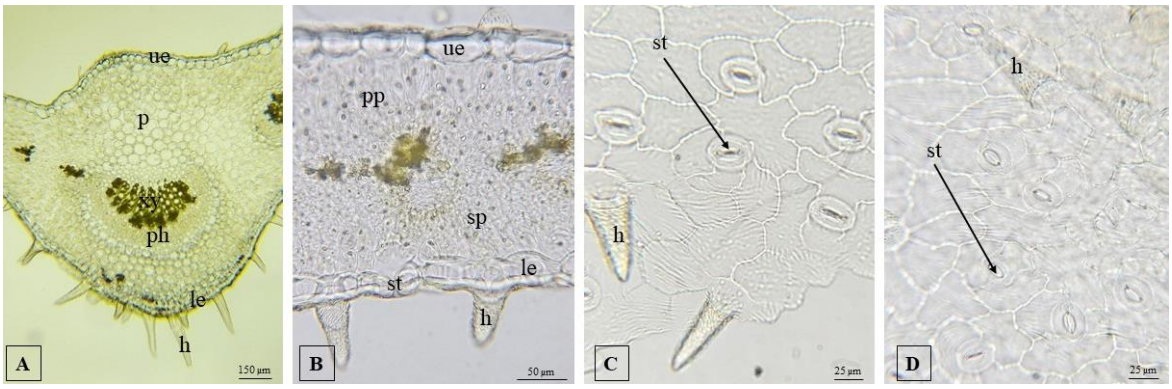

Figure 5. Cauline leaf anatomical features of *A. limonifolium* subsp. *pestalozzae* (A: Midrib cross section, B: Lamina cross section, C: Lamina upper surface section, D: Lamina lower surface section); h: unicellular hair, le: lower epidermis, p: parenchyma, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem

Asyneuma linifolium subsp. *linifolium*

The leaf is monofacial (Figure 6). The palisade parenchyma has 1-2 rows in the mesophyll. The spongy parenchyma cells are located between the two palisade parenchyma. The upper epidermal cells are larger than the lower epidermal cells and are square or sometimes oval in shape. Unicellular non-glandular hairs are observed sparsely only in the upper epidermis. The anomocytic stomata are located on the both epidermis surfaces, with 3-5 subsidiary cells.

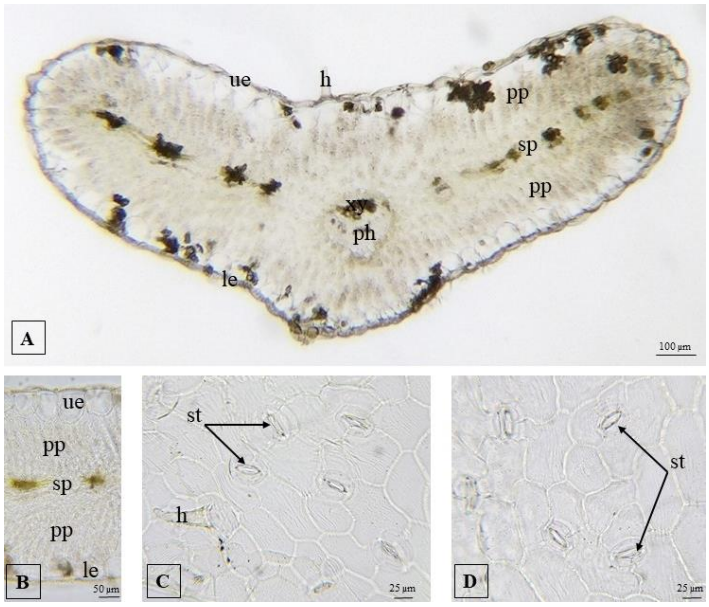

Figure 6. The leaf anatomical features of *A. linifolium subsp. linifolium* (A: Leaf cross section, B: Lamina cross section, C: Lamina upper surface section, D: Lamina lower surface section); h: unicellular hair, le: lower epidermis, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem

Asyneuma linifolium subsp. *nallihanicum*

The leaf is bifacial (Figure 7). The upper and lower epidermal cells are the same size, mostly oval, rarely square. The spongy parenchyma is located under the 1-2 row palisade parenchyma in the lamina. The phloem accompanies to the xylem on the abaxial side in the main vascular bundle. Hairs are absent on the both surfaces. The stomata are anomocytic type and have 3-5 subsidiary cells and are observed in the both epidermis.

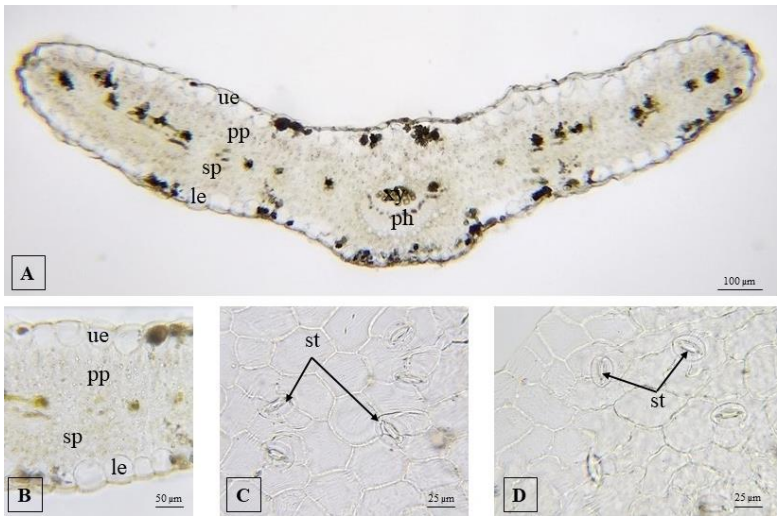

Figure 7. The leaf anatomical features of *A. linifolium subsp. nallihanicum* (A: Leaf cross section, B: Lamina cross section, C: Lamina upper surface section, D: Lamina lower surface section); le: lower epidermis, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem

The results showed that, the anatomical structures of the basal and cauline leaves of *Asyneuma limonifolium* subsp. *limonifolium* and *A. limonifolium* subsp. *pestalozzae* were similar. The leaves are bifacial, the palisade parenchyma 1-2 rows. In addition, the unicellular, non-glandular hairs are located on the both epidermal layers. The anomocytic type stomata are located on the both surfaces. The leaf of *A. linifolium* subsp. *linifolium* is monofacial and the leaf of *A. linifolium* subsp. *nallihanicum* is bifacial. Also, on epidermal layer of *A. linifolium* subsp. *linifolium* unicellular hairs present, on epidermal layer of *A. linifolium* subsp. *nallihanicum* absent. The anomocytic stomata were found on the upper and lower leaf surfaces of the both subspecies. Previous studies have reported the presence of dorsiventral leaf, 1-2 rows of palisade, ranunculaceae type stomata on the both surfaces, and bundle sheath [40-41]. These data are consistent with our results. In addition, Metcalfe and Chalk (1965) reported the presence of epidermal cells with sinuous anticlinal walls, also acicular crystals and yellowish sphaerocrystals for the Campanulaceae family anatomical structures. In addition, the existence of unicellular, non-glandular hairs with papillae-like swelling is known [41-42].

ACKNOWLEDGEMENTS

This work was produced as a part of Şeyda Yayla's Ph.D. thesis. Şeyda Yayla thanks the financial support from the Scientific and Technological Research Council of Türkiye (TUBITAK) under the BIDEB/2211-A Ph.D. The author would like to thank Dr. Kurtuluş Özgişi and Dr. Barış Özüdoğru for locality informations.

AUTHOR CONTRIBUTIONS

Concept: Ş.Y., M.M.H.; Design: Ş.Y., M.M.H.; Control: Ş.Y., M.M.H.; Sources: Ş.Y., M.M.H.; Materials: Ş.Y., M.M.H.; Data Collection and/or Processing: Ş.Y., M.M.H.; Analysis and/or Interpretation: Ş.Y., M.M.H.; Literature Review: Ş.Y.; Manuscript Writing: Ş.Y.; Critical Review: Ş.Y., M.M.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., 47(3): 1020-1032, 2023
Doi: 10.33483/jfpau.1280492

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SYNTHESIS AND PROPERTIES OF *S*-ALKYL 4-(4-CHLOROPHENYL)-5-(PYRROLE-2-YL)-1,2,4-TRIAZOLE-3-THIOL DERIVATIVES

S-ALKİL 4-(4-KLOROFENİL)-5-(PİROL-2-İL)-1,2,4-TRİAZOL-3-TİYOL TÜREVLERİNİN SENTEZİ VE ÖZELLİKLERİ

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ABSTRACT

Objective: *The aim of the work was to develop effective methods for the synthesis of promising heterocyclic systems based on pyrrole and 1,2,4-triazole. In the process of realizing of this aim, 10 new S-alkyl 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol derivatives were synthesized.*
Material and Method: *Chemical structures of synthesized compounds were characterized with elemental analysis, ^{1}H-NMR, LC-MS techniques. The biological potential of the synthesized substances was estimated by the molecular docking method and ADME analysis.*
Result and Discussion: *An optimum method for the synthesis of S-alkyl 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol has been developed. In molecular modeling studies, the compounds were found to be similar to known drugs in some respects. The interaction of each*

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Submitted / Gönderilme : 12.04.2023
Accepted / Kabul : 01.09.2023
Published / Yayınlanma : 20.09.2023

molecule with the crystal structures of cyclooxygenase-2, lanosterol-14α-demethylase in the active site was considered in silico. Pharmacokinetic parameters for a number of the synthesized compounds have been predicted by ADME analysis.
Keywords: *1,2,4-triazole, ADME analysis, docking, synthesis*

ÖZ

Amaç: *Çalışmanın amacı, pirol ve 1,2,4-triazole dayalı gelecek vaat eden heterosiklik sistemlerin sentezi için etkili yöntemler geliştirmektir. Belirtilen hedefin gerçekleştirilmesi sürecinde 10 yeni S-alkil 4-(4-klorofenil)-5-(pirol-2-il)-1,2,4-triazol-3-tiyol türevi sentezlenmiştir..*
Gereç ve Yöntem: *Sentezlenen bileşiklerin kimyasal yapıları element analiz, ^{1}H NMR, LC-MS teknikleri ile karakterize edildi. Sentezlenen maddelerin biyolojik potansiyeli moleküler yerleştirme yöntemi ve ADME analizi ile tahmin edildi.*
Sonuç ve Tartışma: *S-alkil 4-(4-klorofenil)-5-(pirol-2-il)-1,2,4-triazol-3-tiyol türevlerinin sentezi için optimal bir yöntem geliştirilmiştir. Moleküler modelleme çalışmalarında, bileşiklerin bazı açılardan bilinen ilaçlara benzer olduğu bulunmuştur. Her molekülün kristal yapıdaki siklooksijenaz-2 ve lanosterol-14α-demetilaz aktif bölgesi ile etkileşimi in silico olarak değerlendirildi. Sentezlenen bileşiklerin bir kısmı için farmakokinetik parametreler ADME analizi ile tahmin edildi.*
Anahtar Kelimeler: *1,2,4-triazol, ADME analizi, moleküler modelleme, sentez*

INTRODUCTION

Derivatives of 1,2,4-triazole have a high chemical and pharmacological potential, which makes this class of compounds highly promising in the sense of creating an original drug product [1-8]. The 1,2,4-triazole system has already proven to have significant potential in the development of antifungal drugs (fluconazole, voriconazole, ravuconazole, terconazole, itraconazole and sertaconazole), anticancer drugs (anastrozole, vorozol and letrozole), anti-migraine drugs (rizatriptan), antidotes for metal poisoning (deferasirox), anxiolytic drugs (alprazolam and triazolam), and antiviral drugs (ribavirin). The group of antifungal drugs that were created with the participation of this heterocycle is very significant. For example, such a drug as voriconazole allows you to successfully treat even difficult-to-treat forms of mycosis (Figure 1).

Pyrrole derivatives are represented by a smaller number of medicines. However, there are quite effective drugs among them. For example, amtolmetine guacyl has been used as a non-steroidal anti-inflammatory drug for many years (Figure 1).

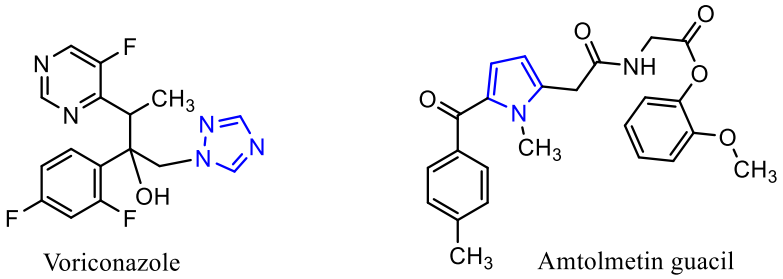

Figure 1. Examples of successful drug design based on 1,2,4-triazole and pyrrole

Among the directions of the first stages of work with this cycle, a special place is occupied by the possibility of its combination with heterocyclic synthons of a different nature [9-12]. The specified approach significantly facilitates the creation of the desired product of chemical transformation, which ultimately has a high probability of formation of a biologically active substance [13]. Of particular interest in the implementation of this strategy is the combination of a triazole fragment with a pyrrole fragment. This interest was also supported by certain achievements of both foreign and Ukrainian scientists [14-16]. In order to obtain new compounds, it was decided to directly combine the cycles of

1,2,4-triazole and pyrrole by a covalent bond and introduce a 4-chlorophenyl substituent into the tandem.

The aim of the work was to synthesize *S*-alkyl derivatives of 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol as potential biologically active compounds. An effective tool that can help model the mechanism of interaction of synthesized substances with biological targets (enzymes, receptors) is molecular docking. The presence in the structure of the synthesized substances of pyrrole and 1,2,4-triazole, which can actively promote the formation of hydrogen, hydrophobic and other bonds, substantiates the need for molecular docking.

An important step in the process of creating a biologically active substance is the preliminary evaluation of pharmacokinetic parameters. ADME (Adsorption, Distribution, Metabolism, Excretion) analysis provides a reasonably accurate assessment of these parameters.

Thus, the combined use of molecular docking and ADME analysis will allow an exact evaluation of the feasibility of further *in vitro* and *in vivo* studies of the synthesized series of substances.

MATERIAL AND METHOD

Chemistry

The intermediate thiol was obtained in several stages. The first step was the synthesis of 2,2,2-trichloro-(pyrrole-2-yl)ethanone. The chemical reaction proceeded easily with the active participation of pyrrole and trichloroacetyl chloride in diethyl ether (Figure 2) [17].

Previously, solutions of pyrrole (**1**) and freshly distilled trichloroacetyl chloride (**2**) in diethyl ether were prepared separately. The resulting pyrrole solution was slowly added dropwise to the trichloroacetyl chloride solution while stirring without heating. After 1 hour, a solution of potassium carbonate in water was added. After that, the organic solvent layer was separated and evaporated. The reaction product in the form of 2,2,2-trichloro-1-(pyrrole-2-yl)ethan-1-one (**3**) was washed three times with *n*-hexane. This stage is more thoroughly described in an earlier work [17].

The next stage was the preparation of pyrrole-2-carbohydrazide (**4**). To implement this stage, the starting substance (**3**) was involved in the process of hydrazinolysis in an alcoholic medium. As a reagent, hydrazine hydrate was used in a fivefold excess. The reaction was carried out in a medium of propan-2-ol with heating for 2 hours.

These chemical transformation products have been synthesized in accordance with the described methods and have physical constants that correspond to the literature data [17].

The obtained pyrrole-2-carbohydrazide (**4**) has been used in the reaction with 4-chlorophenylisothiocyanate. The isolated *N*-(4-chlorophenyl)-2-(pyrrole-2-carbonyl)hydrazine-1-carbothioamide (**5**) was then subjected to intramolecular heterocyclization in an alkaline medium.

The synthesized 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol (**6**) was used in alkylation reactions with halogenated alkanes to further determine the biological potential of the synthesized series of substances (**6.1-6.10**).

The structure of the synthesized substances was confirmed using modern physical and chemical methods of analysis.

All substances used in the experiments were given with accompanying documents confirming their quality and purity.

The structure of synthesized substances, their purity, as well as their individuality of chemical conversion products were confirmed using modern physical and chemical methods of analysis. The melting temperature of all synthesized substances was determined on SRS Inc MPA 100 equipment using the open capillary method. A VarioELcube analyzer (CHNS) was used to establish the nature of the elements that form the studied substances, as well as to determine the quantitative ratio of the elements. ^{1}H NMR spectroscopy spectra were recorded using a Varian Mercury-400 spectrometer. Tetramethylsilane was used as an internal standard. Dimethylsulfoxide-d_6 was used as a solvent. An Agilent "1260 Infinity" high performance liquid chromatography system, supplemented with an Agilent "6120" spectrometer, was used to perform chromatography-mass spectrometry. Spraying in an electric field was used as a method of ionization.

***N*-(4-chlorophenyl)-2-(pyrrole-2-carbonyl)hydrazinecarbothioamide (5) [17]**

To a solution of 2.5 g (0.02 mol) of pyrrole-2-carbohydrazide in 80 ml of ethanol cooled with ice was added an equivalent amount of 3.39 g (0.02 mol) of 4-chlorophenylisothiocyanate. The resulting mixture is heated at 110°C for 2 hours. On cooling, a yellow-white crystalline precipitate, insoluble in water and soluble in organic solvents, is formed.

It is purified by crystallisation from propan-2-ol and treatment of the hot saturated solution with activated carbon followed by filtration. The yield is 83%. Melting point = 162 - 164ºC.

4-(4-Chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol (6)

5.89 g (0.02 mol) of *N*-chlorophenyl-2-(pyrrole-2-carbonyl)hydrazinecarbothioamide is added to an equivalent amount of sodium hydroxide that has been previously dissolved in a small amount of water. The resulting mixture is heated for one hour. Cool it down. Add dilute acetic acid until the solution is completely neutralized, accompanied by the formation of a white precipitate, which is separated by filtration. The reaction product is insoluble in water, soluble in organic solvents and in an aqueous alkali solution.

S-alkyl derivatives of 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol (6.1-6.10).

To a mixture of 0.005 mol of 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol and 0.005 mol of sodium hydroxide dissolved in 40 ml of ethanol, 0.005 mole of haloalkane (iodomethane, bromoethane, 1-bromopropane, 1-bromobutane, 1-bromopentane, 1-bromohexane, 1-bromoheptane, 1-bromooctane, 1-bromononane, 1-bromodecane). Heat for 2 hours, cool, filter the sediment, wash with purified water. For analysis, crystallize from methanol. White substances, insoluble in water, soluble in organic solvents.

4-(4-Chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol (6). Yield (%): 69, m. p. (°C): 267 – 269. ^{1}H-NMR (400 MHz), δ (ppm): 13.93 (s, 1H, SH), 11.74 (s, 1H, pyrrole NH), 7.71-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.89 (d, J = 3.0 Hz, H-3, pyrrole), 5.97 (t, J = 3.1 Hz, H-5, pyrrole), 5.35-5.29 (m, H-4, pyrrole). ESI MS (m/z): $[M+H]^+$ at 277.5. Elemental analysis (EA) ($C_{12}H_9ClN_4S$), calculated, %: C - 52.08, H - 3.28, N - 20.25, S - 11.58; obtained, %: C - 51.94, H - 3.29, N - 20.20, S - 11.61.

4-(4-Chlorophenyl)-3-(methylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.1). Yield (%): 74, m. p. (°C): 194 – 195. ^{1}H-NMR (400 MHz), δ (ppm): 11.74 (s, 1H, pyrrole NH), 7.71-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.89 (d, J = 3.0 Hz, H-3, pyrrole), 5.97 (t, J = 3.1 Hz, H-5, pyrrole), 5.33-5.28 (m, H-4, pyrrole), 2.74 (s, 2H, CH_3). ESI MS (m/z): $[M+H]^+$ at 291,5. Elemental analysis (EA) ($C_{13}H_{11}ClN_4S$), calculated, %: C - 53.70, H - 3.81, N - 19.27, S - 11.03; obtained, %: C - 53.57, H - 3.82, N - 19.22, S - 11.06.

4-(4-Chlorophenyl)-3-(ethylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.2). Yield (%): 69%, m. p. (°C): 163 – 164. ^{1}H-NMR (400 MHz), δ (ppm): 11.74 (s, 1H, pyrrole NH), 7.71-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.51-7.44 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.88 (d, J = 3.0 Hz, H-3, pyrrole), 5.98 (t, J = 3.2 Hz, H-5, pyrrole), 5.34-5.29 (m, H-4, pyrrole), 3.21 (q, J=6.2 Hz, 2H, S-C$\underline{H}_2$-CH_3), 1.35 (t, J=6.0 Hz, 3H, S-CH_2-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 305,5. Elemental analysis (EA) ($C_{14}H_{13}ClN_4S$), calculated, %: C - 55.17, H - 4.30, N - 18.38, S - 10.52; obtained, %: C - 55.02, H - 4.29, N - 18.43, S - 10.55.

4-(4-Chlorophenyl)-3-(propylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.3). Yield (%): 77, m. p. (°C): 158 – 159. ^{1}H-NMR (400 MHz), δ (ppm): 11.73 (s, 1H, pyrrole NH), 7.71-7.65 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.90 (d, J = 3.0 Hz, H-3, pyrrole), 5.98 (t, J = 3.1 Hz, H-5, pyrrole), 5.32-5.26 (m, H-4, pyrrole), 3.15 (t, J=5.2 Hz, 2H, S-C$\underline{H}_2$-C_2H_5), 1.79 (q, J=5.4 Hz, 2H, S-CH_2-C$\underline{H}_2$-CH_3), 1.05 (t, J=7.0 Hz, 3H, S-$(CH_2)_2$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 319.5. Elemental analysis (EA) ($C_{15}H_{15}ClN_4S$), calculated, %: C - 56.51, H - 4.74, N - 17.57, S - 10.06; obtained, %: C - 56.65, H - 4.73 , N - 17.61, S - 10.04.

4-(4-Chlorophenyl)-3-(butylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.4). Yield (%): 81, m. p. (°C): 163 – 164. ^{1}H-NMR (400 MHz), δ (ppm): 11.74 (s, 1H, pyrrole NH), 7.71-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.90 (d, J = 3.1 Hz, H-3, pyrrole), 5.96 (t, J = 3.2 Hz, H-5, pyrrole), 5.33-5.27 (m, H-4, pyrrole), 3.17 (t, J=6.6 Hz, 2H, S-C$\underline{H}_2$-C_3H_7), 1.82 – 1.77 (m, 2H, S-

CH_2-C<u>*H*</u>$_2$-C_2H_5), 1.39-1.34 (m, 2H, S-$(CH_2)_2$-C<u>*H*</u>$_2$-CH_3), 0.93 (t, *J*=6.9 Hz, 3H, S-$(CH_2)_3$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 333,5. Elemental analysis (EA) ($C_{16}H_{17}ClN_4S$), calculated, %: C - 57.74, H - 5.15, N - 16.83, S - 9.63; obtained, %: C - 57.59, H - 5.16, N - 16.79, S - 9.66.

4-(4-Chlorophenyl)-3-(pentylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.5). Yield (%): 74, m. p. (°C): 166 – 167. ^{1}H-NMR (400 MHz), δ (ppm): 11.73 (s, 1H, pyrrole NH), 7.70-7.65 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.89 (d, *J* = 3.0 Hz, H-3, pyrrole), 5.97 (t, *J* = 3.1 Hz, H-5, pyrrole), 5.35-5.30 (m, H-4, pyrrole), 3.25 (t, *J*=6.0 Hz, 2H, S-C<u>*H*</u>$_2$-C_4H_9), 1.76 – 1.69 (m, 2H, S-CH_2-C<u>*H*</u>$_2$-C_3H_7), 1.42 – 1.34 (m, 4H, S-$(CH_2)_2$-(C<u>*H*</u>$_2$)$_2$-CH_3), 0.96 – 0.88 (m, 3H, S-$(CH_2)_4$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 347.5. Elemental analysis (EA) ($C_{17}H_{19}ClN_4S$), calculated, %: C - 58.86, H - 5.52, N - 16.15, S - 9.24; obtained, %: C - 59.01, H - 5.53, N - 16.11, S - 9.22.

4-(4-Chlorophenyl)-3-(hexylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.6). Yield (%): 69, m. p. (°C): 168 – 169. ^{1}H-NMR (400 MHz), δ (ppm): 11.74 (s, 1H, pyrrole NH), 7.70-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.89 (d, *J* = 3.0 Hz, H-3, pyrrole), 5.97 (t, *J* = 3.1 Hz, H-5, pyrrole), 5.34-5.29 (m, H-4, pyrrole), 3.24 (t, *J*=6.4 Hz, 2H, S-C<u>*H*</u>$_2$-C_5H_{11}), 1.71 – 1.65 (m, 2H, S-CH_2-C<u>*H*</u>$_2$-C_4H_9), 1.41 – 1.34 (m, 2H, S-$(CH_2)_2$-C<u>*H*</u>$_2$-C_3H_7), 1.34 – 1.26 (m, 4H, S-$(CH_2)_3$-(C<u>*H*</u>$_2$)$_2$-CH_3), 0.92 – 0.85 (m, 3H, S-$(CH_2)_5$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 361.5. Elemental analysis (EA) ($C_{18}H_{21}ClN_4S$), calculated, %: C - 59.90, H - 5.87, N - 15.52, S - 8.88; obtained, %: C - 60.07, H - 5.86, N - 15.48, S - 8.90.

4-(4-Chlorophenyl)-3-(heptylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.7). Yield (%): 76, m. p. (°C): 174-175 ºC. ^{1}H-NMR (400 MHz), δ (ppm): 11.73 (s, 1H, pyrrole NH), 7.71-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.50-7.43 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.88 (d, *J* = 3.0 Hz, H-3, pyrrole), 5.99 (t, *J* = 3.1 Hz, H-5, pyrrole), 5.34-5.28 (m, H-4, pyrrole), 3.23 (t, *J*=6.2 Hz, 2H, S-C<u>*H*</u>$_2$-$(CH_2)_5$-CH_3), 1.72 – 1.65 (m, 2H, S-CH_2-C<u>*H*</u>$_2$-C_5H_{11}), 1.42 – 1.36 (m, 2H, S-$(CH_2)_2$-C<u>*H*</u>$_2$-C_4H_9), 1.32 – 1.21 (m, 6H, S-$(CH_2)_3$-(C<u>*H*</u>$_2$)$_3$-CH_3), 0.92 – 0.85 (m, 3H, S-$(CH_2)_6$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 375.5. Elemental analysis (EA) ($C_{19}H_{23}ClN_4S$), calculated, %: C - 60.87, H - 6.18, N - 14.94, S - 8.55; obtained, %: C - 60.73, H - 6.17, N - 14.97, S - 8.57.

4-(4-Chlorophenyl)-3-(octylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.8). Yield (%): 73, m. p. (°C): 182-183 ºC. ^{1}H-NMR (400 MHz), δ (ppm): 11.73 (s, 1H, pyrrole NH), 7.71-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.51-7.44 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.88 (d, *J* = 3.1 Hz, H-3, pyrrole), 5.98 (t, *J* = 3.2 Hz, H-5, pyrrole), 5.33-5.27 (m, H-4, pyrrole), 3.21 (t, *J*=6.3 Hz, 2H, S-C<u>*H*</u>$_2$-C_7H_{15}), 1.71 – 1.65 (m, 2H, S-CH_2-C<u>*H*</u>$_2$-C_6H_{13}), 1.38 – 1.22 (m, 10H, m, 2H, S-$(CH_2)_2$-(C<u>*H*</u>$_2$)$_5$-CH_3), 0.93 – 0.86 (m, 3H, S-$(CH_2)_7$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 389.5. Elemental analysis (EA) ($C_{20}H_{25}ClN_4S$), calculated, %: C - 61.76, H - 6.48, N - 14.40, S - 8.24; obtained, %: C - 61.93, H - 6.49, N - 14.36, S - 8.22.

4-(4-Chlorophenyl)-3-(nonylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.9). Yield (%): 82, m. p. (°C): 161-162 ºC. ^{1}H-NMR (400 MHz), δ (ppm): 11.74 (s, 1H, pyrrole NH), 7.71-7.65 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.51-7.42 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.90 (d, *J* = 3.0 Hz, H-3, pyrrole), 5.97 (t, *J* = 3.1 Hz, H-5, pyrrole), 5.33-5.28 (m, H-4, pyrrole), 3.25 (t, *J* = 6.5 Hz, 2H, S-C<u>*H*</u>$_2$-$(CH_2)_7$-CH_3), 1.73 – 1.64 (m, 2H, S-CH_2-C<u>*H*</u>$_2$-C_7H_{15}), 1.37 – 1.31 (m, 2H, S-$(CH_2)_2$-C<u>*H*</u>$_2$-C_6H_{13}), 1.28 – 1.19 (m, 10H, S-$(CH_2)_3$-(C<u>*H*</u>$_2$)$_5$-CH_3), 0.90 – 0.84 (m, 3H, S-$(CH_2)_8$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 403.5. Elemental analysis (EA) ($C_{21}H_{27}ClN_4S$), calculated, %: C - 62.59, H - 6.75, N - 13.90, S - 7.96; obtained, %: C - 62.43, H - 6.76, N - 13.87, S - 7.98.

4-(4-Chlorophenyl)-3-(decylthio)-5-(pyrrole-2-yl)-1,2,4-triazole (6.10). Yield (%): 85, m. p. (°C): 144-145 ºC. ^{1}H-NMR (400 MHz), δ (ppm): 11.74 (s, 1H, pyrrole NH), 7.70-7.64 (m, 2H, H-2,6, 4-Cl-C_6H_4), 7.48-7.42 (m, 2H, H-3,5, 4-Cl-C_6H_4), 6.90 (d, *J* = 3.0 Hz, H-3, pyrrole), 5.98 (t, *J* = 3.1 Hz, H-5, pyrrole), 5.34-5.28 (m, H-4, pyrrole), 3.23 (t, *J*=6.5 Hz, 2H, S-C<u>*H*</u>$_2$-C_9H_{19}), 1.71 – 1.66 (m, 2H, S-CH_2-C<u>*H*</u>$_2$-C_8H_{17}), 1.38 – 1.23 (m, 12H, S-$(CH_2)_3$-(C<u>*H*</u>$_2$)$_6$-CH_3), 0.88 – 0.82 (m, 3H, S-$(CH_2)_9$-C<u>*H*</u>$_3$). ESI MS (m/z): $[M+H]^+$ at 417.5. Elemental analysis (EA) ($C_{22}H_{29}ClN_4S$), calculated, %: C - 63.37, H - 7.01, N - 13.44, S - 7.69; obtained, %: C - 63.53, H - 6.99, N - 13.41, S - 7.71.

Molecular Docking

The next stage of scientific work involved molecular docking. This action is necessary to justify possible further studies of the biological potential of the synthesized substances.

The choice of the direction of *in silico* studies has been defined by the nature of structural

fragments of the synthesized substances. The presence of the pyrrole cycle determined the possibility of testing the presence of anti-inflammatory activity. For this purpose, a model of cyclooxygenase-2 has been chosen, inhibition of which can lead to anti-inflammatory activity. The 1,2,4-triazole cycle and the alkyl fragments represent certain structural elements that may be associated with antifungal activity. To test this hypothesis, it was chosen to test lanosterol 14α-dimethylase, whose inhibition may be associated with antifungal activity.

Standard ligands with the above enzymes were downloaded from Protein Data Bank [18].

The first stage of molecular docking involved the preparation of a ligand. For this purpose, the programs MarvinSketch-6.3.0, Chem 3D and AutoDockTools-1.5.6 were used [19,20].

The second stage provided for the active use of both software products

Discovery Studio 4.0 and AutoDockTools-1.5.6, which made it possible to prepare the enzyme model.

The final stage of molecular docking included the use of the Vina program and AutoDockTools-1.5.6, which made it possible to visualize the location of the studied ligands in the active sites of the enzymes.

ADME Analysis

The graphical interface of the SwissADME website was used to calculate the main pharmacokinetic parameters. The available descriptors and molecular parameters were calculated according to the explanations given in [21].

Among the main descriptors and parameters that were determined were the lipophilicity index (Log P), the topological polar surface area of molecules (TSPA), the Csp^3 fraction, the number of rotating bonds and the molar refraction. They are directly related to the most important properties of molecules, including size, lipophilicity, conformational mobility and the ability to form hydrogen bonds.

In addition, the determination of TSPA allows for predicting adsorption, bioavailability and blood-brain barrier permeability. For the express evaluation of drug-like properties, pellet diagrams were used, which allow for taking into account the parameters of lipophilicity, size, polarity, solubility and flexibility.

RESULT AND DISCUSSION

Chemistry

The general sequence of chemical transformations that allow obtaining the target reaction products (**6.1-6.10**) is shown in Figure 2.

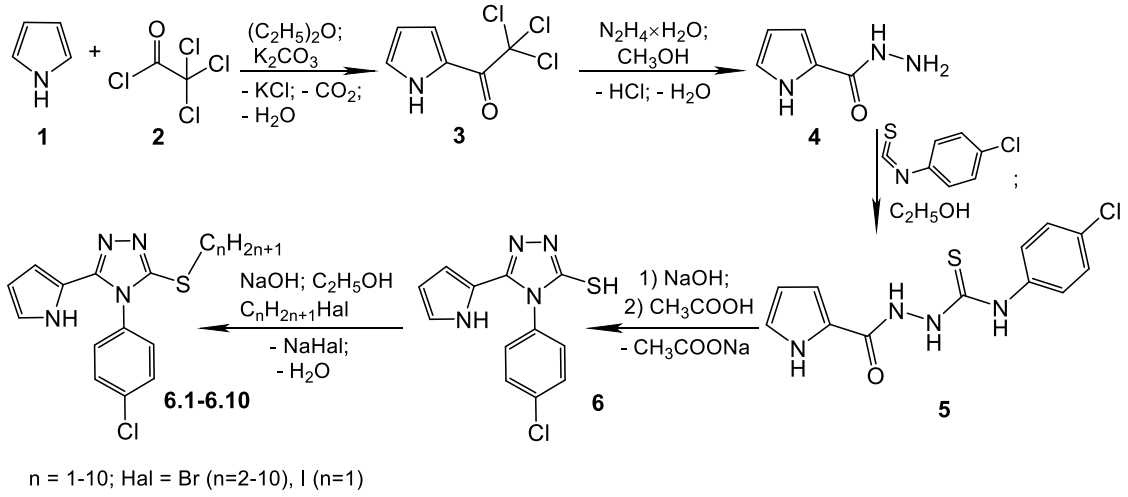

Figure 2. The scheme for the synthesis of target products of chemical transformation

All synthesized S-alkyl derivatives of 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol (**6.1**-**6.10**) are white crystalline substances that are practically insoluble in water, soluble in monoatomic alcohols when heated, well soluble in DMF and DMSO.

^{1}H NMR spectra of the synthesized compounds (**6**, **6.1**-**6.10**) are characterized by signals of NH protons, which are registered as a singlet at 11.74-11.73 ppm. Proton signals of the 4-chlorophenyl substituent are recorded as multiplets: H-2 and H-6 at 7.71-7.64 ppm, H-3 and H-5 at 7.51-7.42 ppm. The signals of the protons of the pyrrole substituent are easily interpreted and are represented by a doublet (H-3) at 6.90-6.88 ppm, a triplet (H-5) at 5.99-5.96 ppm, and a multiplet (H-4) at 5.35-5.26 ppm. Protons of S-alkyl fragments resonate in the strong part of the field in the form of signals with various intensities in the region of 3.25-0.82 ppm. For example, singlet signals of methyl protons of the thiomethyl fragment (-S-CH_3) are present at 2.74 ppm (**6.1**). Multiplet signals from protons of methylene fragments (-S-(C$\underline{H}_2$)$_n$-CH_3) are recorded at a stronger field (1.82-1.65 ppm and 1.42-1.19 ppm).

A gradual increase in the length of the S-alkyl chain leads to the appearance of signals of methyl group protons (-S-$(CH_2)_n$-C$\underline{H}_3$) in the range of 0.93-0.82 ppm.

Molecular Docking

The analysis of docking interactions of the synthesized compounds allows us to conclude that hydrophobic contacts predominate in a number of synthesized compounds with the active site of cyclooxygenase-2, which makes the synthesized substances similar in type of contacts to celecoxib (Figure 3). For example, compound **6.7** with the participation of a hexyl substituent forms alkyl interactions with amino acid residues of Leu A: 93, Tyr A: 116, Tyr A: 356, Val A: 89, Val A: 117. In turn, phenyl, pyrrole and triazole fragments make a significant contribution to the formation of π-alkyl interactions with residues Leu A: 353, Leu A: 532, Phe A: 519, Val A: 524 (Table 1). These contacts are enhanced by the π-σ-interactions of the 1,2,4-triazole fragment with residues Ala A: 528 and Val A: 350. Additionally, the presence of an intermolecular hydrogen bond of π-donor nature involves the pyrrole fragment of the studied compound and residue Ser A: 531 (Figure 3).

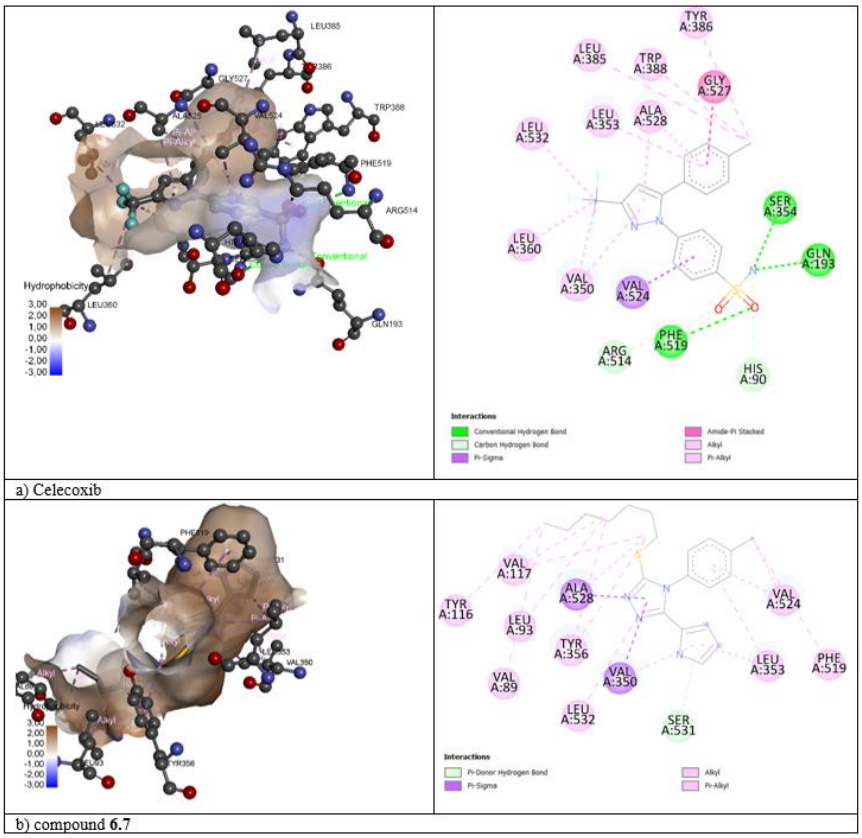

Figure 3. Visualization scheme for binding celecoxib and compound **6.7** to COX-2

Table 1. The result of visualization of the interaction of the synthesized substances with the active site of COX-2

№	Amino acid fragments
6	Ala A: 528, Gly A: 527, Leu A: 385, Ser A: 354, Trp A: 388, Tyr A: 356, Tyr A: 386, Val A: 350, Val A: 524
6.1	Ala A: 528, Leu A: 353, Leu A: 532, Phe A: 519, Ser A: 531, Tyr A: 356, Val A: 350, Val A: 524
6.2	Ala A: 528, Leu A: 353, Leu A: 360, Leu A: 532, Tyr A: 356, Val A: 117, Val A: 350, Val A: 524
6.3	Ala A: 528, Arg A: 121, Glu A: 525, Leu A: 93, Leu A: 532, Pro A: 529, Ser A: 120, Tyr A: 116, Tyr A: 356, Val A: 89, Val A: 117
6.4	Ala A: 528, Arg A: 121, Glu A: 525, Leu A: 93, Leu A: 360, Leu A: 532, Phe A: 358, Pro A: 529, Ser A: 120, Tyr A: 116, Tyr A: 356, Val A: 89, Val A: 117,
6.5	Ala A: 528, Arg A: 121, Leu A: 353, Leu A: 532, Phe A: 519, Pro A: 529, Val A: 117, Val A: 350, Val A: 524,
6.6	Ala A: 517, Arg A: 514, His A: 90, His A: 95, Pro A: 515, Tyr A: 91
6.7	Ala A: 528, Leu A: 93, Leu A: 385, Leu A: 532, Phe A: 519, Ser A: 531, Tyr A: 116, Tyr A: 356, Val A: 89, Val A: 117, Val A: 350, Val A: 524
6.8	Ala A: 528, His A: 90, Leu A: 93, Leu A: 353, Leu A: 532, Tyr A: 116, Tyr A: 356, Val A: 89, Val A: 117
6.9	Ala A: 528, His A: 90, Leu A: 93, Leu A: 353, Leu A: 532, Ser A: 531, Tyr A: 116, Tyr A: 356, Val A: 89, Val A: 117, Val A: 350
6.10	Ala A: 528, Arg A: 121, His A: 90, Leu A: 353, Leu A: 532, Ser A: 120, Tyr A: 356, Val A: 89, Val A: 117, Val A: 350, Val A: 524, Val A: 532

The minimum free binding energy of the synthesized compounds with COX-2 is in the range of -0.4...-8.5 kcal×mol^{-1}, which allows us to indirectly assess the probability of anti-inflammatory activity as low (Table 2).

Table 2. Energy of interaction with COX-2

№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$
6	-8.3	6.4	-6.6	6.8	-0.4
6.1	-8.2	6.5	-8.4	6.9	-0.7
6.2	-7.4	6.6	-4.8	6.10	-7.3
6.3	-6.6	6.7	-8.5	*Celecoxib*	-13.4

* ε_{min} - The minimum energy of complex formation.

The nature of the amino acid residues involved in the formation of bonds with fluconazole and the studied compounds in most cases was identical (Figure 4). However, in terms of the number of bonds formed, a number of the synthesized compounds are superior to fluconazole (Table 3). The active role here is played by the S-alkyl substituent of the 1,2,4-triazole cycle, with which most hydrophobic interactions are formed. For example, the heptyl substituent of the most promising compound **6.7** is involved in the formation of alkyl interactions with residues Ala A: 256, Arg A: 96, Leu A: 100, Leu A: 152, Phe A: 399 and Val A: 395 (Figure 4). It is also necessary to note the possibility of π-alkyl interaction involving phenyl, pyrrole and triazole fragments and residues Cys A: 394, Leu A: 324 and Arg A: 96, respectively. This interaction is enhanced by π-π T-stacking contacts, which can occur with the active role of the π-chemical bond of the pyrrole synthon and the residue Tyr A: 76. The presented picture of interactions is qualitatively complemented by intermolecular hydrogen bonds that arise between the Hydrogen atom of the NH group of the pyrrole and the residue Gln A: 72.

The minimum energy of affinity of the synthesized compounds for lanosterol 14α-demethylase is expectedly high, although none of them exceeds fluconazole in this respect (Table 4).

The development of a biologically active substance with high activity and low toxicity is one of the main tasks of modern medicinal chemistry. The level of pharmacological activity, as well as toxicity, is determined by a number of factors, including the speed and completeness of absorption, distribution, metabolism and elimination. It is possible to preliminarily assess these indicators using the SwissADME computer service, which allows calculating physicochemical, pharmacokinetic, drug-like properties and related parameters.

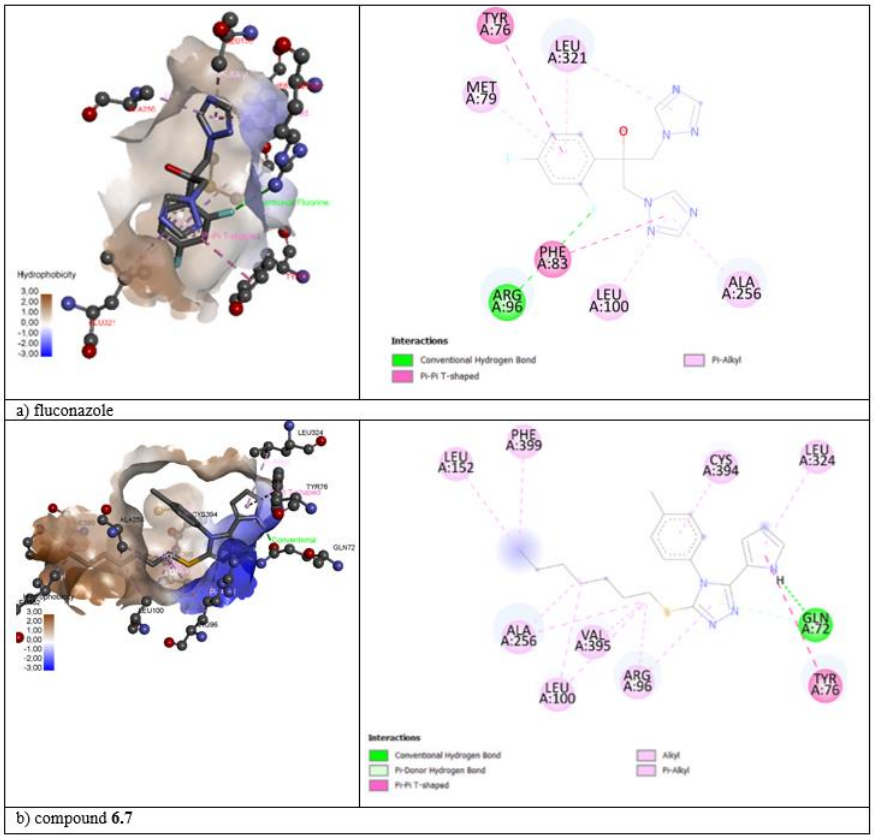

Figure 4. Imaging scheme for binding fluconazole and compound **6.7** to lanosterol 14α-demethylase

Table 3. The result of visualization of the interaction of the synthesized substances with the lanosterol 14α-demethylase active site

№	Amino acid fragments
6	Ala A: 256, Ala A: 400, Cys A: 394, Leu A: 315, Phe A: 387, Pro A: 320, Thr A: 260
6.1	Ala A: 76, Ala A: 88, Lys A: 79, Lys B: 79, Ser A: 80, Phe A: 84, Tyr A: 92, Ile A: 64
6.2	Ala A: 256, Arg A: 96, His A: 259, Leu A: 321, Leu A: 324, Phe A: 78, Tyr A: 76, Val A: 434
6.3	Ala A: 256, Arg A: 96, Cys A: 394, His A: 259, Leu A: 100, Leu A: 321, Met A: 79, Phe A: 78, Phe A: 83, Tyr A: 76, Val A: 434
6.4	Ala A: 76, Ala B: 76, Ala A: 88, Glu A: 83, Leu A: 100, Leu A: 152, Leu A: 321, Ser A: 80, Ser B: 80, Phe A: 84, Tyr A: 92
6.5	Ala A: 256, Arg A: 96, His A: 392, Leu A: 321, Leu A: 324, Met A: 79, Val A: 395
6.6	Ala A: 256, Arg A: 96, His A: 392, Leu A: 321, Leu A: 324, Met A: 79, Val A: 395
6.7	Ala A: 256, Arg A: 96, Cys A: 394, Gln A: 83, Leu A: 100, Leu A: 152, Leu A: 321, Phe A: 399, Tyr A: 76, Val A: 395
6.8	Ala A: 256, Cys A: 394, Leu A: 321, Leu A: 324, Pro A: 320, Phe A: 78, Tyr A: 76
6.9	Ala A: 256, Arg A: 96, Cys A: 394, Leu A: 100, Leu A: 321, Met A: 79, Met A: 99, Phe A: 83, Tyr A: 76
6.10	Ala A: 256, Arg A: 96, Cys A: 394, Leu A: 100, Leu A: 321, Met A: 79, Met A: 99, Phe A: 83, Tyr A: 76

Table 4. Energy of interaction with lanosterol-14α-demethylase

№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$
6	-7.4	6.4	-8.0	6.8	-7.3
6.1	-6.7	6.5	-8.2	6.9	-7.4
6.2	-6.8	6.6	-8.3	6.10	-7.6
6.3	-6.8	6.7	-8.7	*Fluconazole*	-10.9

* ε_{min} - The minimum energy of complex formation.

ADME Analysis

The development of a biologically active substance with high activity and low toxicity is one of the main tasks of modern medicinal chemistry. The level of pharmacological activity, as well as toxicity, is determined by a number of factors, including the speed and completeness of absorption, distribution, metabolism and elimination. It is possible to preliminarily assess these indicators using the SwissADME computer service, which allows calculating physicochemical, pharmacokinetic, drug-like properties and related parameters.

Based on the results of this analysis, it was found that the synthesized compounds meet the required criteria in terms of molar mass (the criteria from 150 to 500 g/mol), molecular refraction (the criteria from 40 to 130) and topological polar surface area (the criteria from 20 to 130 Å2) (Table 5). At the same time, three compounds (**6.8, 6.9** and **6.10**) do not meet the required value in terms of the number of rotating bonds (there should be no more than 9 rotating links) (Table 5). Similarly, compounds **6.1, 6.2, 6.3** do not have the required degree of saturation, which is expressed by the presence of a fraction of sp^3-hybridized Cabons not less than 0.25 (Table 5).

The arithmetic mean of the five indicators (iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT) allows to estimate the required level of lipophilicity. For example, according to the Log Po/w (MLOGP) index, compounds **6.7-6.10** do not meet the required value (should be < 4.15), according to the Log Po/w (XLOGP3) index - compounds **6-10** (should be from -0.7 to + 5.0), according to the Log Po/w (WLOGP) index - compounds **6.7-6.10** (must be < 5.88) (Table 5). Thus, in a number of the synthesized compounds, derivatives with the number of carbon atoms in the alkyl substituent not exceeding six have the required lipophilicity (Table 5). Most of the synthesized compounds (**6.1-6.7**) successfully pass the Lipinski, Ghose, Veber, Egan and Muegge filters (Table 5). In addition, given the bioavailability criterion of 0.55 for these seven compounds, *in vivo* bioavailability using rats can be expected to be 10% or higher (Table 5).

The probability of gastrointestinal absorption for most of the synthesized compounds (**6.1-6.8**) is high (Table 5). However, only three of the obtained substances (**6.1-6.3**) have a chance to cross the blood-brain barrier (Table 5). The range of values of the molecular permeability index through the skin (log Kp) is from -5.9 to -3.49 cm/s, which suggests a satisfactory possibility of realizing this property (Table 5). If the log Kp value is > -2.5 cm/s, then it is concluded that the ability to penetrate the skin is relatively low.

According to the prediction results, most of the compounds (**6.1-6.8**) are not substrates of P-glycoprotein, an ATP-binding cassette transporter that removes toxins and xenobiotics from the cell (Table 5). Therefore, the risk of multidrug resistance to these compounds is low.

Synthetic accessibility is estimated in the range from 1 (very easy to synthesize) to 10 (very difficult to synthesize). In the range of synthesized compounds, this indicator forms an interval of 2.67 - 3.82, i.e., the realization of the synthesis of these compounds is quite realistic (Table 5).

Table 5. Results of ADME analysis of synthesized compounds

Molecular descriptor/ constant	**Compound**									
	6.1	**6.2**	**6.3**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**6.10**
Fraction Csp^3	0.08	0.14	0.20	0.25	0.29	0.33	0.37	0.40	0.43	0.45
Number of rotatable bonds	3	4	5	6	7	8	9	10	11	12
Molar refractivity	77.88	82.68	87.49	92.30	97.10	101.91	106.72	111.53	116.33	121.14
TPSA, $Å^2$	71.80	71.80	71.80	71.80	71.80	71.80	71.80	71.80	71.80	71.80
Log Po/w (iLogP)	2.64	2.64	3.15	3.30	3.51	3.66	3.76	4.02	4.33	4.69
Log Po/w (XLogP3)	3.04	3.41	3.94	4.29	4.84	5.38	5.92	6.46	7.00	7.54
Log Po/w (WLogP)	3.64	4.03	4.42	4.81	5.20	5.59	5.98	6.37	6.76	7.15
Log Po/w (MLogP)	2.83	3.08	3.33	3.58	3.81	4.05	4.27	4.50	4.72	4.93
Log Po/w (SILICOS-IT)	3.22	3.57	3.93	4.30	4.68	5.06	5.45	5.84	6.24	6.63
Consensus Log Po/w	3.07	3.35	3.75	4.06	4.41	4.75	5.08	5.44	5.81	6.19
Lipinski filter	+	+	+	+	+	+	+	-	-	-
Ghose filter	+	+	+	+	+	+	+	-	-	-
Veber filter	+	+	+	+	+	+	+	+	-	-

Table 5 (*continue*). Results of ADME analysis of synthesized compounds

Molecular descriptor/ constant	Compound									
	6.1	**6.2**	**6.3**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**6.10**
Egan filter	+	+	+	+	+	+	+	-	-	-
Muegge filter	+	+	+	+	+	+	+	-	-	-
Bioavailability Score	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Gastrointestinal absorption	High	High	High	High	High	High	High	High	Low	Low
Blood-brain barrier is permeable	+	+	+	-	-	-	-	-	-	-
P-glycoprotein substrate	-	-	-	-	-	-	-	-	+	+
Log Kp, cm/s	-5.92	-5.74	-5.45	-5.28	-4.98	-4.68	-4.38	-4.09	-3.79	-3.49
PAINS alert	0	0	0	0	0	0	0	0	0	0
Brenk's alert	0	0	0	0	0	0	0	0	0	0
Leadlikeness	+	+	-	-	-	-	-	-	-	-
Synthetic accessibility	2.67	2.85	3.03	3.14	3.25	3.36	3.48	3.48	3.70	3.82

The SwissADME radar can also be used for rapid bioavailability assessment. According to the results of this method, compounds **6.4** and **6.5** can be considered drug-like. It is for these compounds that the radar plot falls within the required physicochemical range, which is determined by lipophilicity, size, polarity, solubility, saturation and flexibility (pink area in the figure) (Figure 5).

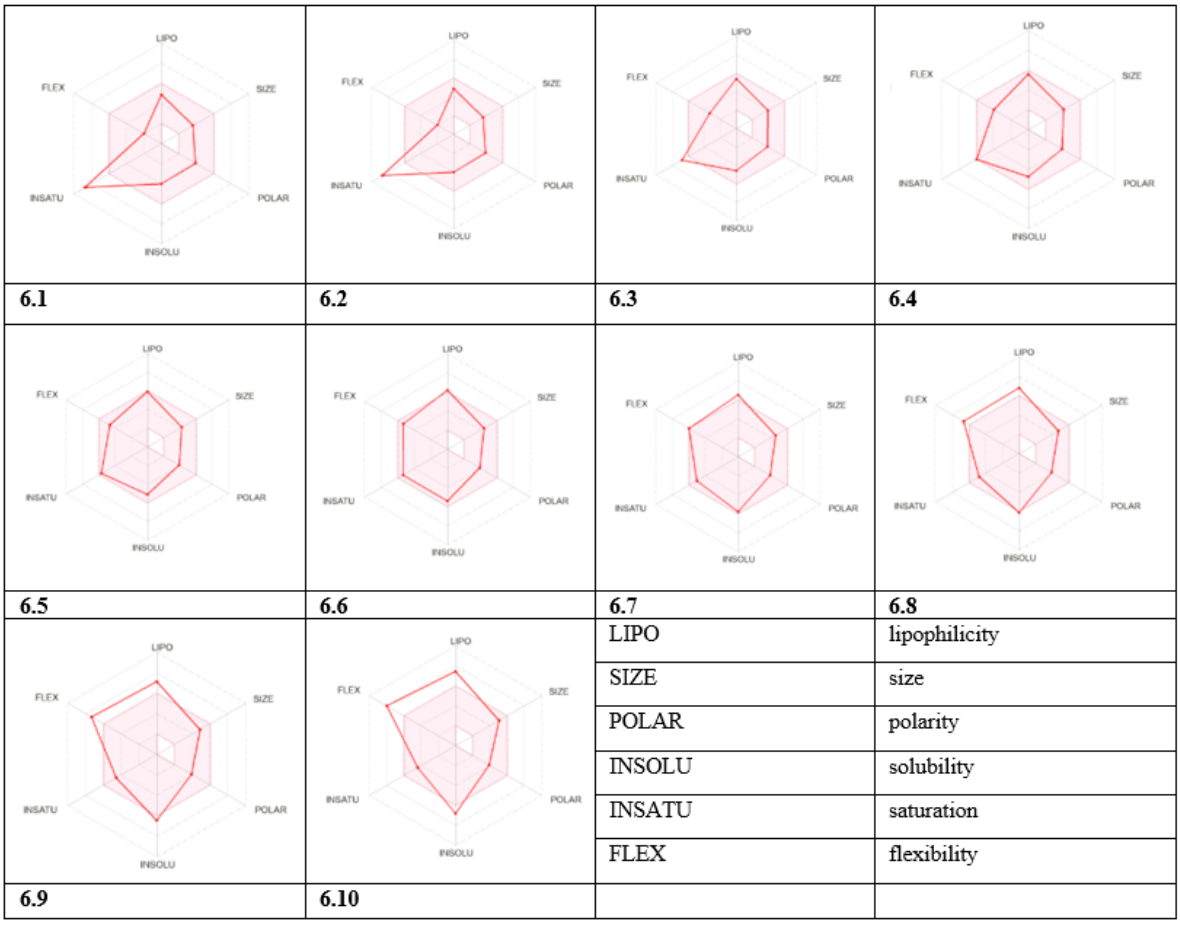

Figure 5. SwissADME bioavailability radar

The synthesis of new derivatives of *S*-alkyl 4-(4-chlorophenyl)-5-(pyrrole-2-yl)-1,2,4-triazole-3-thiol has been successfully carried out. The structure and identity of all substances have been confirmed. The results of the ADME analysis and molecular modelling helped to confirm the prospects for further more in-depth studies of a number of synthesized compounds. The most attractive for further biological studies is the antifungal activity.

AUTHOR CONTRIBUTIONS

Concept: A.G., S.F.; Design: A.G., S.F.; Control: A.G.; Sources: A.G., S.F.; Materials: S.F., O.Z., T.T., T.B.; Data collection and/or Processing: S.F., O.Z., T.T.; Analysis and/or Interpretation: A.G., S.F.; Literature Review: A.G., O.Z., T.B. T.T.; Manuscript Writing: A.G., S.F.; Critical Review: T.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., 47(3): 1033-1040, 2023
Doi: 10.33483/jfpau.1352203

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT OF AMPHOTERICIN-B LOADED NANOPARTICLES AND EVALUATION THE ANTIMICROBIAL POTENCY

AMFOTERİSİN-B ENKAPSÜLE EDİLMİŞ NANOPARTİKÜLLERİN ANTİMİKROBİYAL POTENSİNİN DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *The aim of this study is the development of Amphotericin B loaded polymeric nanoparticles and the determination of the potency of Amphotericin B nanoformulation samples and commercially supplied Amphotericin B samples in comparison with reference Amphotericin B standard, according to the protocol detailed in the United States Pharmacopoeia.*

Material and Method: *Amphotericin B nanoparticles were fabricated using single emulsion method. The comparison of the potencies of the AmB nanoformulation and commercial Amphotericin B with the antimicrobial potency of the reference Amphotericin B standard was performed using the disk diffusion method specified in the United States Pharmacopeia.*

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Submitted / Gönderilme : 29.08.2023
Accepted / Kabul : 04.09.2023
Published / Yayınlanma : 20.09.2023

Result and Discussion: *Amphotericin B loaded poly(ethylene glycol)-b-poly(ε-caprolactone) nanoparticles successfully developed having the average hydrodynamic diameter of 215.14±0.72 nm and PDI value of 0.18±0.02. The Amphotericin B encapsulation efficiency, which was determined using an HPLC method, was 66.4±1.42%. The % potency of commercial Amphotericin B was calculated as 95.7%, while the % potency of the nanoformulation of Amphotericin B was calculated as 99.1%, indicating the favor of utilizing polymeric nanoparticles as delivery systems.*
Keywords: *Amphotericin B, antimicrobial potency, pharmacopoeia method, polymeric nanoparticles*

ÖZ

Amaç: *Bu çalışmanın amacı, Amfoterisin B yüklü polimerik nanopartiküllerin geliştirilmesi ve Amfoterisin B nanoformülasyon örneklerinin ve ticari olarak temin edilen Amfoterisin B örneklerinin potensinin, Amerika Birleşik Devletleri Farmakopesi'nde ayrıntılı olarak açıklanan protokole göre referans Amfoterisin B standardı ile karşılaştırmalı olarak belirlenmesidir.*
Gereç ve Yöntem: *Amfoterisin B nanopartikülleri tek emülsiyon yöntemi kullanılarak üretilmiştir. Referans Amfoterisin B standardının antimikrobiyal potensi ile ticari Amfoterisin B ve Amfoterisin B'nin nanoformülasyonunun potenslerinin kıyaslanması, Amerika Birleşik Devletleri Farmakopesinde belirtilen disk difüzyon yöntemi kullanılarak gerçekleştirilmiştir.*
Sonuç ve Tartışma: *Ortalama hidrodinamik çapı 215.14±0.72 nm ve PDI değeri 0.18±0.02 olan Amfoterisin B yüklü poli(etilen glikol)-b-poli(ε-kaprolakton) nanopartikülleri başarıyla geliştirilmiştir. HPLC yöntemi kullanılarak belirlenen Amfoterisin B enkapsülasyon etkinliği %66.4±1.42 olarak bulunmuştur. Ticari Amfoterisin B'nin % potensi %95.7 olarak hesaplanırken, Amfoterisin B'nin nanoformülasyonunun % potensi %99.1 olarak hesaplanmış olup bu bulgu, taşıyıcı sistem olarak polimerik nanopartiküllerin kullanılmasının avantajını ortaya koymaktadır.*
Anahtar Kelimeler: *Amfoterisin B, antimikrobiyal potens, farmakope yöntemi, polimerik nanopartiküller*

INTRODUCTION

Polymeric nanoparticles, which are comprised of synthetic or natural polymers, are one of the most frequently utilized forms of nanoparticles for drug delivery. Polymeric based particles have the ability to encapsulate medicinal molecules within their core or matrix, or to attach them to their surface. In order to enhance their interaction with biological systems, polymeric nanoparticles may be made with a variety of forms, sizes, charges, and surface features [1]. Polymeric nanoparticles can also be modified with functional groups or ligands to improve their targeting, controlled release, or stimulus sensitivity. Polymeric nanoparticles, for example, can be coated with antibodies, peptides [2], or aptamers capable of recognizing particular receptors on the surface of target cells or tissues [3]. Polymeric nanoparticles can also be programmed to release medications in response to pH, enzymes, temperature, light, or magnetic field changes. Polymeric nanoparticles for drug molecules encapsulation have many advantages over conventional drug delivery systems. They can increase the loading capacity and stability of drugs, reduce their toxicity and side effects, prolong their circulation time and half-life, and improve their pharmacokinetics and pharmacodynamics. Polymeric micro and nanoparticles for drug molecules encapsulation have been used for delivering various types of drugs, such as anticancer agents [4], anti-inflammatory agents [5], antibiotics, antiviral agents, vaccines, gene therapy vectors, and imaging contrast agents [6]. Polymeric nanoparticles for drug molecule encapsulation have shown promising results in preclinical and clinical studies for treating various diseases and disorders [7].

Amphotericin B (AmB) is an antifungal drug used at antifungal treatments. Coccidioidomycosis, aspergillosis, mucormycosis, candidiasis, and blastomycosis are all significant, life-threatening fungal diseases that can be treated with it. It's also efficient against protozoan parasites like leishmaniasis. AmB acts by binding to ergosterol, a sterol presents in fungal and certain bacteria cell membranes. This impairs the integrity and function of the cell membrane, resulting in cellular content leakage and cell death [8]. Because of its low oral absorption, AmB is commonly taken intravenously. It does, however, have a number of adverse effects, including fever, chills, headache, nausea, vomiting, renal damage, and low blood potassium. As a result, it is reserved for severe or potentially fatal infections that do not

respond to other antifungal medications. AmB is also available in a liposomal formulation, which decreases toxicity and enhances absorption [9]. AmB is a powerful antibacterial drug with a broad spectrum of action that may be used to treat a variety of fungal and parasite illnesses. It does, however, have substantial side effects that necessitate careful monitoring and dose modification. Because of this reason we aimed to formulate AmB with polymeric nanoparticles.

Antimicrobial potency determination is critical for quality control and assurance of antibiotic formulations. Therefore, it is necessary to choose practical and economical methods for quality control of antibiotics. Potency determination can be determined by chemical and biological methods such as microbiological, chemical and immunological tests. The measurement of antibiotic components is usually done by chemical methods including UV and HPLC based analyses. However, these methods cannot reflect real biological activity. Compared to chemical methods, microbiological testing allows to measure the actual effects of antibiotics on biological systems. Microbiological methods are accepted as the standard method because they can reveal small changes in antimicrobial activity that cannot be determined by chemical methods [10].

Determination of antibiotic potency by the microbiological method is a method in which varying concentrations of antibiotics are tested against a living microorganism. Factors such as test microorganism, incubation conditions, amount of inoculum, dose of antibiotic to be tested, preparation of antibiotic standards and equipment are influential on test results. Antibiotic potency determination by microbiological method can be done by plate (cylinder-plate or diffusion) or tube (turbidimetric) methods. While inhibition zones are observed and measured during plate method, turbidity is determined during tube method. The obtained results are evaluated by tabulating and integrating them into a linear regression curve [11].

The aim of this study is the development of AmB loaded polymeric nanoparticles and also the determination of the potency of AmB nanoformulation samples and commercially supplied AmB samples in comparison with reference AmB standard, by disk diffusion method according to United States Pharmacopoeia 2023 (USP) [12].

MATERIAL AND METHOD

Commercial Amphotericin B was purchased from Cayman Chemical and reference Amphotericin B standard (United States Pharmacopoeia reference standard) was purchased Sigma-Aldrich. DMSO, poly(vinyl alcohol) (PVA), and acetonitrile were supplied from Sigma-Aldrich. Poly(ethylene glycol)$_{5000}$-*b*-poly(ε-caprolactone)$_{15000}$ (PEG-PCL) was synthesized and characterized at Yıldız Technical University.

Preparation of AmB Loaded Nanoparticles

The single emulsion-solvent evaporation technique was employed to prepare nanoparticles [13]. Basically, 0.5 mg of AmB is dissolved in 30 µl of DMSO and this solution is mixed with 0.5 ml of chloroform containing 20 mg of PEG-PCL polymer. This solution is added onto the 2.5 ml of PVA solution (1%) and sonicated (35W) on an ice bath for 15 seconds to obtain an emulsion form. Then, obtained emulsion was injected, quickly without making any bubbles, into the 18 ml of PVA solution stirring (1%) on a magnetic stirrer. After 2.5 hours of injection, nanoparticle dispersion was centrifuged at 30.000 rpm for 1 hour, collected, and washed once again then stored for further use.

Determination of the Size and Zeta Potential of Nanoparticles

The zeta potential and particle size distribution of produced nanoparticles were measured using the dynamic light scattering technique. The nanoparticles were diluted at a ratio of 1:50 using pure water and all measurements were done at room temperature using a Zetasizer Nano ZS (Malvern Instruments). The samples were examined three times. Electrophoretic light scattering was used to detect the zeta potential as well.

Determination of the Encapsulated AmB into Nanoparticles

A modified HPLC technique was used to determine the quantity of AmB that was encapsulated

[14]. Briefly, an HPLC equipment with a DAD detector (wavelength set at 388 nm) and a C18 column (Gemini 1004.6 mm, 3m, Phenomenex) was used to determine the quantity of AmB in the sample. Acetonitrile:Buffer (30:70) system was used as the mobile phase, and the injection volume was 20 l. (Buffer: 0.01M Potassium dihydrogen phosphate solution at pH 3.0). Before analysis, the nanoparticle supernatant (0.5 ml), obtained after centrifugation, was mixed 1:1 with ethanol. The analysis was carried out in triplicate at a flow rate of 1.5 ml/min in a column set to 30°C for 10 minutes. The AmB's encapsulation efficiency was presented as percentage encapsulation efficiency.

Morphology Analyses of Nanoparticles

Transmission Electron Microscopy (TEM, FEI Tecnai G2 Spirit) at 120 kV was used to examine the morphology of nanoparticles. The TEM imaging was done on phosphotungstic acid stained nanoparticles. For 2 minutes, 7 µl of nanoparticle dispersion (1.0 mg/ml) was deposited onto carbon coated grids. For 6 seconds, nanoparticles were stained with a 0.75% (w/v) phosphotungstic acid staining solution at pH 7.4. The extra solutions were removed using filtration paper, and the grids were vacuum dried.

Test Microorganisms

In this study, *Saccharomyces cerevisiae* ATCC 9763 strain was used as a test microorganism. *S. cerevisiae* strain was incubated for 48 hours at 29-31°C in M19 medium prepared according to USP. The potency determination was carried out in accordance with the procedure specified in USP 2023 [12].

Preparation of AmB Samples

Potency determination of AmB samples was measured and evaluated according to the median reference standard (S3) and standard curve values (S1, S2, S4, and S5). The samples concentrations were determined according to the USP 2023 [12]. The median concentrations of standard, commercially supplied, and nanoformulation of AmB samples were determined as 25 µg/ml. The tested concentrations of all AmB samples are given in Table 1.

Table 1. The tested concentrations of Amphotericin B samples

Reference Standart Amphotericin B	**S1** 16 µg/ml	**S2** 20 µg/ml	**S3** 25 µg/ml	**S4** 31.25 µg/ml	**S5** 39.0625 µg/ml
Commercial Amphotericin B		**M2** 20 µg/ml	**M3** 25 µg/ml	**M4** 31.25 µg/ml	
Nanoformulation of Amphotericin B		**F2** 20 µg/ml	**F3** 25 µg/ml	**F4** 31.25 µg/ml	

Determination of Potency

The disk diffusion method was used for the determination of potency. Firstly, 25% transmittance at 580 nm in saline was obtained from *S. cerevisiae* ATCC 9763 cultures incubated in M19 medium at 29-31°C for 48 hours. Appropriate amounts of *S. cerevisiae* suspension were added to M19 media cooled to 45-50°C. Then, the media were poured into petri dishes and allowed to solidify. Meanwhile, the samples prepared at the concentrations indicated in Table 1 were absorbed in blank discs. Then the discs were placed on the media as in Figure 1.

After the discs were placed, the petri dishes were incubated at 29-31°C for 48 hours. After incubation period, inhibition zone diameters were measured and evaluated. The study was carried out in triplicate.

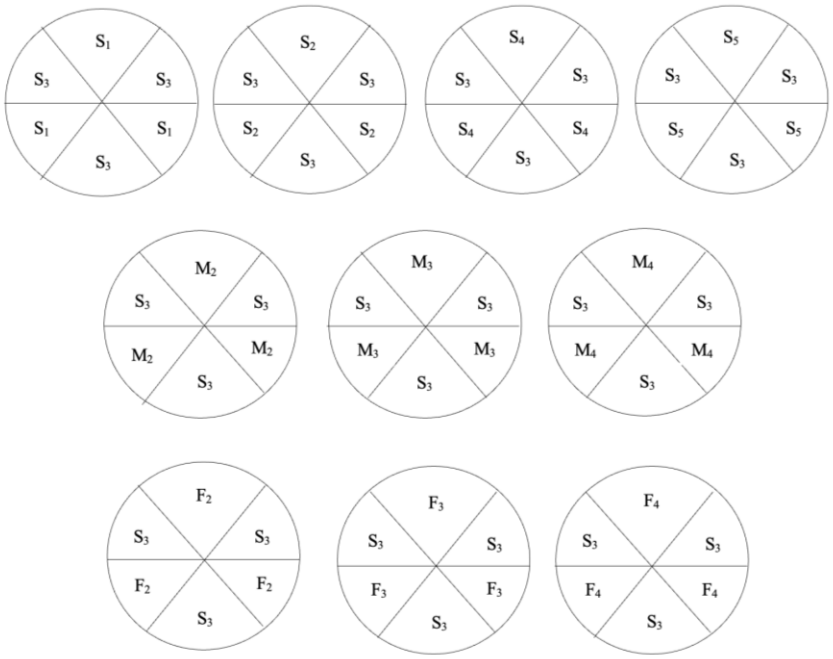

Figure 1. Agar design for determination of AmB potency (S: Reference AmB Standard, M: Commercial AmB, F: Nanoformulation of AmB)

RESULT AND DISCUSSION

AmB loaded nanoformulations were fabricated using single emulsion-solvent evaporation technique which is quite preferred to encapsulate hydrophobic molecules [15]. PEG-PCL copolymer was used to form nanoparticles because of its biodegradation ability and FDA clearance (10.1039/D1CC04941K). Besides, PEG layer limits the undesired interaction with proteins and acts as a steric barrier.

After the nanoformulations were produced, they were subjected to a holistic characterization process. Firstly, the hydrodynamic diameter of the nanoparticles, which indicate their size, were analyzed by dynamic light scattering (DLS) technique. As can be seen from the graph presented in Figure 2A, the average hydrodynamic diameter was determined as 215.14±0.72 nm. The size distribution of the obtained nanoparticles is also in a monomodal narrow distribution as presented in the graph in Figure 2A and the PDI value was found to be 0.18±0.02. In the correlation function graph presented in Figure 2B, the Y-axis intercept value was observed between 0.5-0.9 which indicates that the signal-to-noise ratio in the DLS measurement is at the desired level. The fact that the slope of the same graph is smooth and monomodal (not biphasic and consisting of a single profile) shows that the distribution of the nanoparticles obtained is homogeneous, and the smooth fit with the baseline shows that there are no large aggregates among the nanoparticle population. In addition, the AmB encapsulation efficiency of the nanoparticles was determined as 66.4±1.42% by HPLC analysis and the zeta potential as -17.5±2.3mV which was determined by electrophoretic light scattering method.

After the incubation period of reference standard AmB solution, commercial AmB solution, and AmB naformulation in *Saccharomyces cerevisiae* ATCC 9763, the inhibition zone diameters formed were measured in millimeters. Then mean, standard deviation and RSD% values were calculated. As a result of the study, the R^2 value was calculated as greater than 0.95 and the RSD% value was calculated as less than 10%. The study was conducted at a 95% confidence interval. Inhibition zones formed as a result of the experiment are given in Figure 3.

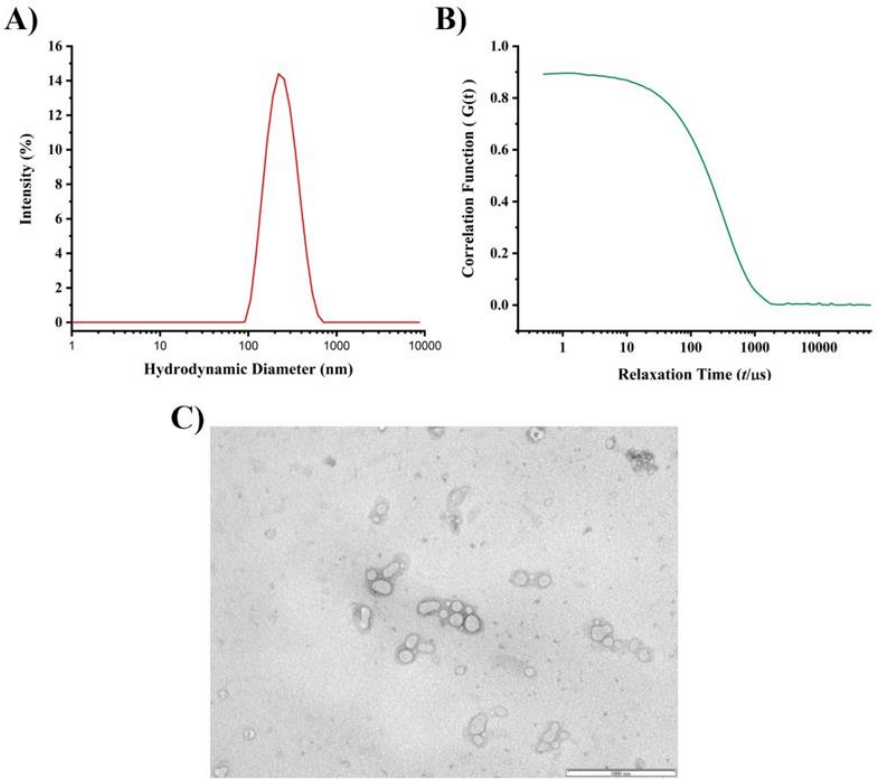

Figure 2. Dynamic light scattering data showing the hydrodynamic diameter (A) and correlation function (B) of the AmB loaded nanoparticles. TEM image of the AmB loaded nanoparticles (C)

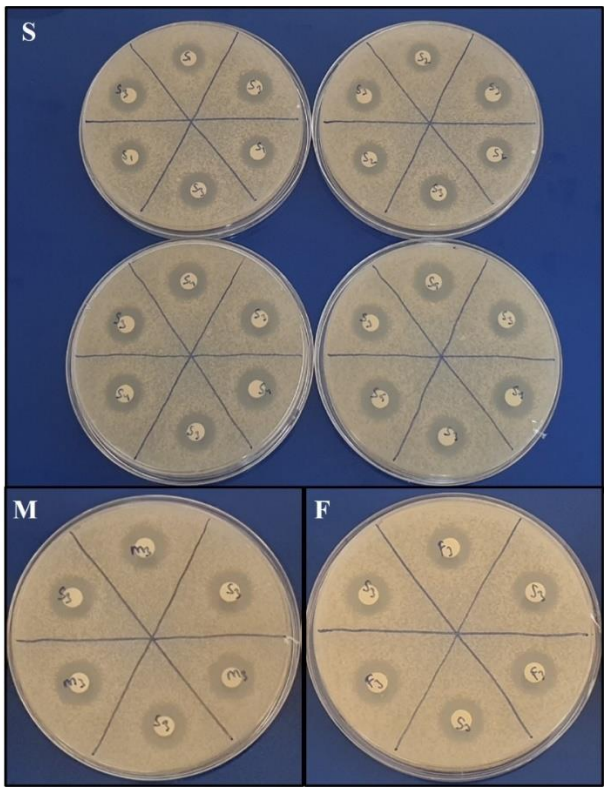

Figure 3. Inhibition zones of reference AmB standard (S), commercial AmB (M), and nanoformulation of AmB (F) samples against *Saccharomyces cerevisiae* ATCC 9763 standard strain

As a result of the experiment, the % potency of commercially supplied AmB was calculated as 95.7%, while the % potency of the nanoformulation of AmB was calculated as 99.1%. These finding indicate the enhanced antimicrobial potency of AmB nanoformulations compared to AmB solution. The utilization of nanoparticles to formulate AmB ending up with synergistic activity might dependend on the increased uptake of of AmB molecules into microbial cell, via nanoparticles [16].

Consequently, AmB loaded PEG-PCL nanoparticle successfully developed and characterized in terms of hydrodynamic diameter, poly dispersity index, zeta potential, and AmB content. The effect of nanoformulation procedure on AmB's antimicrobial potency was unknow while the antimicrobial potency tests detailed in various pharmacopoeias are prerequisite to show formulations efficicacy. Here in this work, we have not only shown that the AmB molecule keeps its antimicrobial potency after the harsh nanoparticle production process, but we also determined that the AmB nanoformulation has a higher antimicrobial effect compared to pristine AmB.

ACKNOWLEDGEMENTS

The authors are thankful to the Technological Research Council of Türkiye for the financial funding of this research (TUBITAK, Grant Number 216S612).

AUTHOR CONTRIBUTIONS

Concept: U.C.Ö., S.S.R., E.Ş.A., G.C., M.E., A.B.; Design: U.C.Ö., S.S.R., E.Ş.A., H.B., S.D., M.E., A.B.; Control: H.B., S.D.; Sources: E.Ş.A., A.B.; Materials: E.Ş.A., H.B., S.D.; Data Collection and/or Processing: U.C.Ö., S.S.R., G.C.; Analysis and/or Interpretation: U.C.Ö., S.S.R., E.Ş.A., G.C., H.B., S.D., M.E., A.B.; Literature Review: U.C.Ö., S.S.R.; Manuscript Writing: U.C.Ö., S.S.R.; Critical Review: E.Ş.A., H.B., S.D., A.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., 47(3): 1041-1052, 2023
Doi: 10.33483/jfpau.1319262

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND *IN VITRO* CHARACTERIZATION OF LIDOCAINE LOADED *ALOE VERA* GEL FORMULATION FOR THE TREATMENT OF BURN WOUNDS

YANIK TEDAVİSİNDE KULLANILMAK ÜZERE LİDOKAİN İÇEREN ALOE VERA JEL FORMÜLASYONUNUN HAZIRLANMASI VE İN VİTRO KARAKTERİZASYONU

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ABSTRACT

Objective: *In this study, topically applied in situ gel formulations were aimed to design for the modulation of burns, with the active ingredient lidocaine and the gel gained from the Aloe vera plant. The prepared in situ gels were in the liquid form at the room temperature and gelled at the body temperature and adhered to the wound surface, resulting in higher drug residence time. By improving the characteristic properties of the in situ gels, it is aimed to improve patient compliance by obtaining higher local lidocaine concentration.*

Material and Method: *In situ gel formulations separated by giving different gel codes were examined with characteristic analyses. Within the scope of these examinations, measurement of gelation temperature, pH measurement, in vitro lidocaine release, viscosity and rheological properties and the texture profile of the formulations were determined.*

Result and Discussion: *Poloxamer 407 based in situ gels designed for topical treatment containing Aloe vera gel and lidocaine have been shown to increase skin residence time. Among the formulations prepared with different content ratios of the polymers Poloxamer 407, Poloxamer 188, HPMC and CMC, the gels coded F_5 and A_{21} showed acceptable gelation temperature for topical use and sustained lidocaine release for 24 hours. According to these findings, it can be revealed that Poloxamer 407-HPMC based in situ gel formulation may be an effective alternative for topical burn*

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Submitted / Gönderilme : 23.06.2023
Accepted / Kabul : 05.09.2023
Published / Yayınlanma : 20.09.2023

treatment.
Keywords: *Aloe vera, burn treatment, in situ gel, lidocaine, topical formulation*

ÖZ

Amaç: *Bu çalışma kapsamında Aloe vera bitkisinden elde edilen jelin, lidokain etken maddesi ile birlikte yanık tedavisinde topikal olarak uygulanacak in situ jel formülasyonlarının geliştirilmesi amaçlanmıştır. Formülasyonların karakteristik özellikleri iyileştirilerek, oda sıcaklığında sıvı formda olan in situ jellerin vücut sıcaklığında jelleşerek yara yüzeyine yapışma suretiyle daha yüksek lokal ilaç konsantrasyonu elde edilerek hasta uyuncunun artırılması hedeflenmiştir.*
Gereç ve Yöntem: *Farklı jel kodları verilerek ayrılan in situ jel formülasyonlarının karakteristik özellikleri analiz edilmiştir. Bu incelemeler kapsamında jelleşme sıcaklığının tespiti, pH ölçümü, in vitro lidokain salım çalışması gerçekleştirilmiş ve in situ jellerin viskozite ve reolojik özellikleri ile tekstür profil analizleri değerlendirilmiştir.*
Sonuç ve Tartışma: *Aloe vera jeli ve lidokain içeren, topikal tedavi için tasarlanmış Poloxamer 407 bazlı in situ jellerin deri üzerinde kalış süresini uzattığı ve lokal lidokain konsantrasyonunu artırabileceği belirlenmiştir. Poloxamer 407, Poloxamer 188, HPMC ve CMC'nin farklı oranları ile hazırlanan in situ jel formülasyonları arasında F_5 ve A_{21} kodlu jeller topikal kullanım için kabul edilebilir jelleşme sıcaklığı göstermiş ve 24 saat lidokain salımı gerçekleştirmiş olup, Aloe vera ve lidokain içeren Poloxamer 407-HPMC bazlı in situ jel formülasyonunun topikal yanık tedavisinde etkili bir alternatif olabileceği sonucuna varılmıştır.*
Anahtar Kelimeler: *Aloe vera, in situ jel, lidokain, topikal formülasyon, yanık tedavisi*

INTRODUCTION

Burn is a significant injury trauma that may result from heat, freezing, electricity, chemicals or radioactive factors and can lead to life-threatening injuries depending on the extent and depth of the damage [1,2]. Burn injuries are the fourth most common trauma sources around the world [3].

In treatment of burn wounds, application of systemic or topical agents are well studied. Main cause of the usage of topical antimicrobial agents is to diminish the development of burn wound sepsis and its related morbidity and mortality [4]. In addition to clinical treatment, medicinal plants take a significant role in the healing of burn wounds due to the various content of alkaloids, flavonoids, terpenoids, tannins, saponins and phenolic compounds [5].

From the literature, it is well established that phytochemicals in medicinal plants show positive effect in the healing process of burn wounds with different burn degrees. This positive effect may be resulted from the antimicrobial, anti-inflammatory, antioxidant, astringent, collagen synthesis stimulator and perfusion enhancing properties of the medicinal plants.

Aloe vera is from the Asphodelaceae family, which is native to Africa, Madagascar and the Arabian Peninsula and cultivated in the South-west coasts of Turkey [5,6]. It is a every green perennial, shrubby plant with rosette leaves. The leaves are thick and fleshy, have grey to green color range, with some variations showing white stains on their upper and lower stem surfaces. The margin of the leaf is milled and has small white teeth. The flowers have yellow color and leaves' color is in green grey spectrum [7,8].

Aloe vera contains high amount of water (99-99.5 %), and solid content (0.5-1 %) is composed of minerals, vitamins, enzymes, polysaccharides, organic acids and phenolic compounds which are soluble in the water or oil [8]. Various parts of the *Aloe vera* contains approximately 70 nutrients as well as 200 active compositions including amino acids, saponins, anthraquinones, lignin, salicylic acid [9]. Anti-inflammatory, laxative, antihistaminic, fibroblast proliferative, burns and wound healing properties of topical *Aloe vera* application in gel form have also been reported [10,11]. It is also used in treatment of skin traumas, as well as, frostbite, rashes, cold sores, dry skin, skin ulcers, psoriasis and seborrheic eczema [6,12,13].

Aloe vera gel is frequently used in the topical treatment of minor burns, sunburns and X-ray burns [6]. The anti-inflammatory property of the *Aloe vera* contributes to the improvement of the inflammatory process caused by the burn injury. Since blocking the formation of vasoactive prostanoids prevents vasoconstriction thrombosis and the progressive ischemic necrosis known to ocur in thermal and electric

burns as a result of thromboxane production, the application of *Aloe vera* can prevent the progressive nature of thermal injury and the provides control of the bacterial growth in the burn wound [14].

Local anesthetics has activity on the sodium ion channels to decrease the permeability of cell membranes; by preventing depolarization and the conduction of electric impulses [15]. Lidocaine belongs to the amide class local anesthetics, and preferred in inhibiting sense of pain with nerve blockade, as stabilizing the neuronal membrane by blocking the ionic fluxes of initiation and transmission of impulses [16,17].

Topical application of an agent refers to a method in which the formulation is applied to superficial regions; such as the skin or ocular, otic and vaginal tissues for the treatment of local diseases [18]. Despite the diversity of formulation systems, semi-solid formulations are frequently used in topical applications [9]. Topical administration provides a great advantage as avoiding the risks associated with intravenous therapy [19,20]. Gels are semi-solid formulations prepared with a suitable gelling agent, possesses the viscosity varying between 1000 and 100000 mPa.s. Gels provide higher solubilization of drugs due to its higher water content in comparison to the creams and ointments. Additionally, gels can hydrate skin and facilitate the drug transport by retaining considerable amount of transepidermal water [21].

In situ gelling formulations are polymeric carriers that are in solution form before contacting to the body, but transform into the gel construction at the physiological conditions [22-24]. The transition from solution to gel phase is dependent on one or more of different stimuli; such as pH shift, temperature menagement, solvent change, ultraviolet radiation, and the content of particular ions or molecules. Thermogels transform from solution to gel with temperature modulation; while they are in liquid form at the room temperature (20-25°C), they turn into gel form when they contact to the body fluids (32-37°C) [25]. *In situ* gel formulations have gained great interest in the last few years as they provide an advantage over conventional delivery systems to achieve plasma drug concentration [22,26]. A rising number of *in situ* gel forming systems have been studied and many patents have been reported for their application in a variety of biomedical fields, including drug delivery [26].

MATERIAL AND METHOD

Chemicals

Lidocaine (Sigma Aldrich, Germany) as an active agent, Poloxamer 407® (P-407) (Sigma-Aldrich, Germany), Poloxamer 188® (P-188) (Sigma-Aldrich, Germany), hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich, Germany), carboxymethyl cellulose (CMC) (Sigma-Aldrich, Germany) as the polymer for *in situ* gelling systems. Benzalkonium chloride (Sigma Aldrich, Germany) is preferred as the preservative and distilled water is preferred as solvent.

Plant Material

Aloe vera samples were obtained from Çukurova University Ali Nihat Gökyiğit Medicinal and Aromatic Plants Garden, in January 2022. Collected *Aloe vera* samples were separated from leaves and homogenized in the laboratory to obtain *Aloe vera* gel.

Preparation of Gel Formulations

In situ gel formulations were prepared by the cold method with different polymer concentrations [27,28]. Shortly, weighed amount of Poloxamer 407 (12%-22% w/v) was dissolved in the distilled water and stirred on a magnetic stirrer to obtain a clear solution at least 12 hours at 4°C. Lidocaine concentration (5% w/v) was kept constant for all the formulations and added to the each solution with continuous mixing. HPMC and CMC solutions was prepared separately by incorporating a given amount (0.5% - 5% w/v) in water and then, mixed the with Poloxamer 407 solution. As following, different amount of *Aloe vera* gel (10-20-25-20% w/v) was added to prepared *in situ* gel formulations.

Determination of Sol-Gel Transition Temperatures

The temperature at the phase transition from sol to gel phase is recorded as the sol-gel transition temperature. The gelation temperatures were decided by the tube rotation method [29].

The gel sample was taken into a glass vial and temperature was gradually increased by the water bath. The specific temperature which the sample turn into gel from the sol gel form was recorded. The sol-gel transition temperature studies were done in triplicate for each formulation.

Determination of Lidocaine

Analytic validation by UV for Lidocaine was performed with phosphate buffer (pH 6.8) at the wavelength of 263 nm [13,30]. Partial validation was evaluated in the scope of linearity, precision and accuracy parameters [31]. The standard curves (n=3) were studied at the 50, 100, 150, 200, 250, 300, 400, 500 and 1000 µg/ml of concentrations. Concentrations of 100, 200, 400 µg/ml (n=3) were studied for precision while 50, 300, 500 µg/ml (n=3) were studied for accuracy.

Organoleptic Evaluation and pH Analysis

The color, odor and state of prepared *in situ* gels were evaluated by physical appearance for organoleptic determination. pH values of the *in situ* gels were determined with digital pH meter (WTW Profi Lab. pH 597, Germany) in triplicate and average values with deviations were recorded.

Examination of Rheological Behavior

The dynamic properties of the gels were measured using the Haake Rheometer I (Thermo Fisher Scientific Inc., Essen, Germany) (n=3). The rheologic characteristics of *in situ* gels were measured at 25 ± 0.5°C and 37± 0.5°C. The sample was places on the platform and shear rate evaluation was done between 0-2000 s^{-1}. RheoWin 4.87.0006 (Haake®) software was used to evaluate the results [32].

Texture Profile Analysis (TPA) of Gel Formulations

Mechanical characteristics of the gels including cohesiveness, adhesiveness and hardness were analyzed using Texture Analyzer (TA.XT. Plus C, Stable Micro System, Haslemere, Surry, UK). 10 mm diameter Perspex probe (SNSP/10, h: 10 mm) was used to measurement with 5 kg loading capacity. *In situ* gels were measured by placing 10 g of gels into a 25 mm beaker at 37± 0.5°C. Test parameters are given: Speed Before Test 2 mm/s, Test Speed 2mm/s Speed After Test 2 mm/s, Trigger Force 0.001 N.

***In vitro* Release Study**

In vitro drug release studies were conducted using dialysis membrane (Sigma-Aldrich, Germany; Molecular weight cut-off = 14,000 Da) using phosphate buffer as dissolution medium [33]. 1 ml of *in situ* gel formulation was placed in dialysis bags in 40 ml of dissolution medium of phosphate buffer with 100 rpm rate of stirring. At determined time intervals, 1 ml of samples were collected and were analyzed by UV spectrophotometer. Phosphate buffer was replaced by the same amount of media to remain sink conditions. Experiments were conducted in triplicate.

RESULT AND DISCUSSION

In this study, for the preparation of thermosensitive *in situ* gelling system of Lidocaine and *Aloe vera,* the cold method was used. *Aloe vera* was preferred due to anti-inflammatory, wound and burn healing properties of topical *Aloe vera* application in gel form have been reported, beside its moisturizing and soothing effects [10,11]. *Aloe vera* gel was gained from the leaves of the *Aloe vera* plant. *Aloe vera* gel was obtained by slicing the two leaves of the plant from the base (Figure 1) [34].

All the formulations were visually evaluated in light against alternative black and white backgrounds before and after gelling. Most of *in situ* gels prepared for this study were transparent at all test temperatures (25 and 37°C). The formulations prepared were found to be visually homogenous and clear, with no phase separation.

Sol-gel transition temperature was determined by visual inspection for different concentrations of gel. The measurements of sol-gel transition temperature were conducted by the tube rotation method (n=3). As predicted, it was established that gelation temperature decreased with the increasing content of Poloxamer 407. Some formulations tested did not exhibit gelling properties at any temperature (in the range of 20°C to 50°C). With the aim of ensure gelation of the thermoreversible gel at body

physiological temperature, a gelation temperature 37°C was selected. Compositions and sol-gel transition temperature of the selected *in situ* gel formulations prepared are detailed in Table I. Characterization studies were carried out with formulations F_5 and A_{21}.

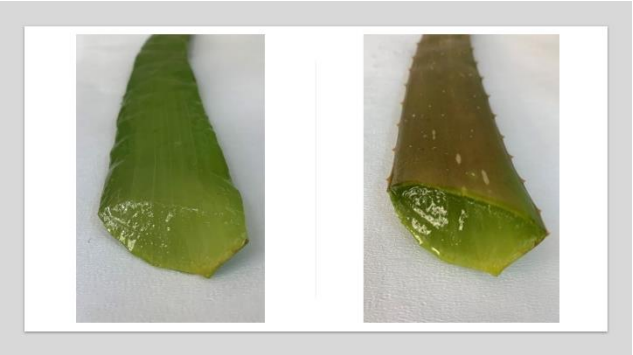

Figure 1. Leaves of the *Aloe vera* plant

Table 1. Composition percentage of *in situ* gel formulations and sol-gel transition temperatures

Code	P407 (%)	HPMC (%)	*Aloe vera* gel (%)	Lidocaine (%)	Benzalkonium chloride (%)	Sol-Gel Temp. (±0.5°C, n=3)
F_1	18	0.5	-	5	0.01	30.5°C
F_2	18	1	-	5	0.01	32.8°C
F_3	18	2.5	-	5	0.01	Solid Below 5°C
F_4	18	5	-	5	0.01	Solid Below 5°C
F_5	20		-	5	0.01	35.3°C
F_6	20	0.5	-	5	0.01	28.8°C
F_7	20	1	-	5	0.01	28.3°C
F_8	20	2.5	-	5	0.01	Solid Below 5°C
F_9	20	5	-	5	0.01	Solid Below 5°C
F_{10}	22	0.5	-	5	0.01	25.5°C
F_{11}	22	1	-	5	0.01	25.6°C
F_{12}	22	2.5	-	5	0.01	Solid Below 5°C
F_{13}	22	5	-	5	0.01	Solid Below 5°C
A_1	18	0.5	10	5	0.01	31.8°C
A_2	18	0.5	20	5	0.01	Liquid Above 50°C
A_3	18	0.5	25	5	0.01	Liquid Above 50°C
A_4	18	1	10	5	0.01	Liquid Above 50°C
A_5	18	1	20	5	0.01	Liquid Above 50°C
A_6	18	2.5	10	5	0.01	Solid Below 5°C
A_7	18	2.5	20	5	0.01	Solid Below 5°C

Table 1 (*continue*). Composition percentage of *in situ* gel formulations and sol-gel transition temperatures

Code	P407 (%)	HPMC (%)	*Aloe vera* gel (%)	Lidocaine (%)	Benzalkonium chloride (%)	Sol-Gel Temp. (±0.5°C, n=3)
A_8	18	2.5	50	5	0.01	Solid Below 5°C
A_9	18	5	10	5	0.01	Solid Below 5°C
A_{10}	18	5	20	5	0.01	Solid Below 5°C
A_{11}	18	5	50	5	0.01	Solid Below 5°C
A_{12}	20	0.5	10	5	0.01	Liquid Above 50°C
A_{13}	20	0.5	20	5	0.01	Liquid Above 50°C
A_{14}	20	0.5	25	5	0.01	Liquid Above 50°C
A_{15}	20	1	10	5	0.01	31.8°C
A_{16}	20	1	15	5	0.01	Liquid Above 50°C
A_{17}	20	1	20	5	0.01	Liquid Above 50°C
A_{18}	20	1	25	5	0.01	Liquid Above 50°C
A_{19}	20	5	50	5	0.01	Solid Below 5°C
A_{20}	22	0.5	10	5	0.01	28.5°C
A_{21}	22	0.5	20	5	0.01	36.5°C
A_{22}	22	0.5	25	5	0.01	Liquid Above 50°C
A_{23}	22	1	10	5	0.01	27.5°C
A_{24}	22	1	20	5	0.01	31.8°C
A_{25}	22	1	22.5	5	0.01	33.3°C
A_{26}	22	1	25	5	0.01	Liquid Above 50°C
A_{27}	22	5	10	5	0.01	Solid Below 5°C
A_{28}	22	5	20	5	0.01	Solid Below 5°C
A_{29}	22	5	50	5	0.01	Solid Below 5°C

The quantification analyses of the polymeric gel formulations were conducted using a UV spectrophotometer. For partial validation of the analytical method for the determination of lidocaine content; linearity, accuracy, precision and selectivity properties were evaluated [31].

To obtain the calibration curve 50, 100, 150, 200, 250, 300, 400 and 500 µg/ml concentrations were studied. The equation and the curve of the Lidocaine concentration/Absorbance values were obtained.

The precision of the method was evaluated by recovery studies done in three concentration levels of 100, 200, 400 µg/ml. Results were calculated as 0.111±0.018; 0.278±0.019 and 0.584±0.014 in order. The analyses were conducted on the same day to evaluate repeatability or intra-day variability and on different days to determine the intermediate precision or inter-day variability. Samples were prepared in three concentration levels of 50, 300, 500 µg/ml The results were calculated with the equation

obtained from calibration curve and compared to the known concentrations, and the mean (%) recovery of samples were found to be 95.929%.

pH values of F_5 and A_{21} formulations prepared with Lidocaine were measured and mean and standard deviation values were calculated. The pH values of F_5 and A_{21} formulations were determined 5.864±0.020 and 5.567±0.032, respectively.

Rheological evaluation was performed to determine the flow properties of the formulation. The rheograms of the F_5 and A_{21} formulations containing lidocaine are presented at Figure 2. Measurements were done at both 25 and 37°C.

Mechanical properties of the *in situ* gels including cohesiveness, adhesiveness and hardness were determined and the results are presented at the Figure 3.

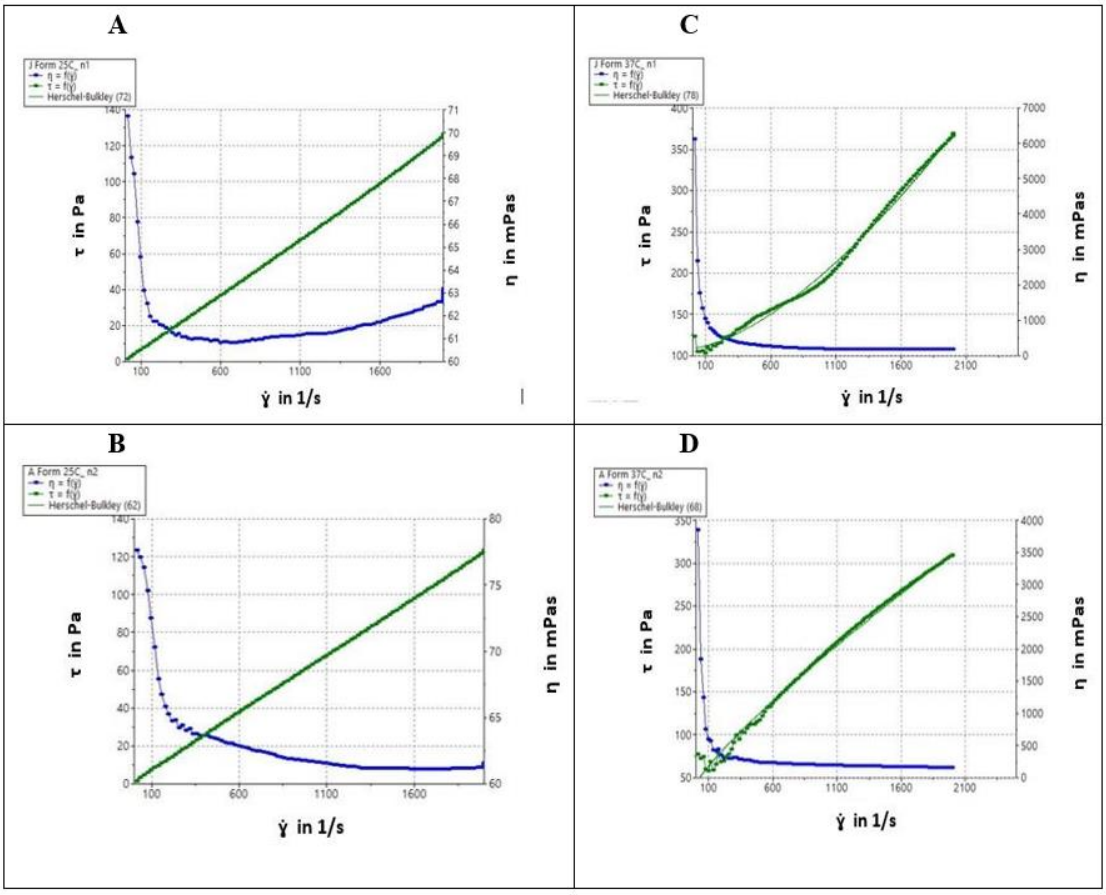

Figure 2. Rheograms of the F_5 and A_{21} formulations measured at 25°C and 37°C. (**A**: F_5 at 25°C, **B**: A_{21} at 25°C, **C**: F_5 at 37°C, **D**: A_{21} at 37°C). (τ: shear stress, γ̇: shear strain, Pa: stress in pascals; x axis = γ̇ in 1/s, y axis = τ in Pa)

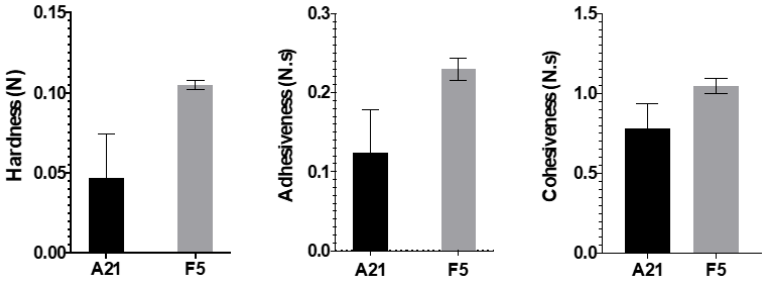

Figure 3. Texture profile analysis of the A_{21} and F_5 formulations (Mean ± SD; n = 3)

The cumulative drug release (%) of lidocaine from the *in situ* gel and lidocaine solution were calculated from the calibration curve of lidocaine. As shown in Figure 4, lidocaine solution has reached to 97.61 ± 2.14% just after 1.5 hours depending on solubility in the medium. For the formulation F_5 and A_{21} percentages of cumulative release at 24 hours were 73.39 ± 3.54%, 84.54 ± 2.66%, respectively.

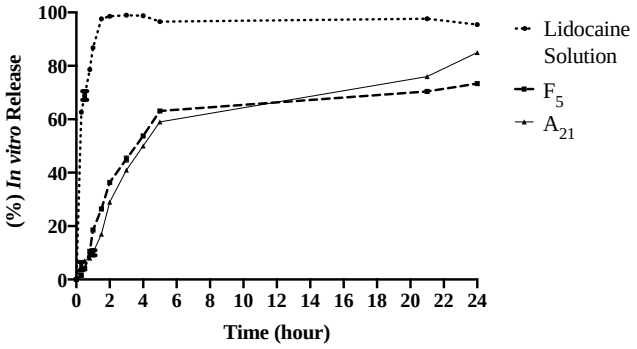

Figure 4. *In vitro* release profiles of lidocaine solution, F_5 and A_{21} *in situ* gel formulations (Mean ± SD; n = 3)

In this study, a well known anesthetic molecule, lidocaine, which is used in clinic for years; was combined with the *Aloe vera* plant due to its known activity in burn lesions, in order to improve therapeutic efficiency. Novel topical delivery of lidocaine and *Aloe vera* plant was designed as an *in situ* gelling system for modulation of burn wounds.

A significant reason why *in situ* gel systems gain great interest as a local application system in dermal diseases is that they can hold on to the injured skin surface for a long time, release the active substance continuously, and provide higher local drug concentration than conventional semi-solid dosage forms [35,36]. *In situ* gel systems provide ease of application on injured surfaces due to their flow properties at the solution form at room temperature [37,38]. In this study, P-407 and P-188 were used as temperature dependent gelling agents, and HPMC and CMC were preferred as conventional gelling agents. Poloxamer *in situ* gels are low viscous solutions at 25°C but turn into gels at physiological temperature. This property makes them optimum polymers for topical applications. In addition, their biodegradability, low toxicity and high stability advantages makes P-407 and P-118 potential polymers that can be used in temperature sensitive gelation systems [39,40]. HPMC is a polymer that is widely used in oral and topical pharmaceutical formulations and varies according to viscosity, molecular weight and degree of substitution. Due to its high water absorption capacity and gelling properties, HPMC has become an important material for drug delivery systems. It is chemically inert, compatible with packaging components, physically stable under physiological conditions and easily available [41]. CMC is preferred due to its high water solubility, high biocompatibility and low price, and is widely used in many areas [42].

In this study, the gel obtained from the *Aloe vera* plant was used regarding to its anti-inflammatory, moisturizing, soothing and wound healing effects. The effect of *Aloe vera* on sunburns, X-ray burns, thermal and electrical burns have been proven in various literature [43].

In addition to the therapeutic effect of *Aloe vera,* lidocaine, a local anesthetic belonging amide class, has been added to prevent sense of pain. It is aimed to develop a topical *in situ* gelling formulation using the anesthetic effect of lidocaine and the wound healing properties of the *Aloe vera* [14,15].

In situ gel formulations were prepared as a topical drug delivery system and gelation temperatures were evaluated to determine appropriate polymer ratios. The transition temperatures from solution to gel state depending on the polymer concentrations were evaluated. Since topical use was aimed in our study, the rates of gelation at body temperature were accepted as ideal [44,45]. Gelation temperatures above 25°C and below 37°C have been considered suitable. For gelation temperatures below the 25°C, a gel might easily be formed at room temperature; obstructing the preparation, handling and administration of the formulation. On the contrary, at gelation temperatures above the 37°C, a liquid

state may maintain after topical application [43,44,46]. It was found that the gelling temperature increased with the addition of *Aloe vera* in the formulations. For this reason, the formulations to be added *Aloe vera* were selected from gels with a gelling temperature below 36°C or formulations being in solid state below 5°C (Table 1). Some of the formulations remained in the solid state below 5°C (F_3, F_4, F_8, F_9, F_{12}, F_{13}, A_6, A_7, A_8, A_9, A_{10}, A_{11}, A_{19}, A_{27}, A_{28}, A_{29}) while some formulations remained in the liquid state above 50°C (A_2, A_3, A_4, A_5, A_{12}, A_{13}, A_{14}, A_{16}, A_{17}, A_{18}, A_{22}, A_{26}); thus eliminated from the further characterization studies. Remaining formulations (F_1, F_2, F_5, F_6, F_7, F_{10}, F_{11}, A_1, A_{15}, A_{20}, A_{21}, A_{23}, A_{24}, A_{25}) had sol-gel transition temperatures between 25-37°C. Characterization tests were carried out on F_5 and A_{21} coded gels due to their ideal gelling temperatures, 35.3 and 36.5°C respectively; providing appropriate sol-gel transition properties for physical application.

For partial validation of the analytical method for the quantification of lidocaine content; linearity, accuracy, precision and selectivity properties were evaluated [29].

As result of the linearity, the r^2 value of the standard curve is close to one, indicating the reliability of the obtained data. The linearity of the method was determined in the range of 50-500 μg/ml, and showed perfect correlation within the concentration range.

The (%) relative standard deviation values of the data obtained from the accuracy assay were found to be less than 2%. It has been shown that the method determined for quantification gives accurate results. The precision study was carried out in term of repeatability. Since the (%) relative standard deviations of the resulting data are less than 2%, the reproducibility of the study has been proven. None of the placebo formulations interfered at the wavelength of 263 nm, where lidocaine showed maximum absorbance. This data shows that the quantification method is specific to lidocaine and provides the required selectivity.

Skin pH is approximately in the range of 4-6 [47]. Despite the strong buffering capacity of the skin, the pH of topical formulations should be between 5.0 and 7.0 for safe application. When formulations with acidic pH value are applied, the patient may experience discomfort and skin irritation, while microbial growth may develop at the alkaline pH. The pH values of the formulations we prepared were found to be appropriate [48].

Evaluation of the rheological characteristics of *in situ* gels is one of the most significant parameters to predict their *in vivo* behavior. Dynamic viscosity (η′) is defined as the flow resistance of the formulation against oscillating motion. A higher dynamic viscosity value refers to higher resistance to flow [49]. In this study, it was observed that the viscosity of the gels in solution form was low at room temperature, while the viscosity of the gels increased at 37°C. In rheological measurements, Newtonian flow model is observed in shear stress versus shear velocity measurement at room temperature, while Non-Newtonian flow model is observed in measurements made at 37°C (Figure 2). Viscosity and rheology results support that the formulation behaviors differ depending on the temperature.

It has been shown that parameters of hardness, adhesion and cohesion are related to the ease of removal of the topical formulations from the packaging in which they are placed; the convenience of application to the surface on which they are applied, and the retention of the product in place. Therefore, texture profile analysis is frequently applied to identify formulations that may be appropriate for clinical application [45]. In this study, the hardness, adhesion and cohesion parameters of the texture profile analysis were evaluated. Hardness is defined as the force required for a predetermined deformation; with this parameter the degree of deformation of the sample is measured [47]. A low gel stiffness is desirable for providing the gel to be easily removed from the container and spread over the skin. In our study, the hardness values were found to be ideal, and it was determined that the hardness of the formulation containing *Aloe vera* was lower than the formulations which *Aloe vera* was not added (Figure 3). The adhesion parameter is established as the work required to overcome the attractive forces between the surface of the sample and the probe [51]. This parameter is related to the adhesive characteristics of the formulation; higher adhesion value provides more adhesion on the tissue surface, and this improves the desired retention time of the drug [49]. Cohesion is defined as the internal structural strength that maintains strong interconnections with a certain level of resistance to rupture during application [50]. It is defined as the structural deformation and strength of the internal bonds in the sample after shear stress [51,52]. Adhesion and cohesion values are high in F_5 gel and lower in A_{21} gel. HPMC and poloxamer polymers show mucoadhesive properties. However, although the polymer concentration is

higher in the F_5 formulation, the lower adhesion and cohesion values are thought to be related to *Aloe vera* gel content (Figure 3).

Diffusion through a dialysis membrane is a conventional technique to evaluate the drug release from colloidal dispersions and topical formulations [30]. *In vitro* release study shows that poloxamer based thermoresponsive *in situ* gel could significantly decrease the drug release compared to lidocaine solution (Figure 4). At the same time, the lidocaine and *Aloe vera* loaded *in situ* gel might display a higher burn wounds therapy effect compared with the conventional lidocaine solution due to quick and constant drug release profile.

In conclusion, lidocaine and *Aloe vera* containing *in situ* gel formulations were developed and characterized for treatment of burn wounds. Different content ratios of Poloxamer 407, Poloxamer 188, HPMC and CMC were used as gelling agents, and suitable gelation temperature for topical use was examined. F_5 and A_{21} coded formulations showed appropriate sol-gel transition temperature and characterized with further studies including pH, rheological properties, texture profile analysis and drug release profiles. Poloxamer 407 based *in situ* gels showed increased skin residence time, and provided *in vitro* lidocaine release for 24 hours. According to these results lidocaine and *Aloe vera* containing Poloxamer 407-HPMC based *in situ* gel formulations can be concluded as an effective alternative for topical treatment of burn wounds.

AUTHOR CONTRIBUTIONS

Concept: U.M.G., T.Ç., S.D.K.; Design: U.M.G., T.Ç., S.D.K.; Control: U.M.G.; Sources: U.M.G., S.D.K.; Materials: U.M.G., S.D.K.; Data Collection and Processing: U.M.G., T.Ç., S.S.; Analysis: U.M.G., T.Ç., S.S.; Literature Review: U.M.G., S.S.; Manuscript Writing: U.M.G., T.Ç.; Critical Review: U.M.G., T.Ç., S.D.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., 47(3): 1053-1057, 2023
Doi: 10.33483/jfpau.1340128

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANATOMY OF THE ENDEMIC *ALYSSUM PATERI* SUBSP. *PATERI*

ENDEMİK ALYSSUM PATERI SUBSP. PATERI'NİN ANATOMİSİ

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ABSTRACT

Objective: *Alyssum pateri Nyár. subsp. pateri is an endemic plant known as "kanatlı kevke" in Türkiye. The plant is perennial and in semi-shrub form. The plant is traditionally used externally in the treatment of rheumatism in Bingöl (Türkiye). Microscopic analysis of the anatomical structures of plants can provide useful information for taxonomic classification. Light microscopy analysis is a widely used and effective method for the identification of medicinal plants. In this study, the anatomical features of the A. pateri subsp. pateri were examined.*

Material and Method: *The plant material was collected from Ankara (Türkiye). The samples were protected in 70% alcohol. The cross and surface sections were cut by hand with a razor blade into microscopic preparation form. A Leica DM 4000B microscope was used for anatomical analysis and micro photographs.*

Result and Discussion: *The anatomical characters of the leaf, petiole and stem of the Alyssum pateri subsp. pateri were revealed. The leaf is dorsiventral and contains 1-2 rows of palisade parenchyma. Cruciferous stomata and stellate hairs are located on the lower epidermis. The petiole cross-section is sulcate and stellate hairs are observed in the epidermal layer. The main vein is arc-shaped and is accompanied by lateral veins. The stem is disc-shaped. Stem epidermis contains stellate hairs. The interfascicular tissue between the xylem strands of the vascular bundles is composed lignified cells. Vascular bundles are surrounded by a pericyclic sclerenchymatous cap.*

Keywords: *Alyssum pateri subsp. pateri, Brassicaceae, medicinal plants, pharmaceutical botany, plant anatomy*

ÖZ

Amaç: *Alyssum pateri Nyár. subsp. pateri Türkiye'de "kanatlı kevke" olarak bilinen endemik bir bitkidir. Bitki çok yıllık ve yarı çalı formundadır. Bitki geleneksel olarak Bingöl (Türkiye)'de romatizma tedavisinde haricen kullanılmaktadır. Bitkilerin anatomik yapılarının mikroskobik analizi, taksonomik sınıflandırma için yararlı bilgiler sağlayabilir. Işık mikroskobu analizi, tıbbi*

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Submitted / Gönderilme : 09.08.2023
Accepted / Kabul : 05.09.2023
Published / Yayınlanma : 20.09.2023

bitkilerin tanımlanmasında yaygın olarak kullanılan ve etkili bir yöntemdir. Bu çalışmada A. pateri subsp. pateri'nin anatomik yapıları incelenmiştir.
Gereç ve Yöntem: *Bitki materyali Ankara (Türkiye)'dan toplandı. Numuneler %70 alkol içinde korunmuştur. Enine ve yüzey kesitleri jilet yardımıyla elle kesilerek mikroskobik preparat formuna getirildi. Anatomik analiz ve mikro fotoğraflar için Leica DM 4000B mikroskop kullanıldı.*
Sonuç ve Tartışma: *Alyssum pateri subsp. pateri'nin yaprak, yaprak sapı ve gövdesinin anatomik karakterleri ortaya konulmuştur. Yaprak dorsiventraldir ve 1-2 sıra palizat parankiması içerir. Crucifer tip stomalar ve stellat tüyler alt epidermiste bulunur. Yaprak sapının enine kesiti sulkattır ve epidermal tabakada stellat tüyler görülür. Ana damar yay şeklindedir ve buna yan damarlar eşlik eder. Gövde disk şeklindedir. Gövde epidermisi stellat tüyler içerir. İletim demetlerinin ksilem şeritleri arasındaki interfasiküler doku odunlaşmış hücrelerden oluşur. İletim demetleri, perisiklik bir sklerenkimatik başlık ile sarılıdır.*
Anahtar Kelimeler: *Alyssum pateri subsp. pateri, bitki anatomisi, Brassicaceae, farmasötik botanik, tıbbi bitkiler*

INTRODUCTION

Brassicaceae Burnett consists of herbaceous and rarely small shrubs with 346 accepted genera in the world. The leaves are alternate, rarely opposite and without stipule. The flowers are usually hermaphrodite and hypogynous. Sepals and petals 4 and free. Stamens are usually 6 and tetradynamous. Ovary is syncarpous and fruit is a capsule. The *Alyssum* L. has a wide native range from North Africa to temperate Eurasia. The genus contains 113 species worldwide, annual, biennial or perennial plants. The Genus is characterized by stellate indumentum and simple, entire leaves. The petals are yellow or sometimes whitish [1-2]. Many members of the Brassicaceae contain mustard oil glycosides, and the seeds often contain mucilage and fixed oil [3]. The family plants show antimicrobial, anticancer, antimutagenic, anti-inflammatory, neuroprotective and antioxidant activities [4].

Alyssum pateri Nyár. subsp. *pateri* is an endemic plant known as "*kanatlı kevke*" in Türkiye [5]. The plant is perennial and in semi-shrub form. Leaves are obovate or oblanceolate. Petals bright yellow, fruits with stellate hairs and seeds narrowly winged [1]. In recent studies, the taxonomic update of *A. pateri* has been made and "*Alyssum pateri* Nyár." is used as a synonym for "*Odontarrhena pateri* (Nyár.) Španiel, Al-Shehbaz, D.A.German & Marhold". The plant is traditionally used externally in the treatment of rheumatism in Bingöl (Türkiye) [6].

Türkiye is one of the richest countries in the world in terms of plant diversity, and 30% of its approximately 10.500 plant species are endemic [7]. This extraordinarily rich vegetation has traditionally developed a folk medicine culture in the society [6-7]. The majority of the people living in rural areas traditionally use plants in the treatment of diseases. As in other countries of the world, plants traditionally used for therapeutic purposes in recent years attract the attention of researchers [6,8]. It is very important to fully identify the plants that are candidates for herbal medicine and continue to be used in traditional folk medicine. Microscopic analysis of the anatomical structures of plants can provide useful information for taxonomic classification. Light microscope analysis is a widely used and effective method for the identification of medicinal plants [9-12].

In this study, the anatomical features of the *Alyssum pateri* Nyár. subsp. *pateri* were examined with a light microscope and micro-photographs were taken. The results obtained are important in the long term due to the increasing demand for herbal medicines.

MATERIAL AND METHOD

The plant material was collected from Ankara (Türkiye) (*mh23005*) (Figure 1). The samples were protected in 70% alcohol. The cross and surface sections were cut by hand with a razor blade into microscopic preparation form. The Sartur solution [13] was used in microscopic examinations. A Leica DM 4000B microscope was used for anatomical analysis and micro photographs.

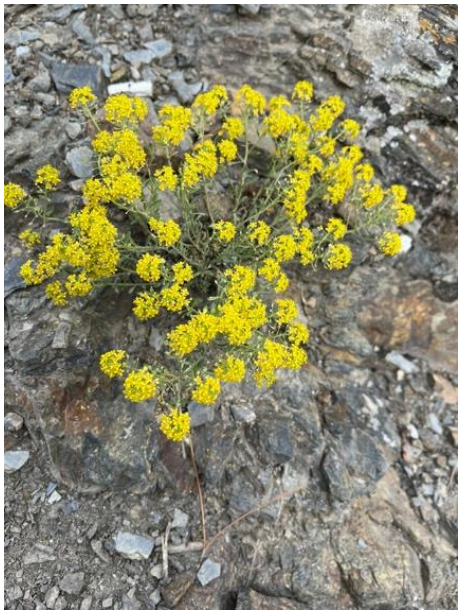

Figure 1. *Alyssum pateri* subsp. *pateri*

RESULT AND DISCUSSION

Leaf

The leaf is dorsiventral. The upper epidermis is composed of square-rectangular cells. The lower epidermis cells are square, sometimes oval, and smaller than the upper epidermis cells. The mesophyll consists of 1-2 rows of palisade parenchyma and 4-7 rows of spongy parenchyma. The bundle sheath surrounds the vascular bundles. The stomata and hairs are found only on the lower surface. The stomata are cruciferous, surrounded by 3 subsidiary cells and the hairs are stellate (Figure 2).

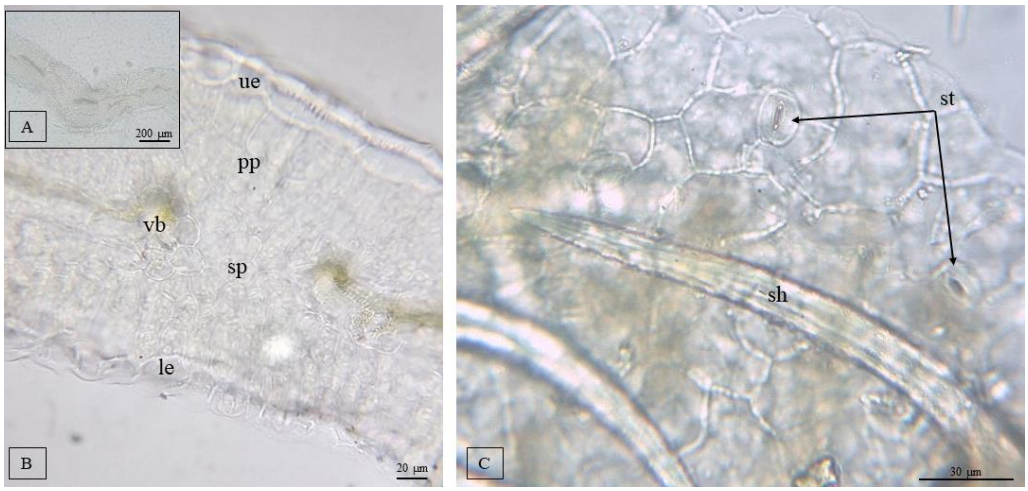

Figure 2. Leaf anatomical features (A: general view, B: lamina cross-section, C: lamina lower surface-section); le: lower epidermis, pp: palisade parenchyma, sh: stellate hair, sp: spongy parenchyma, st: stomata, ue: upper epidermis, vb: vascular bundle

Petiole

The cross-section of the petiole is sulcate and the abaxial side is protruding. The epidermal cells of the adaxial side are oval shaped, and the cells of the abaxial side are square and smaller. The petiole has a main vein and is arc-shaped. Also, lateral veins are also present. The main vein embedded in thin-walled, oval parenchymatous cells is clearly surrounded by bundle sheath. Numerous stellate hairs are found on the epidermal layer of the petiole (Figure 3).

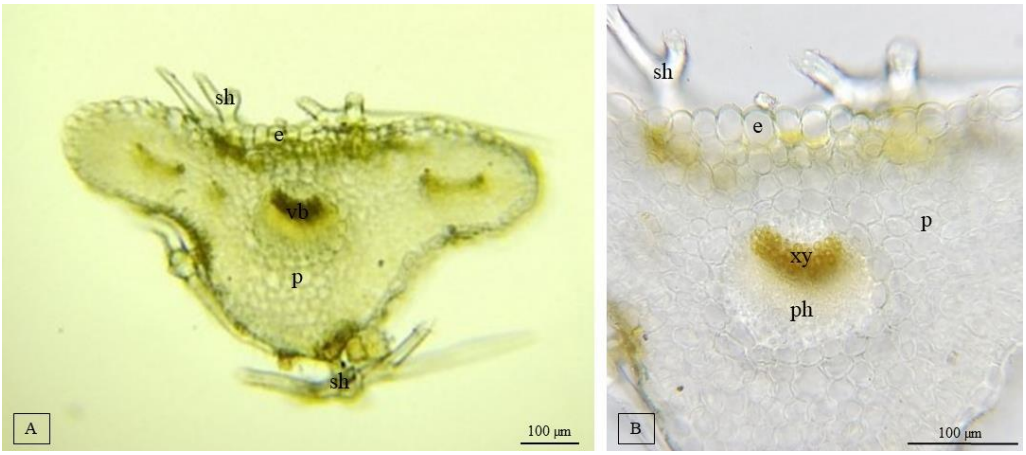

Figure 3. Petiole anatomical features (A: general view, B: detailed view); e: epidermis, p: parenchyma, ph: phloem, sh: stellate hair, vb: vascular bundle, xy: xylem

Stem

The stem cross-section is disc-shaped. The epidermis layer consists of a single row of epidermal cells and covered with stellate hairs. The cortex parenchyma cells are in 8-10 rows. The endodermis consists of a single row of regularly arranged cells. The interfascicular tissue between the xylem strands of the vascular bundles is composed lignified cells. Vascular bundles are surrounded by a pericyclic sclerenchymatous cap. The pith is composed of thin-walled parenchymatous cells with large intercellular spaces (Figure 4).

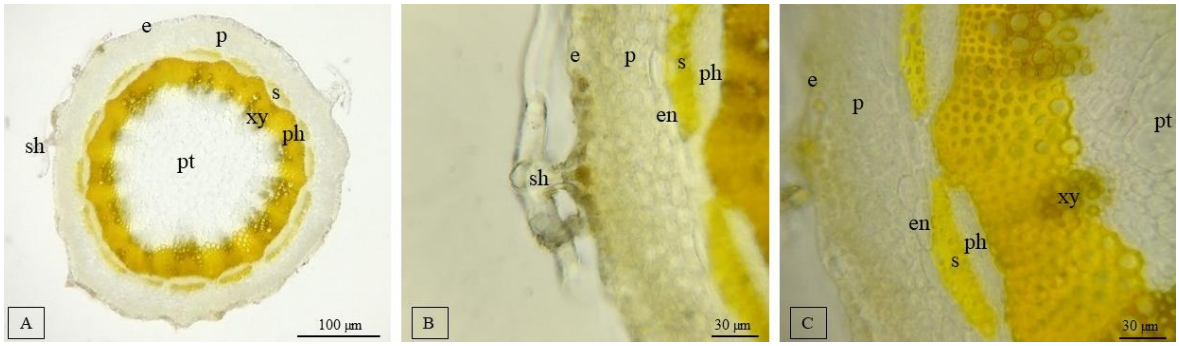

Figure 4. Stem cross-section (A: general view, B-C: detailed view); e: epidermis, en: endodermis, sh: stellate hair, p: cortex parenchyma, ph: phloem, pt: pith, s: sclerenchyma, xy: xylem

In previous studies on the anatomical structures of the family, it has been reported that the leaves are monofacial or bifacial, the palisade parenchyma has 1-3 rows, different forms of cover hairs, cruciferous type stomata and the stem contains a closed cylindrical xylem [14]. In this study, the anatomical characters of the leaf, petiole and stem of the *Alyssum pateri* subsp. *pateri* were revealed.

The leaf is dorsiventral and contains 1-2 rows of palisade parenchyma. Cruciferous stomata and stellate hairs are located in the lower epidermis. The petiole cross-section is sulcate and stellate hairs are observed on the epidermal layer. The main vein is arc-shaped and is accompanied by lateral veins. The stem is disc-shaped. Stem epidermis contains stellate hairs. The interfascicular tissue between the xylem strands of the vascular bundles is composed lignified cells. Vascular bundles are surrounded by a pericyclic sclerenchymatous cap.

ACKNOWLEDGEMENTS

Şeyda Yayla thanks the financial support from the Scientific and Technological Research Council of Türkiye (TUBITAK) under the BIDEB/2211-A Ph.D.

AUTHOR CONTRIBUTIONS

Concept: Ş.Y., M.M.H.; Design: Ş.Y., M.M.H.; Control: Ş.Y., M.M.H.; Sources: Ş.Y., M.M.H.; Materials: Ş.Y., M.M.H.; Data Collection and/or Processing: Ş.Y., M.M.H.; Analysis and/or Interpretation: Ş.Y., M.M.H.; Literature Review: Ş.Y., M.M.H.; Manuscript Writing: M.M.H.; Critical Review: M.M.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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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 47(3): 1058-1070, 2023
Doi: 10.33483/jfpau.1225743

DERLEME MAKALE / REVIEW ARTICLE

FARMASÖTİK ENDÜSTRİDE FARMA 4.0 YAKLAŞIMI

PHARMA 4.0 APPROACH IN PHARMACEUTICAL INDUSTRY

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ÖZ

Amaç: *Bu derlemede, teknolojideki gelişmelerin farmasötik endüstri bakış açısı ile değerlendirilmesi ve gelişen teknoloji ile ortaya çıkan Farma 4.0 yaklaşımının farmasötik üretim ve Ar-Ge alanlarına etkisinin tartışılması amaçlanmıştır.*
Sonuç ve Tartışma: *Farmasötik endüstri, teknolojik gelişmelerin öncelikle uygulandığı en önemli endüstri alanlarından biridir. Bununla birlikte, kaliteli, etkili ve güvenli ilaç üretme gerekliliği çoğu zaman farmasötik endüstrinin teknolojik gelişmeleri eş zamanlı takibi açısından kısıtlayıcı bir etken olarak görülmüştür. İlaç üretiminde konvansiyonel sistemlerin kullanılması ile yaşanan zorluklar, ilaç üreticilerini kaçınılmaz olarak ve teknolojik gelişmeler doğrultusunda verimli alternatifler aramaya yöneltmiştir. Endüstri 4.0 kavramının ortaya çıkması ile bu yaklaşımın farmasötik endüstrideki uygulanabilirliği tartışılmaya başlanmıştır. Yapay zeka, nesnelerin interneti, makine öğrenimi ve bulut sistemler, Endüstri 4.0'ın temel bileşenlerini oluşturmaktadır. Veri ve deney tasarımlarının oldukça önemli olduğu ilaç keşfi ve formülasyon geliştirme alanlarında bu bileşenlerin kullanımı verimlilik, etkililik ve güvenlik açısından büyük bir potansiyele sahiptir. Endüstri 4.0 kavramının farmasötik alandaki karşılığı olarak tanımlanan Farma 4.0, verimliliği artırmasının yanında çevreci üretim sistemleri kurmayı da vadeden ve böylece sürdürülebilirliği destekleyen bir yaklaşımdır. Yeni endüstri devrimi ile tüm dünyanın büyük bir dönüşüm içerisine girdiği teknoloji çağında, farmasötik endüstrinin de bu gelişmelere en kısa sürede uyum sağlaması gerekmektedir.*
Anahtar Kelimeler: *Dijitalleşme, endüstri 4.0, farma 4.0, makine öğrenimi, yapay zeka*

ABSTRACT

Objective: *The purpose of this review is to evaluate developments in technology from the perspective of the pharmaceutical industry and to discuss the impact of Pharma 4.0 approach, which emerged with developing technology, on pharmaceutical production and R&D areas.*
Result and Discussion: *Pharmaceutical industry is one of the important industrial areas. However, the need to produce quality, effective and safe products has often been seen as a limiting factor in*

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Gönderilme / Submitted : 29.12.2022
Kabul / Accepted : 29.04.2023
Yayınlanma / Published : 20.09.2023

the pharmaceutical industry's ability to keep pace with technological advances. With the emergence of Industry 4.0 concept, the applicability of this approach in the pharmaceutical industry has started to be discussed. The use of key components of Industry 4.0 in the pharmaceutical industry, where data and experimental designs are crucial, has great potential for efficiency, efficacy, and safety. Pharma 4.0, which is defined as the pharmaceutical equivalent of Industry 4.0, promises to establish environmentally friendly production systems as well as increase efficiency and thus support sustainability. In the age of technology, where the whole world is undergoing a major transformation with the new industrial revolution, the pharmaceutical industry needs to adapt to these developments as soon as possible.
Keywords: *Artificial intelligence, digitalization, industry 4.0, machine learning, pharma 4.0*

GİRİŞ

Bilgi ve bilginin doğru kullanılması insanlık tarihinde önemli değişimlerin yaşanmasını sağlamıştır. Tarihte yaşanan bu önemli değişimlerin en önemli örneklerinden biri de endüstri devrimleridir. Endüstri devrimleri tarihsel, sosyal ve teknik açıdan farklılıklar göstermektedir. Bu ana farklılıklar dikkate alındığında endüstri devrimlerini dört döneme ayırabiliriz. 1700'lü yılların sonuna doğru buhar kullanımının keşfedilmesi ile başlayan birinci endüstri devrimi, devamında yaşanacak gelişmelerin öncüsü olmuştur. Endüstrileşmenin ilk aşaması olarak da görülen birinci sanayi devrimi ile insanların çalışma ortamı ve yaptığı işlerin niteliği endüstriye kaymıştır. 1860-1915 yılları arasında yaşandığı kabul edilen ikinci endüstri devriminde ise elektrik enerjisi belirleyici olmuştur. Elektriğin makinelere aktarılması ile bu dönemde seri üretime geçilmiştir. Dünya savaşları ve yaşanan çeşitli olumsuzlukların ardından dünya devletleri, üretimde daha verimli yaklaşımlar geliştirmek için çalışmalara başlamıştır. Bu çalışmaların sonucunda, bilgi teknolojilerinin de endüstriye entegre edilmesi ile üçüncü endüstri devrimi başlamıştır. Bu dönemde özellikle otomasyonun artması ile üretim hacimleri artırılmış ve yeni gereksinimlerin doğması kaçınılmaz olmuştur. 2000'li yıllara gelindiğinde artık bilgisayarların ve dijital sistemlerin kendine her alanda yer bulduğu görülmektedir. Üzerinde yoğun çalışmalar yapılan insan benzeri sistemlerin endüstriyel açıdan kullanımı değerlendirilmeye başlanmıştır. 2011 yılında Almanya'da yapılan bir fuarda ilk kez sözü edilen dördüncü endüstri devrimi, içinde bulunduğumuz dönemi ifade etmektedir. Endüstri 4.0 terimi, *Internet of Things* (IoT-Nesnelerin İnterneti), yapay zeka, robotik gibi hızla gelişen teknolojileri bir araya getiren endüstri devrimini ifade eder. Bütünleşmiş, otonom ve kendi kendini organize eden üretim sistemleri ile karakterize edilmektedir [1]. Üretim ve Ar-Ge faaliyetlerinin büyük önem taşıdığı farmasötik alanda da Endüstri 4.0 etkileri görülmeye başlanmıştır. Farma 4.0, Endüstri 4.0 uygulamalarına ve veri bütünlüğüne dayalı, geleceğin ilaç endüstrisi için bütünsel bir işletim modelidir [2].

Farmasötik üretimin sıkı denetim altında olması gerekliliği farmasötik endüstrinin gelişmesini pek çok açıdan kısıtlamaktadır. İkinci endüstri devriminden kalma bir alışkanlıkla hâlâ seri üretimin ve kalitenin test edilmesinde ampirik yaklaşımların kullanılması farmasötik endüstrinin durumunu açıklamada oldukça önemli bir ölçüttür. Seri üretimden sürekli üretime geçiş, tasarımla kalite (*Quality by Design-QbD*) ve proses analitik teknolojisi (*Process Analytical Technology* -PAT) gibi yenilikçi yaklaşımlara Farma 4.0 bileşenlerinin entegrasyonu ile ilaç endüstrisi büyük gelişim gösterme potansiyeline sahiptir. İlaç geliştirme ve üretim işlemlerinin daha anlaşılır olması PAT araçlarının kullanımı ile mümkündür. Farma 4.0 bileşenlerinin de PAT araçlarına entegrasyonu ile bir molekülün ilaç haline gelene kadar geçirdiği tüm aşamalar kolaylıkla izlenebilir ve kontrol edilebilir olacaktır. Biyoteknoloji alanında yaşanan gelişmeler tedavilerin bireyselleşmesine doğru yola çıkıldığını göstermektedir. Böyle bir araştırma ortamında, farmasötik endüstrinin ampirik yaklaşımlara ve seri üretimlere devam etmesi uygun yaklaşımlar değildir. Ar-Ge ve üretim faaliyetlerinde verimliliği artırmak, yeni teknolojilerin var olan sistemlerle bütünleşmesi ile mümkündür. Endüstri 4.0 dijitalleşmeye verdiği önemle verimliliği artırmanın yanı sıra çevreci sistemler geliştirmeyi de hedeflemektedir. Sunduğu yeni yaklaşımlarla karbon ayak izi ve atıkların son derece fazla olduğu bir endüstri alanı olan farmasötik endüstride çevre dostu sistemler geliştirilmesine katkıda bulunacaktır [3]. Günümüzde birçok endüstriyel alanda çevre ve insan odaklı bir yaklaşım olan Endüstri 5.0'dan

bahsedilmeye başlandığı göz önüne alındığında farmasötik endüstrinin de bu atmosfere uyum sağlaması için Farma 4.0'ın gerekli bir adım olduğu açıktır.

Farma 4.0, klinik araştırmalardan ilaç firmaları ve yasal otoriteler arasındaki iletişime kadar oldukça geniş bir alanda uygulanmaktadır ancak bu derleme kapsamında Ar-Ge ve endüstriyel üretim konuları ağırlıklı olarak verilmiştir.

Endüstri Devrimleri

İnsanlık tarihinden bu yana ekonomik ve teknolojik gelişmeler endüstri devrimlerini de beraberinde getirmiştir.

Endüstri 1.0

18. yüzyılda kimya alanındaki gelişmelerle birlikte, gazların davranışını açıklayan bilimsel bilgiler ortaya atılmış, enerji kaynağı olarak kullanılan su ve rüzgârın yerini alabilecek buharı kullanma olasılığı bu bağlamda değerlendirilmiştir. İlk buhar makinesi 1600'lerin sonlarında Hollanda'da Fransız bir mülteci tarafından icat edilmiş, bu icat birinci endüstri devriminin başlangıcı olarak kabul edilmiştir [4].

Endüstri 1.0, farmasötik endüstrinin başlangıç noktasıdır [5]. Bitkisel müstahzarların ilaç olarak kullanımı insanlık tarihine kadar uzanmaktadır. Endüstri 1.0 döneminde, bitkisel, mineral ve hayvansal kaynaklı maddeler elle kontrol edilebilen basit araçlar ve ticari ölçekte makineler yardımıyla öğütülmüş, karıştırılmış ve sıkıştırılabilen ilaç ürünlerine dönüştürülmüştür [6]. Birinci endüstri devriminde ortaya çıkan makinelerden bazıları -tablet baskı makinesi gibi- günümüzde de yaygın olarak kullanılmaktadır.

Endüstri 2.0

İkinci endüstri devrimi, elektriğin keşfi ve üretim hattında yeni teknolojilerin kullanılmasıyla başlamıştır. Teknolojik devrim olarak da bilinen ikinci endüstri devrimi 1860-1915 yılları arasındaki hızlı bir sanayileşme ve standardizasyon dönemidir [7]. Teknolojik devrim, farmasötik endüstride, özellikle üretim hattında, makineleşmeye imkan vermiş ve eleme, karıştırma, tablet baskı gibi işlemlerin büyük ölçekte gerçekleştirilmesini, kalite ve süreçlerin izlenebilirliğini mümkün hale getirmiştir [5].

Endüstri 3.0

İkinci Dünya Savaşı'ndan sadece birkaç yıl sonra programlanabilir bilgisayar teknolojilerini kapsayan üçüncü endüstri devrimi gerçekleşmiştir [7]. Bilgisayar ağı, kablosuz iletişim, internet gibi iletişim teknolojileri ile farmasötik üretimde süreçlerin yüksek derecede otomasyonunu içeren sürekli üretim ve aktif kontrol gibi kavramların ortaya çıkmasını sağlamıştır.

Endüstri 3.0 ile farmasötik endüstride süreç ve ürün kalitesi verilerini gerçek zamanlı olarak sağlamayı amaçlayan Proses Analitik Teknolojisi (*Process Analytical Technology*- PAT) ve kalite hedefli ürün profillerini kontrol etmeyi amaçlayan Tasarımla Kalite (*Quality by Design*- QbD) yer almaya başlamıştır [8-10]. Ancak PAT ve QbD gibi yaklaşımların farmasötik endüstride tam potansiyeline ulaşması için daha fazla teknolojik gelişmeye gereksinim vardır.

Endüstri 4.0

IV. Sanayi Devrimi'nden ilk olarak 2011 yılında Almanya'nın Hannover kentinde düzenlenen Hannover Fuarı'nda söz edilmiştir. Almanya, üretim endüstrisinde dünyanın en rekabetçi ülkelerinden biridir ve bu nedenle Endüstri 4.0 yaklaşımından ilk olarak Almanya'da bahsedilmiş olması tesadüf değildir [11]. Endüstri 4.0'ın ana hedefi, bilgi ve dijitalizasyonu merkeze alarak üretim, ulaşım, sosyal ve diğer tüm alanlarda dönüşümü sağlamaktır. Son yıllarda hızla gelişen bilgi teknolojileri ve yazılım sektörü, dördüncü sanayi devrimini kaçınılmaz kılmıştır. Geleceğin endüstrisinde, dijital sistemlerin kullanımı ile daha bilgiye dayalı, akılcı üretim yöntemleri benimsenerek üretimde verimliliğin artması hedeflenmektedir [12].

Endüstri 4.0, üretim sürecinde yer alan birimlerin ve paydaşların birbiriyle etkileşiminin, üretilen verilere gerçek zamanlı erişimin, bu sayede katma değer üretiminin sağlanmasını hedeflemektedir [13]. Endüstri 3.0'ın otomatik ve dijital ortamında kazanılan deneyim, farmasötik endüstrinin de Farma 4.0'a geçiş sürecini desteklemektedir [14].

Farmasötik Endüstrinin Mevcut Durumu

Farmasötik endüstride kısa bir zaman öncesine kadar sadece yardımcı ve etkin maddelerin test edilerek, üretimde sabit işlemler kullanılarak, ara ürün ve bitmiş ürünler test edilerek kalitenin sağlandığı gösterilmekteydi [15]. Bu yaklaşımda ana hedef, sabit ve tekrarlanabilir süreçlerin oluşturulmasıdır. Üreticiler tüm üretim ve test süreçlerini otoriteye sunmakla yükümlü olduğu için yapılan küçük bir değişiklik tüm sürecin ve bu sürece ait verilerin yeniden üretilmesini gerektirmektedir. Bu durum da hem yasal otorite hem de ilaç endüstrisi bileşenleri için işgücü kaybına neden olmaktadır. Aynı zamanda geleneksel yaklaşımda tüm süreçler iyi anlaşılarak ve her aşamada ilgili analizler yapılarak ilerlenmediği için olası bir sorunun kaynağını belirlemek oldukça zordur [15]. Tüm bu kısıtlayıcı etkenler, üreticileri yeni seçenekler aramaya teşvik etmiştir.

21. yüzyılın sonlarına doğru rekabetin ve teknolojinin artan etkisiyle birlikte ilaç endüstrisinin ürün kalitesini artırma, maliyeti azaltma, ürünü pazara sunma süresini kısaltma gibi arayışlar söz konusudur. Bu gereksinimleri karşılamak için ilaçla ilgili otoriteler QbD ve PAT gibi yeni yaklaşımları gündeme getirmiştir [8,9].

QbD, kalitenin bitmiş üründe test edildiği ampirik yaklaşımdan farklı olarak, ürünün henüz geliştirilme aşamasında kalitenin sürece dahil edilmesi gerektiğini savunmaktadır [16]. QbD'nin temel unsurları ICH kılavuzlarında şu şekilde açıklanmıştır. Kalite hedefli ürün profili (QTPP) ürün tasarımının temelini oluşturmaktadır [10,16]. Kritik kalite özellikleri (CQA) amaçlanan ürün kalitesini sağlayabilmek için belirli aralıklarda bulunması gereken tüm özellikleri kapsamaktadır [10]. CQA'lere bağlı kritik işlem parametreleri, karıştırma hızı, sıcaklık, su miktarı gibi özellikleri kapsar ve bu parametreler ürün kalitesine değişen derecelerde etki etme potansiyeline sahiptir [16]. QTPP'nin belirlenmesi ile başlayan ve ürünün yaşam döngüsü süresince geliştirilmeye devam eden tasarım aralığı, İşlem girdileri (materyal özellikleri ve işlem parametreleri) ile kritik kalite özellikleri arasındaki ilişkiyi açıklamaktadır [17]. QbD'nin önemli bir diğer unsuru olan risk değerlendirilmesi ile materyal özelliklerinden ve işlem parametrelerinden hangilerinin ürünün CQA'ları üzerinde kritik etkiye sahip olduğu belirlenebilmektedir. Neden-sonuç, balık kılçığı veya bilinen diğer ismiyle Ishikawa diyagramı ürün kalitesi üzerinde etkili olan parametreleri tüm yönleriyle göstermeye yarayan bir yaklaşımdır [16,18].

Farmasötik üretim süreci için FDA ve ICH'in mevcut yaklaşımı "Kalite ürünle test edilemez, tasarım ile oluşturulmalıdır." şeklindedir. FDA tanımına göre PAT; üretim sırasında kritik parametrelerin gerçek zamanlı ölçümleriyle üretimi tasarlamak, analiz ve kontrol etmek; işlem sırasında kullanılan maddeler ve işlemin performans özelliklerini saptamak amacıyla geliştirilmiş ve hedeflenen ürün kalitesini sağlamak olan bir sistemdir [9]. PAT, üretim döngülerinin kısaltılmasında, reddedilen ürün sayısının azaltılmasında, gerçek zamanlı analizlerin uygulanmasında ve sürekli üretim işlemlerine geçişte yararlı olabilecek bir yaklaşımdır [16]. Ancak, mevcut tesislerin ekipman ve veri yönetim eksikliği, PAT uygulamasında aksaklıklar yaşanmasına neden olmaktadır.

Geleneksel yöntemle birlikte farmasötik endüstride seri üretim benimsenmiş ve uzun yıllar boyunca değiştirilmeden sürdürülmüştür. Seri üretimde, hammaddelerin doldurulduğu andan üretimin tamamlandığı ana dek hiçbir sistem parametresinde değişiklik yapılmamaktadır. Sürekli üretimde ise üretim süreci boyunca hammadde yüklemesi ve ürünün sistemden alınması devam etmektedir. İşlem parametreleri anlık olarak denetlenebilmekte ve geliştirilebilmektedir [19]. Günümüzde, ilaç üretim işlemi seri üretim ve sürekli üretim operasyonlarının kombinasyonundan oluşmaktadır [20].

Kalitenin ürünle değil tasarım ile test edilmesi gerekliliğini destekleyen QbD ve PAT yaklaşımı, sürekli üretime de katkıda bulunmaktadır. PAT araçlarının kullanımı ile üretim süreci sistematik hale getirilebilmektedir. PAT kılavuzuna göre kritik işlem parametreleri, işlem değişkenleri ve kalite özelliklerinin izlenmesi farklı yöntemlerle gerçekleştirilebilmektedir.
Bu yöntemlere *at-line* (hat üzerinde) ölçüm, *on-line* (çevrimiçi) ölçüm ve *in-line/in-situ* (yerinde) ölçüm örnek olarak verilebilir [21-23].

Gerçek zamanlı serbest bırakma testleri *(Real time release testing* -RTRT), ICH kılavuzunda "tipik olarak işlem verilerine dayanan, işlem içi ve/veya sonuç ürünün kalitesini değerlendirme ve garanti etme yeteneği" olarak tanımlanmaktadır. RTRT, kalite güvencesini artırarak, iyileştirerek ve ayrıca üretkenliği de artırarak farmasötik üretimde süreci daha anlaşılır kılmaktadır [9,10,24,25]. *At-*

line, on-line ve *in-line* ölçüm gibi gerçek zamanlı testler ve diğer sistematik PAT araçlarının kullanımı sayesinde gerçek zamanlı işlem kontrolü gerçekleştirilebilmektedir. Sürekli üretim için iyi bir örnek olan PAT yaklaşımının benimsenmesi ile sürekli gerçek zamanlı kalite güvencesi ve gerçek zamanlı serbest bırakma sağlanabilir. Bu doğrultuda, ilaç endüstrisinin üretim sistemlerini yenileme ve gerçek zamanlı işlem kontrolü araçlarını üretim yöntemlerine entegre etme çalışmaları hızlanmıştır.

İlaçların üretim aşamasında, tasarımla kalitenin sağlanması ve ürün optimizasyonunda kendi kendine öğrenen ve deneysel sonuçları modelleyebilen yapay sinir ağları sıklıkla kullanılmaktadır [26]. Yapay sinir ağları, insan beyninin bilgiyi işleme işlemini taklit etmek için modellenmiş bilgisayar programlarıdır. Tıpkı insanlar gibi deneyim yoluyla öğrenir veya eğitilirler [27]. Önformülasyon aşamasında bağımsız kritik materyal özellikleri (CMA) ve CPP değişkenleri ile bağımlı QTPP değişkeni arasında ilişki kurmak için yapay sinir ağları kullanılabilir. Farmasötik endüstride formülasyon geliştirmek ve geliştirilen formülasyonu optimize etmek için uzun bir süredir yapay sinir ağları kullanılmaktadır [28,29]. Örneğin, yaş granülasyon işleminde partikül büyüklüğü, akış hızı, sıkıştırılmış ve yığın dansite gibi özellikleri yapay sinir ağları aracılığıyla tahmin etmek için modeller oluşturulmuştur [30]. Ürün kalitesinin önemli bir göstergesi olan çözünme çalışmalarında yapay sinir ağlarının kullanıldığı yaklaşımlar da bulunmaktadır. Farklı yapay sinir ağı yapılarıyla tabletlerde polimer etkisi [31] ve tablet baskı özellikleri gibi işlem parametrelerinin çözünmeye etkisi modellenmiştir [32].

Farma 4.0

Farma 4.0, farmasötik endüstrinin üretim alanında ulaşmayı hedeflediği dijitalizasyon temelli akıllı üretim stratejisidir [33]. Farma 4.0 ilk olarak Endüstri 4.0'ın ilaç endüstrisindeki uygulaması olarak *The International Society for Pharmaceutical Engineering (ISPE)* tarafından kullanılmış, Farma 4.0 ile ilgilenen özel bir grup kurulmuştur [34]. Bu gruba göre; Farma 4.0, dijitalizasyon ve ICH Q10 rehberinin bir kombinasyonudur [35], ICH Q10 ve Farma 4.0'dan oluşan operasyon modeli farmasötik kalite sistemi ve Farma 4.0 bileşenlerinden oluşmaktadır. Ürün yaşam döngüsü ve kalite yönetim sistemi kavramları, rehbere göre uygulanmış, Farma 4.0 ile etkin bir şekilde birleştirilmiştir. Bu sayede kesintisiz bir ürün yaşam döngüsü oluşturmak mümkün olmuştur.

Endüstri 4.0 ve dolayısıyla Farma 4.0 bileşenleri aşağıda ayrıntılı olarak belirtilmiştir.

Farmasötik Endüstride Yapay Zeka

Yapay zeka en basit haliyle, insan zekasına benzer yeteneklere sahip olan bilgisayarlar olarak tanımlanabilmektedir [36]. Bilgisayarlar uzun yıllardan beri endüstriyel kullanımda olsa da karar verebilen ve öğrenme yeteneğine sahip olan yapay zeka kavramı tüm endüstri alanları için radikal bir yenilik oluşturmaktadır. Tarihsel ve kültürel olarak insanoğlunun kendini doğa üzerinde karar yetisine sahip tek varlık olarak görmesi nedeniyle, yapay zeka kavramının benimsenmesi kolay olmamıştır. Gösterilen tüm dirençlere rağmen yarar-zarar analizi yapıldığında, yapay zeka teknolojilerinin endüstriyel alana çok büyük katkılar vereceği düşünülmektedir [37].

Yapay zeka, ilaç keşif sürecini hızlandırma, kaliteyi artırma ve maliyetleri azaltma potansiyeline sahiptir [38]. Örneğin, şizofrenide ilaç uyuncunu artırmaya yönelik yapay zeka tabanlı hasta izleme platformu geliştirilmiştir. Akıllı telefonlar aracılığı ile ilaç alımını görsel olarak doğrulayan, ilaç konsantrasyon düzeylerine göre test edilen, klinik olarak doğrulanmış bir uygulamadır. Zorlu bir hasta grubu olan şizofrenide 6 ay boyunca uyuncu %90'a çıkarma yeteneği, platformun klinik araştırma ve klinik uygulamada değerini kanıtlamıştır [39]. Bir başka çalışmada kronik böbrek hastalığı ve idiyopatik pulmoner fibrozis için yeni tedavilerin keşfinde yapay zeka ve makine öğrenimi kullanılması ve böylece genler, proteinler ve hastalıklar arasındaki ilişki ağı çözümlenmesi amaçlanmıştır. Makine öğrenimi, veriler arasındaki sistematik bağlantıları kurarken, yapay zeka tabanlı akıl yürütme sistemleri kullanılmaktadır. Bu platform sayesinde, hastalıkların mekanizmalarının anlaşılması, elde edilen sonuçların yeni ilaç geliştirme ve ilaç hedeflerini belirlemede kullanılması mümkün olacaktır. İlaç şirketlerinin uzun yıllardır yaptığı araştırmalar sonucu elinde bulunan büyük miktardaki veriyi, yapay zeka ve makine öğrenimi yetenekleriyle birleştirerek hastalıkların patofizyolojisini belirlemek de amaçlanmaktadır [40]. Bir başka araştırma ise ilaç adayı moleküllerin farmasötik özelliklerini tahmin etme amacıyla kuantum mekaniği ve makine öğrenimini birleştirmeyi hedeflemektedir [41].

Makine Öğrenimi (Machine Learning- ML)

Makine öğrenimi, yapay zekanın bir dalıdır. Veri gövdelerine dayanan hesaplamalı modelleri eğiterek süreçleri modellemeyi amaçlamaktadır. Örneğin ML, önceki deneylerden elde edilen verileri dikkate alarak belirli bir ilaç formülasyonunun stabilitesini tahmin edebilmektedir [42].

Makine öğrenimi alanındaki son gelişmeler, daha hızlı süreç yönetimi, geniş kullanılabilirlik gibi önemli üstünlükler sunmaktadır. Tüm alanlarda olduğu gibi farmasötik endüstride de makine öğrenimine dayalı uygulamalar kullanılmaya başlanmıştır. Farmasötik endüstrinin mevcut durumuna bakıldığında formülasyon geliştirme sürecinde etkin ve yardımcı tüm maddelerin özellikleri göz önünde bulundurularak optimum formülasyonu elde etmek hedeflenmektedir. Bu süreç pahalı, zahmetli ve zaman alıcıdır. Yapay zeka ve makine öğreniminin tahmin gücünden yararlanarak, mevcut verilerden kritik kalite özellikleri, kritik işlem parametrelerini belirlemek formülasyon geliştirme sürecine önemli katkıda bulunacaktır.

Suda çözünmeyen ilaçlar için etkili bir çözündürme tekniği olan amorf katı dispersiyonların fiziksel stabilite sorunu tekniğin gelişmesinin önünde büyük bir engeldir. Stabilite deneylerinin minimum altı ay süreyle test edilmesi gerekmektedir. Bu, zaman alıcı ve tahmin edilemez bir süreçtir. Han ve ark.'nın yaptığı bir araştırmada katı dispersiyon formülasyonlarının stabilitesi için 8 farklı makine öğrenimi tekniği ile 646 formülasyon verisi kullanılarak tahmin modelleri geliştirilmiştir. Deneysel veriler ve modelleme sonuçlarının karşılaştırılmasıyla tahmin gücü en yüksek olan makine öğrenimi tekniği belirlenmiş ve farklı formülasyonlar için de uygulanabilir olduğu gösterilmiştir [43].

Büyük Veri (Big Data)

Büyük veri, çok çeşitli kaynaklardan hızla oluşturulan ve iletilen büyük, karmaşık veri kümelerini ifade eder [44]. Makine öğrenimi ve yapay zeka gibi veri odaklı yaklaşımların farmasötik endüstri ile uyumlu hale getirilmesiyle, uzun yıllardır elde edilen büyük veriler değerlendirilme fırsatını yakalayacaktır. Aynı zamanda büyük bir hızda üretilmeye devam eden verilerin de sınıflandırılması, depolanması ve kullanımı yenilikçi yaklaşımlar ile sağlanabilir.

Avrupa İlaç Ajansı (EMA) büyük veri kullanımının ilaç geliştirme sürecini hızlandırabileceğine, ilaçların daha etkili ve güvenli bir şekilde kullanılmasını sağlayabileceğine ve sağlık sistemleri üzerinde olumlu etkileri olabileceğine inanmaktadır. Bununla birlikte, EMA, büyük verinin potansiyel risklerinin de farkında olduğunu ve bu nedenle, veri güvenliği ve veri korumasına özel önem verdiğini belirtmektedir. Büyük verinin tıpta kullanımına yönelik stratejiler geliştirmek amacıyla 2016 yılında özel bir birim kurulmuştur [45].

Bulut Bilişim (Cloud Computing)

"Bulut" terimi genellikle internet için bir metafor olarak kullanılır [46]. Çeşitli kaynaklardan gelen verilerin güvenli bir şekilde işlenmesi ve depolanması gerekliliği bulut bilişim kavramının ortaya çıkmasını sağlamıştır. Aynı zamanda oluşturulan çevrimiçi platformlar sayesinde depolama kapasitesi artırılmıştır. Çevrimiçi dünyaya entegre edilen bulut bilişim sistemleri ile farmasötik endüstride ilaç keşfi ve formülasyon geliştirme sürecinde elde edilen çok sayıdaki veriye erişim kolaylaşmaktadır. Bazı ilaç şirketleri yeni ilaçların keşfi ve geliştirilmesi süreçlerini iyileştirmek, tıbbi görüntüleme verileri gibi büyük boyutlardaki verileri depolamak ve işlemek için bulut bilişim servislerini kullanmaya başlamıştır [47,48]. Sağlık sektörünün son eğilimlerinden birisi olan giyilebilir cihazlardan gelen hasta verileri de bulut tabanlı uygulamalarda tedavi amaçlı depolanabilmektedir [49].

Medikal Nesnelerin İnterneti (IoMT)

Medikal nesnelerin interneti, nesnelerin interneti teknolojisinin tıbbi cihazlarda kullanımını yansıtan kavramdır [50]. Nesnelerin interneti kavramının ortaya çıkması sonucu internete bağlanabilen tüm cihazlardan gerçek zamanlı sinyal ve veri almak mümkün olmaktadır. Tıbbi amaçlı kullanımı olan cihazlardan hastaların sağlık verilerini sürekli olarak toplamak önemli bir gelişmedir. Sağlık hizmetlerinde nesnelerin interneti teknolojisi kullanımı konusunda en çok üzerinde durulan cihazlar kalp atışı, vücut sıcaklığı gibi verileri toplayarak uzaktan hasta izlemeye imkan tanıyan cihazlardır.

Giyilebilir cihazlar (*wearable devices*) olarak adlandırılan bu cihazlar hem hasta hem de sağlık sağlayıcılarına önemli üstünlükler sunma potansiyeline sahiptir.

Medikal nesnelerin interneti kavramının bazı uygulamaları aşağıda belirtilmiştir:

Şeker İzleme: Diyabetli hastalarda en sık karşılaşılan sorun, her öğünde şeker düzeyi ölçümüne uyuncun düşük olmasıdır. Var olan cihazlarla şeker düzeylerini ölçmek ve kaydetmek bazı hastalar için zahmetli bulunmaktadır. Aynı zamanda bu ölçümler hastanın sadece test yapılan andaki şeker düzeyini bildirmektedir. Şeker düzeylerinin sürekli değişkenlik gösterdiği hastalarda periyodik ölçümler yeterli görülmemektedir. IoT cihazları hastaların gün içindeki şeker düzeyindeki değişimlerin sürekli olarak izlenmesine olanak tanımaktadır. Bu cihazlar sayesinde manuel olarak kayıt tutulmasına gerek kalmadan veriler kaydedilmektedir. Şeker düzeyinde ciddi bir anormallik tespit edildiğinde hasta cihaz tarafından uyarılabilmektedir [51].

Kalp Ritmi İzleme: Sağlık tesislerinde bulunan hastalar için bile kalp ritmi izlemi zor olabilmektedir. Ayrıca periyodik olarak izlem yapmak, kalp ritmindeki dalgalanmaların gözden kaçırılmasına neden olabilmektedir. Sürekli kardiyak izlem için kullanılan geleneksel cihazlar hastaların sürekli olarak kablolarla makineye bağlanmasını gerektirir ve bu da hastaların hareketlilikleri üzerinde çok büyük bir kısıtlayıcıdır. Günümüzde kalp atış hızı takibi için çeşitli küçük IoT cihazları bulunmaktadır [52].

Depresyon ve Ruh Hali İzlemi: Depresyon belirtileri ve hastaların genel ruh halinin geleneksel sistemlerle elde edilmesi oldukça zor sağlık verileridir. Bu amaçla geliştirilen *"Mood-aware"* IoT cihazları hastanın kalp atışı ve kan basıncı gibi verileri toplayıp analiz ederek hastanın ruhsal durumu hakkında bilgi edinilmesini sağlayabilmektedir. Bu cihazlar, hastalık tanısı için çok net veriler sunmasa da sağlık personeline elde ettikleri verilerle yardımcı olabilmektedirler [53].

2012'de Amerikan Gıda ve İlaç Dairesi (FDA), ikinci nesil bir antipsikotik ajan olan aripiprazolün sensör gömülü bir versiyonu olan Abilify MyCite®'in kullanımına onay vermiştir [54]. Bu ilaç, yasal otorite tarafından onaylanan ilk ilaç-cihaz kombinasyonu olması nedeniyle tüm ilgileri üzerinde toplamıştır. Abilify MyCite®, birbirleriyle bağlantılı tablet, yama, uygulama, gösterge paneli ve iletişim alanından oluşan bir sistemdir ve yutulduktan sonra, giyilebilir bir Bluetooth yaması sayesinde akıllı telefona sinyal göndererek günlük verilerin tümünün tek bir yerde görülmesini sağlamaktadır [55].

Proteus Discover ise yine FDA tarafından 2012 yılında onaylanan ve günümüzde birkaç farklı ilaçla kullanılabilen dijital ilaç izleme sistemi olarak tasarlanmış bir tıbbi üründür. Bu sistem, ilacın içine yerleştirilmiş bir mikroçip ve giyilebilir bir yamadan oluşur. Mikroçip, ilacın alındığını algılar ve bu bilgiyi giyilebilir yamaya gönderir. Yama, bu bilgiyi kullanarak hasta davranışlarını ve ilaç kullanımını takip etmek için tasarlanmış bir uygulama aracılığıyla ilaç alım verilerini gösterir [56].

IoT teknolojisi aynı zamanda farmasötik üretimde de kendine yer bulabilecek potansiyele sahiptir. Verimliliği artırmak ve zaman tasarrufu sağlamak için ilaç endüstrisinde seri üretimden sürekli üretime geçiş konusunda çalışmalar yapılmaktadır. IoT tabanlı üretim cihazları operasyonel verileri gerçek zamanlı olarak diğer cihazlara ve üretim sorumlularına ileterek sürekli üretime katkı sağlamaktadır [57].

Çevresel Sürdürülebilirlik ve Endüstri 4.0

Birleşmiş Milletler, sürdürülebilirliği, gelecek nesiller de dahil olmak üzere herkes için daha sürdürülebilir bir refah sağlamak amacıyla; adaletsizlik, eşitsizlik, iklim değişikliği, çevresel bozulma ve çevre kirliliği gibi küresel sorunları ele almayı hedefleyen hareket olarak tanımlamaktadır [58]. Çevresel sürdürülebilirlik ise esas olarak çevresel sistemlerin dengesine, doğal kaynakların tüketimine ve ekolojik bütünlüğe odaklanmaktadır [59].

Son dönemde üretimde gündemde olan sürdürülebilir üretim kavramı, sürdürülebilir kalkınma hedeflerini endüstri ile bütünleştirmeyi amaçlamaktadır [60]. Sürdürülebilir kalkınma hedefleri ile çevresel, sosyal ve ekonomik verimliliğin artması amaçlanmaktadır.

Tüm bu potansiyeline rağmen Endüstri 4.0'ın ana odak noktası dijitalleşmedir ve çevresel sürdürülebilirlik, yeşil üretim gibi konular ikinci planda kalmaktadır. Endüstri 5.0'ın; sürdürülebilir, insan odaklı, esnek bir endüstriye geçişte araştırma ve inovasyonu merkeze alarak mevcut Endüstri 4.0 yaklaşımını tamamlaması ve kaynakların korunması, iklim değişikliği, sosyal istikrar dahil olmak üzere toplumsal sorunlara çözüm sağlamada aktif bir rol oynaması hedeflenmektedir [61]. Farmasötik

endüstride de Farma 4.0 ile birlikte Farma 5.0 hedefleri de belirlenmeli ve özellikle çevreci ve sürdürülebilir yaklaşımlar üzerinde daha çok durulmalıdır.

Enerji tüketiminde, karbon ayak izinde ve atık üretiminde azalma, farmasötik endüstrinin sürdürülebilirlik çabalarının özü olarak kabul edilmektedir [62]. Katı dozaj şekillerinin üretim işleminde yer alan granülasyon ve kaplama yöntemleri, farmasötik üretim tesislerinden kaynaklanan sera gazı emisyonunu önemli ölçüde artıran yöntemlerdir [63,64]. 2018 yılı istatistiklerine göre 52 milyon ton CO_2 üretimi ile farmasötik endüstri yeşil bir endüstri olmaktan çok uzaktır [65]. Farma 4.0 uygulamalarının kolaylıkla uygulanabileceği sürekli üretim [66], IoT ve yapay zeka tabanlı üretim sistemleri, verimliliği ve esnekliği artırmanın yanında atıkları azaltarak karbon emisyonunun azalmasını sağlayacaktır [67]. Çin ve Alman şirketlerde yapılan bir araştırmaya göre endüstriyel üretimde dijitalleşmenin enerji tüketimini azaltarak çevresel sürdürülebilirliğe katkı sunacağı düşünülmektedir [68].

SONUÇ VE TARTIŞMA

İnsanlık tarihi boyunca yaşanan gelişmeler çeşitli endüstri alanlarında etkisini göstermiştir. Farmasötik endüstri de bu alanlardan biridir. Farmasötik endüstride kaliteli, etkili ve güvenli ilaç üretmek ana hedeftir. Bu gereklilikleri sağlamadan üretim yapan ilaç şirketleri yasal otoritelerden onay alamaz. Bu nedenle ilk olarak bu gereklilikleri yerine getirmek hedeflenmektedir. Bu durum, teknolojik gelişmelerin hız kazandığı son yüzyılda farmasötik endüstrinin diğer alanlara nispeten teknolojik gelişmeleri geriden takip etmesine neden olmuştur. Kalitenin sağlandığını göstermek için uzun yıllar boyunca ampirik yaklaşım kullanılmıştır. Bu yaklaşımda kalitenin sağlandığı test edilerek gösterilmektedir. Kalitenin test edilerek gösterilmesi yaklaşımının yetersizliği gelişen teknolojinin de desteği ile net bir şekilde anlaşılmıştır. Zahmetli ve maliyetli olan ilaç geliştirme faaliyetlerinde kullanılmak üzere proses analitik teknolojisi (PAT) ve tasarımla kalite (QbD) yaklaşımları öne sürülmüştür. Bu yaklaşımlar ile farmasötik ürünlerin kalitesi sonuç ürün üzerinde yapılan testlerle değil süreç boyunca gösterilmektedir. Bu sayede üretim sürecinde yapılan değişikliklerin kalite üzerindeki etkisi erken safhalarda görülerek hızlı aksiyon alınabilmektedir. PAT kavramlarından biri olan tasarım aralığı, farmasötik ürünlerin sağlaması gereken özellikleri belirli limitler arasında tutmayı hedeflemektedir. Bu kavram sayesinde ampirik yaklaşıma kıyasla geniş bir alanda çalışılarak tasarım alanı içerisinde yapılan değişiklik için otoriteden tekrar onay alınması gerekliliği ortadan kaldırılmış olur [17]. Ruhsatlandırma süreçlerinde yaşanan aksaklıklar ve uzun değerlendirme süreleri ilaç endüstrisinin gelişimini yavaşlatan unsurlardan biridir. Benimsenen yeni yaklaşımlarla birlikte sağlık otoriteleri ve endüstri arasında eş zamanlı iletişim ve veri paylaşımının güçlendirilmesi hedeflenmektedir. QbD ve PAT yaklaşımlarına ek olarak Farma 4.0'ın dijital olanaklarından yararlanılarak ruhsatlandırma sürecinin ve böylece ilaçların piyasa çıkışının hızlanması önündeki engellerin sistematik olarak aşılacağı öngörülmektedir.

QbD ve PAT, ampirik yaklaşıma kıyasla çok daha modern ve sistematik yaklaşımlar olmasına rağmen içinde bulunduğumuz teknoloji çağının hâlâ gerisinde bulunmaktadır. Bu sistemleri kullanabilmek için de çok sayıda deney yapılması ve veri elde edilmesi gerekliliği, insan gücüne duyulan ihtiyacın sürmesine neden olmaktadır. Bilgisayar ve yapay zeka tabanlı uygulamaların aksine bu sistemler fiziksel dünya ile daha çok ilgilidir. Bu durum farmasötik endüstrideki üretim süreçlerinin kısalmasını ve farmasötik endüstrinin hızlı gelişim gösterebilen bir endüstri alanı olmasını engellemektedir [69].

Son yıllarda yaşanan teknolojik gelişmeler her alan gibi farmasötik endüstriyi de etkilemiştir. Üretim süreçlerinde verimliliği artırma konusunda büyük vaatlerde bulunan yeni sistemler dikkatleri üzerine çekmeyi başarmıştır. Önceleri bilim kurgu romanları ile hayatlarımıza giren yapay zeka kavramı artık adından ciddi anlamda söz ettirmektedir. Özellikle makine öğrenimi gibi gelişme gösteren sistemler üzerine kurulu olan yapay zeka teknolojisi pek çok sürecin minimum zaman ve maliyetle tamamlanmasını mümkün kılmaktadır [70].

Tüm bu teknolojik gelişmelerin ilaç endüstrisinde kullanımını içeren kavram Farma 4.0 olarak adlandırılmaktadır. Ham maddeden başlayarak hastanın tedavi izlemine kadar her basamakta etkili olması beklenen teknolojik gelişmeler farmasötik endüstrinin geleceği olarak görülmektedir. Son

zamanlarda kişiselleştirilmiş tıptan sıkça söz edilmesi bu teknolojiye duyulan ihtiyacı gözler önüne sermektedir. Yapay zeka destekli gen dizilimi programları sayesinde potansiyel hastalıklar henüz semptom göstermeden teşhis edilebilir duruma gelmiştir [71]. Hastalıkların teşhisi kadar önem taşıyan bir diğer konu ise tedavilerin kişiye özel uygulanabilmesidir. Geleneksel yöntemlerin takip edildiği dönemlerde "ortalama hasta fenotipi" dikkate alınarak tedaviler geliştirilmiş ve ilaç üretimleri yapılmıştır. Farmasötik endüstrinin seri üretim yapmasını mümkün kılan bu anlayış hastaları bireysel olarak değerlendirmek yerine istatistiksel ortalama verilerini kullanır. Farma 4.0 yaklaşımı ile uygulanmasının mümkün olduğu düşünülen esnek üretim sistemleri hasta odaklı tedavilere geçişi sağlayacaktır. Aynı zamanda IoT cihazlarının medikal veri toplayarak tam zamanlı izleme katkı sunması sayesinde tedavi protokolleri hasta odaklı olarak güncellenebilir duruma gelecektir. Tüm bu nedenler göz önüne alındığında bireyselleştirilmiş tedavi kavramı Farma 4.0 uygulamaları ile mümkün kılınabilmektedir [72].

Farmasötik endüstrinin bir anda Farma 4.0'a geçiş yapması gerçekçi bir beklenti olmamakla birlikte tüm dünyanın hızla bu sistemle bütünleşmeye başladığı gerçeği unutulmamalıdır. Mevcut durumda PAT ve QbD yaklaşımları dahi tam olarak uygulanamamaktadır. Var olan sistemleri yenileri ile değiştirmek belirli zaman alacaktır, teknolojik gelişmeler zamanla üretim süreçleri ile birleştirilmelidir.

Farma 4.0'ın içinde barındırdığı nesnelerin interneti ve bulut bilişim gibi kavramlar kaçınılmaz olarak akıllara güvenlik sorunlarını getirmektedir. Özellikle kişisel verilerin korunması konusunda siber güvenlik yetersiz görülmektedir. Son zamanlarda özellikle finans sektöründe sıkça karşılaşılan siber saldırıların, Farma 4.0 uygulaması sırasında sağlık verilerini elde etme amaçlı yaşanabileceği düşünülmektedir. Bu gibi olumsuzlukların üstesinden gelebilmek için siber güvenlik kavramı çok büyük önem taşımaktadır. Bulut sistemlerine aktarılan verilerin güvenli bir şekilde depolanması yine teknolojik gelişmelerin yararlarından biri olarak karşımıza çıkan şifreleme yöntemleri ile mümkün kılınmalıdır [73].

Mühendislik ve eczacılık alanlarının kesişimi gibi düşünülebilecek olan Farma 4.0, farmasötik endüstri açısından önemli üstünlükleri barındırmaktadır. Hem ilaç şirketlerine hem hastalara hem de sağlık otoritelerine kolaylıklar sunması Farma 4.0'ı göz ardı edilmemesi gereken bir konu durumuna taşımaktadır. Ayrıca yeni teknolojilerin endüstriyel alanda kullanımının çevreci faaliyetlerde bulunacağı ve sürdürülebilirlik kavramına katkı vereceği, uygulamaya geçen diğer endüstriyel alanlarda gösterilmiştir. Farmasötik endüstri de Farma 4.0 ile Birleşmiş Milletler'in kalkınma hedeflerinden olan çevresel sürdürülebilirlik ve yeşil kimya (*Green Chemistry*) uygulamaları olarak adlandırılan kimyasal atıkları azaltma politikalarına destek vermiş olacaktır [74].

Gelecek, bugün alınan kararlar ile şekillendirilmektedir. Söğüt ağacından asetilsalisilik asit sentezi ile başlayan farmasötik endüstri serüveni teknoloji çağına girmiş ve ona uyum sağlamaya başlamış durumdadır. Yabancı sermayeli pek çok şirket yapay zeka ve makine öğrenimini çeşitli alanlarda kullanmaya başlamıştır. Birkaç sene içinde bu girişimlerin artması ve telesağlık olarak da adlandırılan sağlık teknolojilerinin hayatımızda daha çok yer alması beklenmektedir.

YAZAR KATKILARI

Kavram: G.B., Y.A.Ç., L.Ö.; Tasarım: G.B., Y.A.Ç., L.Ö.; Denetim: G.B., Y.A.Ç., L.Ö.; Kaynaklar: G.B., Y.A.Ç., L.Ö.; Malzemeler: G.B., Y.A.Ç.; Veri Toplama ve/veya İşleme: G.B., Y.A.Ç.; Analiz ve/veya Yorumlama: G.B., Y.A.Ç., L.Ö.; Literatür Taraması: G.B., Y.A.Ç., L.Ö.; Makalenin Yazılması: G.B., Y.A.Ç.; Kritik İnceleme: G.B., Y.A.Ç., 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, 47(3): 1071-1083, 2023
Doi: 10.33483/jfpau.1261593

DERLEME MAKALE / REVIEW ARTICLE

UYUŞTURUCU MADDE BAĞIMLILIĞI VE TÜRKİYE'DE UYUŞTURUCU MADDE KULLANIMININ İNCELENMESİ

DRUG ADDICTION AND AN INVESTIGATION OF DRUG USE IN TURKEY

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ÖZ

Amaç: *Bu çalışmada faydalanılan birtakım istatistiki değerler ile uyuşturucu madde kullanım oranlarının ortaya konulması amaçlanmaktadır.*
Sonuç ve Tartışma: *Ulaşılan bilgiler ışığında Türkiye'de madde kullanım oranı günden güne arttığı görülmektedir. Emniyet Müdürlüğü ile birlikte kurumlar kaynaklarını uyuşturucu ile mücadele etmek için kullanmaktadır. Yapılan araştırmalara göre istatistiki analizler doğrultusunda mücadele için daha kapsamlı ve caydırıcı önlemler alınması zorunluluğu ortaya çıkmaktadır. Konu ile ilgili olan tüm paydaşlar, uyuşturucu ile mücadele için tüm paydaşlar ve kurumlar aynı mücadeleci yaklaşıma sahip olmalıdır.*
Anahtar Kelimeler: *Uyuşturucu, uyuşturucu madde bağımlılığı, uyuşturucu madde kullanımı*

ABSTRACT

Objective: *In this study, it is aimed to evaluate the drug use situation in Turkey in the light of current data on some statistical indicators.*
Result and Discussion: *It is observed that the number of people addicted to drugs is constantly increasing in Turkey. In this context, Turkey mobilizes all its resources and fights against drugs with all its institutions, especially the Police Department. However, the negative situation in the statistical indicators within the scope of the fight against drugs reveals that Turkey should take more deterrent measures in this regard. All relevant stakeholders should be mobilized for an effective fight against drugs.*
Keywords: *Drug, drug abuse, drug addiction*

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Gönderilme / Submitted : 07.03.2023
Kabul / Accepted : 03.05.2023
Yayınlanma / Published : 20.09.2023

GİRİŞ

Uluslararası anlaşmalar ve ülkelerin ulusal narkotik kanunları tarafından kontrol edilen maddeler morfin, kokain veya esrar benzeri uyuşturucu maddelerdir. Bunların kullanımı ile birey madde bağımlısı olmaktadır. Yine barbitürat ve amfetamin gibi maddeler de madde bağımlılığı ekseninde incelenen uyuşturucu türleridir [1].

Bu maddeler hücresel hedeflere göre değişmektedir. Farklı organ sistemlerini etkileyebilmekteler ve bu etkileri, uyguladıkları etki mekanizmaları açısından farklılık gösterebilmektedir. Bununla birlikte ortak bir özellik olarak tüm bu maddeler, bağışıklık sistemini de hedef almaktadır. Ekzojen olarak vücuda sokulan bu maddeler, merkezi sinir sistemindeki nöronları hedef alarak ve nörotransmisyon yollarının aktivitesini değiştirerek immün hücreler üzerinde dolaylı olarak etki edebilmektedir [2].

Maddenin kötüye kullanımı, kullanılan maddenin 1 yıllık bir süre zarfında tekrarlayacak şekilde yasal ve sosyal sorunlara yol açacak biçimde madde kullanım durumunu ifade etmektedir. Madde bağımlılığı ise bireyin almakta olduğu maddenin kişinin hayatında merkezi bir konuma sahip olduğunu belirtmektedir. Birey, madde kullanımında kontrolü yitirmiştir. Ve tüm bunların sonucunda bedensel ve psikolojik sorunlarla da uğraşıyor olması muhtemeldir. Madde bağımlılığı, tüm bu durumları kapsayan geniş bir kavramdır [3].

Madde bağımlılığı, bir kişide o maddenin periyodik veya sürekli olarak uygulanmasını takiben ortaya çıkan, bir maddeye psişik veya fiziksel olarak veya her ikisini de kapsayan bir bağımlılık durumudur. Böyle bir durumun özellikleri ilgili ajana göre değişecektir. Ve bu özellikler her bir özel durum için belirli bir uyuşturucu türü bağımlılığı belirlenerek açıklığa kavuşturulmalıdır. Amfetamin bağımlılığı ve kokain bağımlılığı bu duruma örnek olarak verilebilir [1].

Madde bağımlılığı, büyük miktarlarda yasal veya yasa dışı bileşen tüketen kullanıcıların bilişsel, davranışsal ve fizyolojik semptomlarında önemli problemleri ve bozuklukları ifade eden klinik bir terimdir. Kavramsal olarak bozukluk, madde bağımlısının vücudunda günden güne artan fiziksel veya psikolojik etkilerin farkına varılmasına rağmen alışılmış madde bağımlılığının sürdürülmesi anlamına gelmektedir [4].

Madde bağımlılığı, bağımlı birey ve çevresindekiler için zararlı sonuçlarına rağmen zorunlu uyuşturucu arama ve kullanımına neden olan kronik, sıklıkla beyin hastalığıdır. Uyuşturucu almaya yönelik ilk karar genellikle gönüllü olarak alınsa da zamanla beyinde meydana gelen değişiklikler bağımlı bir kişinin öz kontrolünü zorlaştırmaktadır ve uyuşturucu alma konusundaki yoğun dürtülere direnme yeteneğini engeller [5].

Maddeler sınıflandırılırken çeşitli kriterler kullanılmaktadır. Yasalara uygunluk temelinde maddeler yasal (alkol, tütün, kahve, hipnotikler, yatıştırıcılar, inhalantlar) ve yasa dışı (afyon, esrar, kokain, sentetik ilaçlar, halüsinojenler vb) olarak sınıflandırılmaktadır [6].

Birleşmiş Milletler Uyuşturucu ve Suç Ofisi'ne (UNODC) göre yasadışı uyuşturucular terimi, bu maddelerin tıbbi olmayan amaçlarla kullanılmasının önüne geçme girişimlerinin yanı sıra bu tür maddelerin üretilme, dağıtılma ve edinilme şekillerini de dikkate alarak bu maddelerin bulundurulması, üretimi, satışı veya tüketimi kanunen yasaklanmış maddeleri belirtmek amacıyla kullanılmaktadır [7]. Yasadışı uyuşturucular ve metabolitleri, insanlarda biyolojik etki için son derece yüksek potansiyele sahip kimyasal maddeler içermektedir [8].

Bu yasal olarak yasaklanmış psikotropik maddelerin kullanımı dünyanın birçok yerinde artıyor gibi görünmektedir, ancak bu davranış genellikle gizli olduğu için artış oranını ölçmek zor olmuştur. Uyuşturucu kullanımının hastalık yüküne dair yaptığı katkının boyutu dikkate değerdir. Önemli ölçüde can ve mal kaybına yol açtığına dair önemli kanıtlar bulunmaktadır [9].

Bu çalışmada faydalanılan birtakım istatistiki değerler ile uyuşturucu madde kullanım oranlarının ortaya konulması amaçlanmaktadır. Çalışmada değerlendirme konusu olan veriler, IHME (Institute for Health Metrics and Evaluation), T.C İçişleri Bakanlığı ve T.C Adalet Bakanlığından elde edilmiştir. Çalışma kapsamında belirtilen amaç doğrultusunda ilk olarak uyuşturucu madde bağımlılığına yol açan faktörler belirtilecek, sonrasında ise uyuşturucu maddelerin fiziksel, psikolojik ve toplumsal etkilerinden bahsedilecektir. Tüm bunlardan sonra ulaşılan veriler çerçevesinde Türkiye'de madde bağımlılığı konusu tartışılacaktır. Nihai olarak ise çalışmada sonuç bölümü yer almaktadır.

Uyuşturucu Bağımlılığına Yol Açan Faktörler

İnsanlar farklı sebeplerden dolayı uyuşturucu alma yoluna giderler. Bireyler iyi hissetmek için uyuşturucu alabilirler. Uyuşturucular yoğun bir zevk duygusu oluşturabilir. Yine sosyal kaygı ve stresten muzdarip olan bireyler daha az kaygılı hissetmek için uyuşturucu kullanmaya başlayabilirler. Stres, uyuşturucu kullanımına başlamada önemli bir faktör olabilir. Yine bazı insanlar odaklarını geliştirmek için üzerinde baskı hissedebilir. Spordaki veya işindeki yeteneklerini yapay olarak artırma adına reçeteli uyarıcıları kullanma isteği geliştirebilir. Yine bazı bireyleri uyuşturucu bağımlılığında merak ve sosyal baskı da etkileyebilmektedir. Akranların baskısı olumsuz etkileyebilir. Ergenlik, uyuşturucu kullanan akranlar gibi risk faktörlerinin varlığının madde kullanımına yol açabileceği gelişimsel bir dönemdir [10].

Bir bireyin bağımlı olabilmesinde biyolojik ve çevresel faktörler etkili olabilmektedir. Bireyin bağımlılık riskini etkileyebilecek biyolojik faktörler arasında genleri, gelişim aşamaları ve hatta cinsiyet veya etnik köken bulunmaktadır. Bilim adamları, epigenetik adı verilen genlerin bir bireyin bağımlılık riskinin %40 ila 60'ını oluşturduğunu tahmin etmektedir [10,11]. Ayrıca zihinsel bozukluğu olan gençler ve insanlar diğerlerine göre daha fazla uyuşturucu kullanımı ve bağımlılığı riski altındadır [10,12].

Depresyon ve anksiyete gibi duygusal sorunlar da uyuşturucu kullanımına yol açan önemli risk faktörlerindendir. Düşük benlik saygısı, özgüven eksikliği, güçsüzlük ve umutsuzluk gibi birtakım olumsuz duygulardan kaçma arzusu, gençlerin uyuşturucu madde alımına yönelmesinde önemli etkenlerdendir [13-15].

Aile yaşamının ve aile ilişkilerinin belirli yönleri, uyuşturucu sorunlarının başlaması, alevlenmesi ve nüksetmesiyle güçlü ve tutarlı bağlantılara sahiptir. Zayıf ebeveyn-ergen ilişkileri gibi faktörler, ergenin uyuşturucu kullanımını artırabilir [16].

Ailevi risk faktörleri arasında çocuklukta kötü muamele (istismar ve ihmal dahil) , ebeveynlerin uyuşturucu madde kullanımları, ebeveynlerin medeni durumu, ebeveynlerin eğitim düzeyi, ebeveyn-çocuk ilişkileri, ailenin sosyoekonomik durumu ve ebeveynlerin çocuklarına madde kullanımı kapsamında onay vermesi gibi durumlar da yer almaktadır [17].

Çocuk ihmalinin yasal tanımı, bir çocuğa bakmakla mükellef olan ebeveynin veya bakıcının koruma, giyim, sağlık bakımı veya yiyecek de dahil olmak üzere yeterli yaşam ihtiyaçlarını sağlamadığı bir durumu içermektedir [18]. İhmal mağduru olan çocukların madde kullanımı açısından yüksek risk altında olduğu, ortaya konulan çalışmalarda elde edilen sonuçlarla da sabittir [19,20].

Ergenlik, ergenlerin gruplarına ve ilişkilerine çok önem verdikleri, kendileri ve aileleriyle çatışmaya girdikleri, onları uyuşturucu kullanımı, suçluluk ve riskli cinsel davranışlar gibi dış durumlara karşı savunmasız bırakan bir keşif ve çatışma aşamasıdır. Genellikle öznitelikler olarak tanımlanan birden çok risk faktörüne maruz kalırlar veya daha yüksek düzeyde uyuşturucu kullanımı ilişkili birey veya bireylere maruz kalabilirler [21].

Ergenlerin uyuşturucu kullanımıyla alakalı olası risk faktörlerinin belirlenmesi, ergenlerin sağlığı ve gelişimi için olası yıkıcı sonuçları erken tespit etmek ve en aza indirmek için kamu politikalarının ve müdahale stratejilerinin geliştirilmesi oldukça büyük önem arz etmektedir [22].

Akran baskısı ve algılanan popülerliğin ergenlerde madde kullanımı için riski artırdığı yapılan çalışmalarda tespit edilmiştir [23-26] Spesifik olarak ergenler madde kullanımıyla beraber akran grubu içerisinde popülerliklerinin artacağına inandıklarından ötürü bu madde kullanımına katılma durumları daha yüksektir [25,26]. Kendini popüler olarak tanımlayan ergenler, bu şekilde tanımlayamayan ergenlere kıyasla madde kullanım yaygınlığının arttığını göstermiştir [26].

Uyuşturucu kullanan ergenler intihar, cinayet ve hastalık riski nedeniyle yaşıtlarına göre daha çok ölüm riskine sahiplerdir. Akıl sağlığı bozuklukları, depresyon, anksiyete, paranoya, halüsinasyonlar, gelişimsel gecikmeler, sanrılar ve duygu durum bozuklukları dahil olmak üzere bunların hepsi yasadışı uyuşturucu kullanımı ile ilişkilidir [27].

Yine fiziksel ve cinsel yönden istismar da uyuşturucu madde kullanımına yol açabilmektedir. Çocukların fiziksel istismarı, bir çocuğun tesadüfi olmayan bir şekilde zarar görmesine neden olan bir eylemi gerektirmektedir [17,18].

Erkeklerin fiziksel istismara uğrama olasılıkları daha yüksek olmakla beraber kadınların cinsel

istismara uğrama olasılıkları daha yüksektir [28]. Literatürdeki çalışmalar incelendiğinde çocuklukta görülen kötü muamelenin madde kullanımına erken başlamak için önemli bir risk faktörü olduğunu göstermektedir [29]. Fiziksel ve cinsel istismarın özellikle madde kullanımına ilişkin ergen davranışları incelenmiş ve fiziksel veya cinsel istismar ile ergenlerin esrar vb maddeler kullanımları arasında istatistiksel olarak anlamlı bir ilişki olduğunu tespit etmişlerdir [17,29,30]. Kokain, eroin ve barbitüratlar dahil olmak üzere daha yüksek düzeyde yasadışı uyuşturucu kullanımının fiziksel ve cinsel istismarla olduğuna dair kanıtlar bulunmaktadır. Fiziksel veya cinsel saldırı mağduru olmak, bir ergenin madde kullanımına bulaşma riskini iki ila dört kat artırmaktadır [30,31,32,33].

Dikkat eksikliği hiperaktivite bozukluğu (DEHB) ve depresyonun da yine uyuşturucu kullanımı kapsamına risk teşkil eden faktörler olduğu ifade edilmektedir [17,34]. Yine travma sonrası stres bozukluğu veya akıl hastalığı teşhisi konan bireyler, ergenlerde madde bağımlılığı için daha büyük risk altında yer almaktadırlar [17].

Uyuşturucu maddelerin bulunabilir olması ve erişilebilir olması, son yıllarda artan uyuşturucu kullanımına katkıda bulunan faktörlerden birisidir. Uyuşturucuya kolay erişim, gençler arasında da uyuşturucu kullanımının artmasına neden olmaktadır. Uyuşturucuya olan erişim zorlaştırılmadıkça insanların bu maddeleri deneme şansları artmakta ve böylelikle uyuşturucu bağımlılığı da artmaktadır. Dolayısıyla uyuşturucu maddelerin erişilebilirliği, olası olumsuz etkileri en aza indirecek veya ortadan kaldıracak eylemlerin gerçekleştirilebilmesi için acil dikkat gerektiren bir konudur [35].

Kanıtlar, hormonların uyuşturucu alma/arama davranışını etkilemek için stres ve ödül sistemleriyle etkileşime girebileceğini göstermektedir. Stres, uyuşturucu bağımlılığında önemli bir savunmasızlık faktörüdür ve hipotalamikpitüiter - adrenal (HPA) ekseni dahil olmak üzere çeşitli nörobiyolojik sistemlerle düzenlenir [36,37]. Nükleus akkumbens ve beynin ventral tegmental bölgelerinde dopamin devresini içeren ödül sistemi, aynı zamanda uyuşturucu bağımlılığı ile ilişkili bağımlılık davranışlarının oluşumu ve sürekliliğinin ayrılmaz bir parçasıdır [36,38-40].

Uyuşturucu bağımlılığının basit bir tedavisi yoktur. Uyuşturucu bağımlılığı bir kez geliştiğinde kronik bir durum olarak devam edebilir ve genellikle nükseder. Biyolojik, sosyokültürel, ekonomik ve psikolojik faktörlerin tümü uyuşturucu kullanımına katkıda bulunur. Uyuşturucu bağımlılığının tedavisi bu nedenle basit bir konu değildir [41]. Uyuşturucu tedavisi, bağımlı bireylerin zorunlu uyuşturucu arama ve kullanımını durdurmalarına yardımcı olmayı amaçlamaktadır. Tedavi çeşitli ortamlarda gerçekleşebilir, birçok farklı şekilde olabilir ve farklı süreler boyunca devam edebilir. Uyuşturucu bağımlılığı tipik olarak ara sıra tekrarlayan kronik bir hastalık olduğundan dolayı kısa süreli ve tek seferlik bir tedavi genellikle yeterli değildir. Birçoğu için tedavi, birden fazla müdahaleyi ve bağımlıyı düzenli olarak izlemeyi içeren uzun vadeli bir süreçtir [42]. Uyuşturucu kullanımı tedavi programlarının uzunluğu, hastanın taburcu olduktan sonraki durumu ile alakalı olarak en iyi fikir veren bileşenlerden birisidir [43]. Zaman içerisinde davranışsal tedavileri sürdürmek için birkaç aylık tedavi gerekli görülmektedir ve opioid bağımlılığının tedavisinde metadon gibi yöntemler için su süre bir yılı bulabilmektedir [44].

Uyuşturucu Maddelerin Fiziksel, Psikolojik ve Toplumsal Etkileri

Madde bağımlılığı, uyuşturucu arama ve alma zorunluluğuna yol açmaktadır. Uyuşturucunun alımındaki sınırlamalar bu kişilerde kontrol kaybına yol açmaktadır. Yine madde bağımlılığı bireyin sosyal ve mesleki işlevlerini yerine getirememesine neden olmaktadır. Madde bağımlılığı kronik olarak tekrarlayan bir bozukluktur [45]. Kronik olarak tekrarlama durumunun yanı sıra madde bağımlılığında tedavi veya iyileşme faaliyetlerine katılım olmazsa bağımlı olma durumu ilerler ve sakatlığa, hatta ötesi erken ölüme yol açabilir [46].

Psikiyatrik bir bakış açısıyla uyuşturucu bağımlılığının hem dürtü kontrol bozukluklarına hem de kompulsif bozukluklara yol açtığı bilinmektedir [47]. Dürtü kontrol bozuklukları, dürtüsel bir eylemin gerçekleştirilmesinden önce artan bir gerginlik veya uyarılma duygusu ile karakterizedir. Eylemin işlendiği sırada birey zevk, memnuniyet veya rahatlama duyguları deneyimleyebilir. Eylemin ardından da pişmanlık, kendisini kınama veya suçluluk duygusu hissedebilir veya bu duyguları hissetmeyebilir [48].

Uyuşturucu kullanımı önemli bir halk sağlığı problemi oluşturmaktadır. Madde kullanımı ve bağımlılığı, suç faaliyetlerine, HIV enfeksiyonunun ve diğer hastalıkların bulaşmasına, tıbbi masraflara,

ölümlere, yerel toplulukların ve hane halklarının rahatsız edilmelerine varana kadar topluma orantısız maliyetler yüklemektedir [49].

Uyuşturucu kullanımı çok ciddi fiziksel sorunlar ortaya çıkartmaktadır. Bu sorunlar arasında kısa vadede akciğer yetmezliği, kalp krizi, kalp yetmezliği ve uzun vadede obezite, akciğer ve kardiyovasküler hastalık, felç ve kansere sebep olabilmektedir. Aynı zamanda bu hastalık hepatit ve diğer hastalıklara yakalanma riskini de artırmaktadır [27].

Uyuşturucu kullanımı beraberinde olumsuz sosyal sonuçlara yol açmaktadır. Uyuşturucu tedavisi için önemli kamu kaynakları tahsis edilebilmektedir. Hükümetler, genel suç oranlarını düşürmek için uyuşturucu kullanımının başka insanlara yayılmasını sınırlamaya ve uyuşturucu kullanımı ile suç davranışı arasındaki bağı koparmaya çalışmaktadırlar [50-52].

Uyuşturucu bağımlılığı ve yasadışı uyuşturucu kullanımı sağlık sorunları, yoksulluk, şiddet, suç davranışı ve sosyal dışlanma ile ilişkilidir. Topluma olan toplam maliyetini tahmin etmek zordur. Uyuşturucu kullanımının sonuçlarına ilişkin sağlık bakımı ve diğer maliyetlere ek olarak uyuşturucu bağımlılığı aynı zamanda verimlilik ve hane halkı gelir kaybına yol açmaktadır. Yine madde bağımlılığı şiddet, güvenlik sorunları, trafik ve işyeri kazaları ve yolsuzluklar gibi sosyal maliyetleri de içerir. Tüm bunlar, çok büyük ekonomik maliyetlere ve kabul edilemez bir insan kaynağı israfına neden olur [53].

Morfin, kokain ve esrar dahil olmak üzere uyuşturucu maddelerin dünyanın her yerinden çok sayıda kişi tarafından eğlence amaçlı kullanımı, bu tür uyuşturucu kullanımının sonuçları hakkında ciddi endişe uyandırmıştır. Örneğin dünya genelinde milyonlar tarafından kullanılan esrar sadece nörolojik değil aynı zamanda immünolojik sistemleri de hem doğrudan hem de dolaylı olarak etkilediği yaygın olarak kabul edilmektedir. Opiatlar, özellikle morfin ve eroin, bağışıklık ve nörolojik sistem üzerindeki etkileri kapsamında geniş çaplı olarak incelenmiştir. Dahası kokain kullanımı artık salgın olarak nitelendirilmektedir [54].

Kokain ve amfetamin gibi uyarıcı maddeler, tipik olarak artmış refah ve coşku duygularına ve artan bir uyarılma durumuna neden olan maddelerdir. Morfin ve kodein gibi afyonlar, ağrının giderilmesi sebebiyle klinik ortamlarda kullanılabilmektedir. Bununla birlikte bunlar, yüksek dozda alındığında kullanıcının iyi olma hissine neden olabilir. Bu da bu maddelerin kötüye kullanılmasına ve nihayetinde bağımlılığa neden olabilmektedir [55].

Bir uyuşturucu madde, merkezi sinir sistemi içerisinde olduğu kadar çevresindeki çeşitli yanıt sistemleri üzerinde de etkili olabilmektedir. Örneğin asetilkolin metabolizmasına etki eden bir uyuşturucu madde, asetilkolin sistemlerinin aracılık ettiği tüm tepkileri etkilemesi beklenebilir. Yine örnek olarak yüksek dozdaki bir morfin, vücut sıcaklığında düşüşe neden olabilir [56].

Uyuşturucu madde bağımlılığı beyin kimyasında önemli ve kalıcı değişiklikler oluşturmaktadır. Uyuşturucu maddeler dışında alkol ve nikotin gibi maddeleri tedavi etmek için etkili ilaçlar mevcuttur. Yine afyon için de aynı şey söylenebilir. Fakat amfetamin gibi uyarıcılar ve esrarın tedavisi için aynı şeyleri söylemek pek olası değildir. Uyuşturucu madde bağımlılığı genellikle akut bir hastalıkmış gibi tedavi edilmektedir [57].

Ulusal Uyuşturucu Kullanımı ve Sağlık Araştırması (NSDUH), yasadışı uyuşturucu kullanımının kısırlık tedavisi gören erkekler arasında yaygın olduğuna dair güçlü kanıtlar ortaya koymaktadır. Erkeğin doğurganlığını olumsuz etkilediği tespit edilen yasadışı uyuşturucular esrar, opioidler, metamfetaminler, kokain ve anabolik androjenik steroidlerdir [58].

Sağlık, emniyet ve sosyal bakım alanlarındaki politika yapıcılara daha iyi rehberlik sağlamak için uyuşturucuların neden olduğu zararların etraflı bir şekilde ele alınması gerekmektedir. Bu uyuşturucu maddelerin olası zararlarının çok çeşitli olması bu durumu biraz zorlaştırsa da büyük önem arzettiği aşikardır [59].

Aşırı dozda esrar kullananların diğer uyuşturucu maddeleri de sıklıkla kullandıkları bilinmektedir. Bu durum potansiyel bir sinerjik etki oluşturarak zaten zarar ifade eden durumun daha fazla sonuçlanmasına yol açabilir [60].

Türkiye'de İstatistiki Göstergelerle Uyuşturucu Kullanımı

Ülkemizin mevcut coğrafi konumundan ötürü uyuşturucu trafiği kapsamında önemli bir geçiş noktası olduğu ifade edilebilir. 1980 yılından sonra hızlanarak artan silah ile eroin değişimi sonrasında ülkemizde de uyuşturucu maddelerin kullanılmaya başlandığı ifade edilebilir [61]. Ülkemize ulaşan

esrar ve eroin geçişinin yarısının kara, diğer yarısının da deniz ve hava yolu ile olduğu ifade edilmektedir. Bu kapsamda Marmara Bölgesi'nin ve bu bölge içerisinde yer alan İstanbul'un birinci derece öncelikli alanlar olduğu belirtilmektedir [62].

Gelişmiş ülkelerde olduğu gibi gelişmekte olan ülkeler için de uyuşturucu satışı önemli bir sorun olarak karşımıza çıkmaktadır. Türkiye de diğer ülkeler gibi uyuşturucu ile mücadele etmektedir. Uyuşturucu suçlarına yönelik düzenlemeler ile alakalı olarak cumhuriyet tarihi incelendiğinde konu ile ilgili olarak yapılan ilk düzenlemenin 1926 yılında olduğu görülmektedir. Mevzubahis düzenleme, 765 numaralı Türk Ceza Kanunu'dur. Yine uyuşturucu ile mücadele kapsamında 5237 sayılı Türk Ceza Kanunu da büyük önem arz etmektedir. Bu kanuna göre uyuşturucu veya uyarıcı madde imal etmek, aynı zamanda ticaretini yapmak, bununla beraber uyuşturucu madde veya uyarıcı maddenin kullanılmasını kolaylaştırmak; kullanım amacı ile uyuşturucu madde veya uyarıcı madde satın alma, kabul etme ve bulundurma durumları yasaklanmıştır [63].

Türkiye'de Emniyet Genel Müdürlüğü, uyuşturucu ile mücadelede aktif bir rol oynamaktadır. Yine Emniyet Genel Müdürlüğü kapsamında yer alan Türkiye Uluslararası Uyuşturucu ve Organize Suçlarla Mücadele Akademisi (TADOC), bu çerçevede ön planda yer almaktadır. 2004 yılında TADOC bünyesinde Türkiye Uyuşturucu ve Uyuşturucu Bağımlılığı İzleme Merkezi kurulmuştur. TUBİM, 08.10.2008 tarihli Resmî Gazete kararıyla TADOC'tan ayrı bir Şube Müdürlüğüne dönüştürülmüştür. Türkiye'de uyuşturucu ile mücadele amaçlı olarak ilk strateji belgesi, 1997 yılında hazırlanmış olup daha sonra bunun kapsamı genişletilerek 2006-2012 yıllarını içeren "Bağımlılık Yapıcı Maddeler ve Bağımlılık ile Mücadelede Ulusal Politika ve Strateji Belgesi" hazırlanmıştır. Bu strateji belgesinden sorumlu kurum olarak TUBİM işaret edilmiştir. Bu strateji belgesi kapsamındaki iki ayrı eylem planı yürütüldükten sonra bu strateji belgesinin geçerliliği 01.01.2013 tarihi itibariyle sonlanmıştır. Daha sonra 2016-2018 yıllarını kapsayan "2016-2018 Ulusal Uyuşturucu İle Mücadele Eylem Planı" yürürlüğe girmiş, akabinde ise şu an yürürlükte olan "2018-2023 Uyuşturucu ile Mücadele Ulusal Strateji Belgesi ve Eylem Planı" 11.05.2018 tarihinde yürürlüğe girmiştir [64].

Türkiye'de madde bağımlılığı ile alakalı güncel verilere ulaşmak için çalışma kapsamında faydalanılan birtakım kaynaklar bulunmaktadır. Bu kapsamda Sağlık Ölçüm ve Değerlendirme Enstitüsü (IHME), başvurulan uluslararası bir kaynak olmakla beraber bunun dışında yine T.C. İçişleri Bakanlığı ve T.C. Adalet Bakanlığı verilerinden faydalanılmıştır. Bu kaynaklar aracılığıyla ulaşılan istatistiki veriler üzerinden gidilerek Türkiye'deki uyuşturucu kullanımının durumu tartışılacaktır.

IHME kaynaklı verilere bakıldığında Türkiye için ilgili verilerin 1990-2017 yılları arası mevcut olduğu görülmektedir. Ulaşılan T.C. Adalet Bakanlığı verilerinin ise 1999-2019 yılları arası dönemi kapsadığı bilinmektedir. T.C. İçişleri Bakanlığı kaynaklı ulaşılan veriler ise 2011-2019 yıllarını kapsayan dönemi işaret etmektedir.

Tablo 1'de uyuşturucu madde kullanım bozukluğu olan nüfusun toplam nüfus içerisindeki % payı yer almaktadır. Tablo dikkatli incelendiğinde 2005 yılına kadarki olan yıl geçişlerinde belirli dönemlerde statik bir durumun var olduğu gözlemlenmekteyken, hatta 2001 yılından 2004 yılına kadar uyuşturucu bağımlısı olan kişilerin toplam nüfus içerisindeki payı sürekli olarak azalmış iken 2005 yılından sonra durumun tersine döndüğü görülmektedir. 2005 yılından itibaren Türkiye'de uyuşturucu kullanıcısı olan kişi sayısının toplam nüfus içerisindeki % payı sürekli olarak artmıştır. 2017 yılına gelindiğinde Türkiye'nin toplam nüfusunun %1.54'ünün uyuşturucu kullanım bozukluğu yaşadığı tespit edilmiştir.

Tablo 2'ye bakıldığında Türkiye'deki amfetamin, kokain, esrar, opioid ve diğer yasadışı uyuşturucuları kullanan kişilerin yıllara göre sayıları görülmektedir. 1990-2017 yılları arası verilerin bulunduğu tabloda belirtilen uyuşturucu türlerinin her birisinin kullanımında 1990 yılından 2017 yılına kadar artış gözlemlenmektedir. Türkiye'de 2017 yılı için amfetamin kullanıcılarının sayısı 25,425.15, kokain kullananların sayısı 34,663.56, esrar kullananların sayısı 87,420.83, opioid kullanıcılarının sayısı 1,11 milyon ve diğer yasadışı uyuşturucuları kullananların sayısı ise 17,381,31 olarak saptanmıştır.

Tablo 3'te ise 2017 yılında uyuşturucu madde kullanım bozukluğundan ölen kişilerin sayıları yer almaktadır. Tabloya bakıldığında farklı uyuşturucu türlerine göre ölümlerin sayısının yıllara göre arttığı gözlemlenmektedir. Amfetamin kaynaklı ölüm sayısı diğer uyuşturucu türlerine göre daha azdır. Yasadışı uyuşturucu kaynaklı ölüm sayısının 2017 yılı için 525 olduğu gözlemlenmektedir. Yine Opioid kaynaklı ölüm sayılarının yüksek olduğu görülmektedir. 2017 yılında Opioid kullananlardan 358 kişi

hayatını kaybetmiştir. Kokain kullanıcıların ölüm sayısı 28'dir. Diğer yasadışı uyuşturucuların kullanıcılarının 126'sı ölmüştür.

Tablo 1. Uyuşturucu madde kullanım bozukluğu olan kimselerin toplam nüfus içerisindeki % payı [65]

Yıllar	Yüzde
1990	1.27
1991	1.27
1992	1.28
1993	1.28
1994	1.29
1995	1.29
1996	1.30
1997	1.31
1998	1.33
1999	1.34
2000	1.34
2001	1.34
2002	1.33
2003	1.32
2004	1.31
2005	1.31
2006	1.32
2007	1.36
2008	1.40
2009	1.44
2010	1.47
2011	1.48
2012	1.49
2013	1.50
2014	1.51
2015	1.52
2016	1.53
2017	1.54

Tablo 2. Uyuşturucu madde kullanım bozukluğu olanların sayısı [65]

Yıllar	Amfetamin	Kokain	Esrar	Opioid	Diğer Yasadışı Uyuşturucular
1990	18,643.75	20,721.36	64,431.40	580,123.63	9,202.26
1991	19,044.32	21,177.93	65,672.66	598,671.10	9,456.61
1992	19,479.39	21,684.35	67,024.11	617,425.53	9,708.84
1993	19,903.22	22,191.99	68,316.30	636,076.24	9,966.98
1994	20,256.68	22,675.84	69,487.79	653,280.38	10,226.17
1995	20,559.10	23,163.99	70,694.50	669,450.47	10,489.23
1996	21,043.38	23,730.14	71,995.06	688,042.51	10,761.08
1997	21,743.66	24,360.74	73,257.05	709,168.80	11,047.06
1998	22,477.49	24,987.30	74,411.38	729,957.46	11,351.19
1999	23,117.02	25,557.54	75,400.56	747,807.39	11,630.45
2000	23,597.44	26,039.17	76,247.74	762,544.68	11,899.09
2001	23,967.17	26,452.99	76,993.17	773,240.52	12,187.70
2002	24,295.54	26,855.25	77,705.12	779,026.12	12,494.00
2003	24,576.54	27,246.17	78,379.03	782,223.56	12,804.80
2004	24,828.92	27,638.13	79,003.81	786,151.94	13,098.09
2005	25,056.88	28,038.34	79,583.19	795,459.06	13,416.40
2006	25,047.41	28,496.81	79,898.06	819,234.61	13,778.73
2007	24,760.54	29,055.11	79,934.50	857,949.53	14,171.93
2008	24,399.25	29,690.55	79,973.36	903,336.75	14,566.35
2009	24,125.73	30,336.92	80,139.03	946,514.27	14,933.08
2010	24,074.49	30,920.79	80,581.29	978,380.65	15,286.51
2011	24,171.58	31,453.24	81,271.00	1.00 milyon	15,636.66
2012	24,300.09	32,005.52	82,083.07	1.02 milyon	15,978.51
2013	24,489.31	32,577.03	83,022.02	1.04 milyon	16,298.31
2014	24,713.03	33,143.59	84,039.29	1.06 milyon	16,578.54
2015	24,939.91	33,696.77	85,150.54	1.08 milyon	16,831.02
2016	25,166.47	34,213.28	86,306.27	1.10 milyon	17,100.87
2017	25,425.15	34,663.56	87,420.83	1.11 milyon	17,381.31

Tablo 3. Uyuşturucu madde kullanım bozukluğundan ölen kişi sayısı [65]

Yıllar	Amfetamin	Kokain	Yasadışı Uyuşturucular	Opioid	Diğer Yasadışı Uyuşturucular
1990	5	9	125	63	47
1991	5	9	133	69	50
1992	5	10	142	75	52
1993	6	10	151	81	54
1994	6	11	159	87	56
1995	6	11	166	91	58
1996	6	11	170	94	59
1997	6	11	172	96	59
1998	6	11	174	98	59
1999	6	12	179	101	60
2000	6	12	180	101	61
2001	6	12	182	102	61
2002	6	12	181	102	60
2003	6	13	194	110	65
2004	6	13	204	117	68
2005	6	14	218	126	72
2006	7	14	231	135	75
2007	7	15	242	143	78
2008	7	15	256	152	81
2009	7	17	282	170	88
2010	8	17	297	181	91
2011	8	19	321	196	98
2012	9	21	350	216	105
2013	10	23	392	250	109
2014	11	25	454	300	119
2015	11	26	485	326	122
2016	12	27	504	342	124
2017	12	28	525	358	126

Tablo 4'te uyuşturucu madde kaynaklı ölümlerin yaş aralıklarına göre dağılımı yer almaktadır. Uyuşturucu madde kullanımından ötürü ölen kişilerin çoğunluğunun 15-49 yaş aralığında olduğu gözlemlenmektedir. Tablo 2017 yılı içerisinde 15 ile 49 yaş aralığında 342 kişinin hayatını kaybettiğini ortaya koymaktadır. 70 üzeri yaş grubu içerisinde uyuşturucu madde kullanımından ötürü hayatını kaybedenlerin sayısı 2017 yılı için 177'dir. Yine 50-69 yaş aralığı içerisinde yer alan kişilerden 226 kişi, 2017 yılında uyuşturucu madde kullanımından ötürü hayatını kaybetmiştir.

Tablo 5'te Adalet Bakanlığı'ndan elde edilen veriler bulunmaktadır. Tabloda uyuşturucu madde bağlantılı suçlardan ceza infaz kurumlarında bulunanların yıllara göre sayıları yer almaktadır. Sayılara bakıldığında 2012 yılından bu yana sürekli olarak hükümlü sayısının arttığı gözlemlenmektedir. 2019 yılında uyuşturucu bağlantılı suçlardan ceza infaz kurumlarında bulunanların sayısının 81540 olduğu görülmektedir.

Tablo 6'ya bakıldığında Türkiye'nin uyuşturucu ile mücadele kapsamında yapmış olduğu harcama tutarları görülmektedir. Bu harcamalar sadece 2013 yılından 2014 yılına gelindiğinde düşme eğilimi göstermiştir. Fakat bunun dışında Türkiye'nin uyuşturucu ile mücadele kapsamında yapmış olduğu harcamaların sürekli olarak arttığı Tablo 6 aracılığıyla gözlemlenmektedir. Ülkemizde uyuşturucu mücadelesi için 1.461.955.782 TL harcama yapılmıştır.

Tablo 4. Uyuşturucu madde kaynaklı ölümlerin yaş aralıklarına göre dağılımı [65]

Yıllar	5 yaş altı	5-14 yaş	15-49 yaş	50-69 yaş	70+ yaş
1990	0	0	237	81	32
1991	0	0	255	87	33
1992	0	0	274	92	34
1993	0	0	295	97	36
1994	0	0	317	102	38
1995	0	0	339	106	40
1996	0	0	355	108	41
1997	0	0	372	109	43
1998	0	0	385	110	45
1999	0	0	353	111	48
2000	0	0	296	107	50
2001	0	0	266	107	53
2002	0	0	254	110	55
2003	0	0	200	110	64
2004	0	0	177	112	71
2005	0	0	169	117	79
2006	0	0	165	123	88
2007	0	0	164	128	94
2008	0	0	167	136	102
2009	0	0	177	150	114
2010	0	0	182	159	123
2011	0	0	194	168	133
2012	0	0	211	180	144
2013	0	0	246	191	149
2014	0	0	295	205	159
2015	0	0	319	213	163
2016	0	0	330	219	170
2017	0	0	342	226	177

Tablo 5. Uyuşturucu madde bağlantılı suçlardan ceza infaz kurumlarında bulunanların sayısının yıllara göre dağılımı [66]

Yıllar	Hükümlü Sayısı
1999	4249
2000	4348
2001	5399
2002	5480
2003	5973
2004	5558
2005	4125
2006	8320
2007	10533
2008	15447
2009	23082
2010	24925
2011	23638
2012	22.445
2013	24.890
2014	27.680
2015	33.261
2016	37.367
2017	50278
2018	57674
2019	81540

Tablo 6. Türkiye'de uyuşturucu ile mücadele kapsamında yapılan kamu harcamaları [64]

Yıllar	Harcamalar (TL)
2011	372.638.683
2012	395.792.280
2013	743.546.907
2014	592.858.200
2015	646.192.888
2016	721.885.355
2017	936.194.109
2018	1.362.728.424
2019	1.461.955.782

SONUÇ VE TARTIŞMA

Diğer dünya ülkelerinde olduğu gibi Türkiye için de uyuşturucu madde bağımlılığı ve uyuşturucu madde ile mücadele büyük önem arz etmektedir. Küreselleşmeyle beraber uyuşturucu maddelere erişim kolaylaşmış ve bu noktada ülkeleri yeni önlemler almaya itmiştir. Türkiye'nin ayriyeten coğrafi konumu, yine bu uyuşturucu madde bağımlılığı ile önemli bir dezavantaj oluşturmaktadır.

Uyuşturucu madde bağımlılığı ile alakalı istatistiki verilere bakıldığında Türkiye'de uyuşturucu madde kullanım bozukluğuna sahip olan kişilerin toplam nüfus içerisindeki payının sürekli olarak arttığı görülmektedir. IHME verilerine göre 2017 yılında Türkiye'de nüfusun %1.54'ü uyuşturucu madde kullanmaktadır. En çok kullanılan uyuşturucu türü ise araştırmalara göre opioidlerdir. Opoidlerden sonra esrar gelmektedir. 2017 yılı ölüm verilerine göre en fazla uyuşturucu madde kullanım kaynaklı olduğu ve resmi rakamlara göre 525 kişinin öldüğü anlaşılmaktadır. Diğer bir orana bakarsak opioid kullanıcılarının 358'inin öldüğü anlaşılmaktadır. Uyuşturucu madde kullanımı nedeniyle ölenlerin yaş aralıkları incelendiğinde 2017 yılında 15 ile 49 yaşları arası kişilerden 342 kişi ölmüştür. Aynı tabloya göre 50 ile 69 yaş arası bireylerin ise 226'sı hayatını kaybetmiştir.

Yurtiçi kaynaklarla ulaşılan veriler Adalet Bakanlığı ve İçişleri Bakanlığı kaynaklarından elde edilmiştir. İlk olarak Adalet Bakanlığı verileri 2019 yılı için uyuşturucu madde ile bağlantılı olduğu düşünülen suçlardan 81540 kişinin hükümlü olduğunu göstermektedir. İçişleri Bakanlığı verilerine bakıldığında Türkiye'nin yıllara göre uyuşturucu ile mücadele kapsamında yapmış olduğu kamu harcamaları bilgisi yer almaktadır. Türkiye'nin aynı yıl uyuşturucu madde kullanımıyla mücadele etmek için 1.461.955.782 TL harcadığı gözükmektedir.

Ulaşılan bilgiler ışığında Türkiye'de madde kullanım oranı günden güne arttığı görülmektedir. Emniyet Müdürlüğü ile birlikte kurumlar kaynaklarını uyuşturucu ile mücadele etmek için kullanmaktadır. Yapılan araştırmalara göre istatistiki analizler doğrultusunda mücadele için daha kapsamlı ve caydırıcı önlemler alınması zorunluluğu ortaya çıkmaktadır. Konu ile ilgili olan tüm paydaşlar, Uyuşturucu ile mücadele için tüm paydaşlar ve kurumlar aynı mücadeleci yaklaşıma sahip olmalıdır.

YAZAR KATKILARI

Kavram: F.C.Ö.; Tasarım: F.C.Ö.; Denetim: F.C.Ö.; Kaynaklar: F.C.Ö.; Malzemeler: F.C.Ö.; Veri Toplama ve/veya İşleme: F.C.Ö.; Analiz ve/veya Yorumlama: F.C.Ö.; Literatür Taraması: F.C.Ö.; Makalenin Yazılması: F.C.Ö.; Kritik İnceleme: F.C.Ö.; Diğer: F.C.Ö.

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, 47(3): 1084-1100, 2023
Doi: 10.33483/jfpau.1270660

DERLEME MAKALE / REVIEW ARTICLE

ANKSİYETE VE UYKU BOZUKLUKLARINDA KULLANILAN TIBBİ BİTKİLER

MEDICINAL HERBS USED IN ANXIETY AND SLEEP DISORDERS

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ÖZ

Amaç: *Anksiyete ve uyku bozuklukları ülkemizde ve dünyada son yıllarda giderek yaygınlaşan psikiyatrik hastalıklardandır. Anksiyete ve uyku bozukluğu olan bireyler hastalıklarının esas tedavisi olan farmakoterapi ve psikoterapiye ek olarak farklı tamamlayıcı tedavi arayışlarına yönelmektedir. Bu tamamlayıcı tedavi yöntemleri arasında en sık başvurulan kaynakların tıbbi bitkiler olduğu kaçınılmaz bir gerçektir. Fakat bu ürünlerin "yan etkisi daha az/daha zararsız/daha etkili" olabileceği düşüncesi gözden geçirilmesi gereken önemli bir konudur. Bu derlemede anksiyete ve uyku bozukluklarında kullanılan tıbbi bitkilerin PubMed, Science Direct, Google Akademik, BioMed Central, TÜBİTAK Ulakbim veri tabanlarında detaylı bir literatür taraması ile belirlenmesi, en sık kullanılanlarının ise hastalıklar üzerindeki potansiyel etki mekanizmalarının incelenmesi ve olumlu/olumsuz etkilerinin ortaya konması amaçlanmıştır.*

Sonuç ve Tartışma: *Literatürdeki çalışmalar değerlendirildiğinde; anksiyete ve uyku bozukluklarında kullanılan tıbbi bitkilerin fazlalığına rağmen birçoğunun potansiyel etki mekanizması ve olumlu/olumsuz etkileri tam olarak ortaya konmamıştır. Bu bitkilerin yanlış ve bilinçsizce kullanımı hastalığın seyrinin kötüleşmesine yol açabileceğinden anksiyete ve uyku bozukluklarında kullanılan tıbbi bitkilerin incelendiği daha fazla araştırmaya ihtiyaç duyulmaktadır.*

Anahtar Kelimeler: *Anksiyete, tıbbi bitkiler, uyku bozuklukları*

ABSTRACT

Objective: *Anxiety and sleep disorders are psychiatric diseases that have become increasingly common in our country and in the world in recent years. Individuals with anxiety and sleep disorders tend to seek different complementary therapies in addition to pharmacological treatment, which is the main treatment of their disease It is an inevitable fact that the most frequently used sources among these complementary treatment methods are medicinal herbs. However, the idea that these products may have "less side effects / less damage/ more effect" is an important issue that needs to be reviewed. In this review, the medicinal plants used in anxiety and sleep disorders are determined*

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Gönderilme / Submitted : 24.03.2023
Kabul / Accepted : 12.05.2023
Yayınlanma / Published : 20.09.2023

by a detailed literature review in PubMed, Science Direct, Google Scholar, BioMed Central, TUBITAK Ulakbim databases, and to examine the potential mechanisms of action of the most frequently used ones on diseases and to reveal their positive/negative effects.
Result and Discussion: *When the studies in the literature are evaluated; Despite the abundance of medicinal herbs used in anxiety and sleep disorders, the potential mechanism of action and positive/negative effects of many of them have not been fully revealed. Since the wrong and irrational use of these herbs may lead to worsening of the course of the disease, more researches are needed to examine examine medicinal herbs used in anxiety and sleep disorders.*
Keywords: *Anxiety, medicinal herbs, sleep disorders*

GİRİŞ

Bitkilerin insanoğlu tarafından tedavi amacıyla kullanımının antik çağlara dayandığı bilinmektedir [1]. Dünya Sağlık Örgütünün verilerine göre bireylerin %70-80'inin temel sağlık hizmetleri uygulamalarında tıbbi bitkilerden yararlandığı belirtilmiştir [2].

Toplumumuzda son yıllarda insidansı ve prevalansı giderek artan anksiyete ve uyku bozuklukları da tıbbi bitkilerin kullanımına sıkça başvurulan hastalıklardandır [3]. Bu bitkiler doğal kaynaklardan elde edildikleri için "Yan etkisi daha az/daha zararsız/daha etkili" olduğu yanılgısı ile bilinçsiz bir şekilde kullanılabilmektedir. Doğru ürün, ancak doğru zamanda, uygun dozda ve uygun formülasyonda kullanıldığı takdirde hastalık üzerinde potansiyel olumlu etkisini gösterebilme özelliğine sahiptir. Aksi takdirde bu ürünler istenmeyen yan etkilere veya hastalığın seyrinin kötüleşmesine yol açabilir.

Bu derlemenin amacı anksiyete ve uyku bozukluklarında sıklıkla kullanılan tıbbi bitkilerin potansiyel etki mekanizmalarını ve olumlu/olumsuz etkilerini güncel veriler ışığında ortaya koymaktır.

Anksiyete Bozukluğu

Dünyanın birçok ülkesinde sosyal adaptasyon, psikolojik baskı ve streste genel bir artış yaşanmakta ve bunun sonucunda bireylerde bilişsel ve davranışsal bozukluklar ve bunlarla ilgili sorunlarla sonuçlanan çok sayıda zorluk ortaya çıkabilmektedir. Anksiyete, hafif düzeydeyken günlük hayattaki olumsuz durumlara karşı verilen bir tepki olabileceği gibi, fiziksel, psikolojik ve sosyal işlevselliği etkilediğinde patolojik bir bozukluk olarak kabul edilir. Anksiyete bozukluğu, aşırı ve kontrol edilemeyen endişe ile karakterize olan ve en sık bildirilen psikiyatrik hastalıklar arasındadır. Diğer psikiyatrik hastalıkların yanı sıra anksiyete bozukluğu olan bireylerde baş ağrısı, kontrolsüz titreme ve terleme, kas gerginliği ve ağrıları gibi birçok fiziksel belirtiler görülebilmektedir. Anksiyete bozukluğu arasında en yaygın olanı en az 6 ay süren, sürekli ve aşırı anksiyete ile karakterize olan yaygın anksiyete bozukluğudur. Kronik anksiyete olarak tanımlanan yaygın anksiyete bozukluğu, bireyleri yaşamları boyunca etkileyebileceğinden üzerinde hassasiyetle durulması gereken bir konudur [4-10].

Anksiyete bozukluklarının yaşam boyu prevalansının dünya çapında %16.6'ya ulaşmasıyla birlikte nedenleri ve tedavileri konusunda devam eden araştırmalar önemli ölçüde hız kazanmıştır [11]. Anksiyete bozukluklarının geleneksel tedavisi, farmakoterapi ve psikoterapiye dayanmaktadır [12]. Tamamlayıcı yöntemler ise tek başına veya diğer yöntemlerle kombine halde kullanılabilmektedir [12,13].

Bununla birlikte, anksiyete bozukluğunun tedavisinde önemli rol oynayan antidepresanlar ve anksiyolitikler hem santral hem de periferik sinir sistemi işlevinde nörotransmitterleri modüle ederek hareket etmektedir [12,13]. Ancak bu tedavi yöntemleri birçok hastayı tedavi etmede yetersiz kalabilmektedir [14]. Ayrıca pek çok hasta benzodiazepinlerin olumsuz etkilerini yaşamakta veya ortaya çıkan semptomlar tam olarak kontrol edilemeyebilmektedir [15]. Bu nedenlerle anksiyete dahil olmak üzere çeşitli psikiyatrik hastalıkları tedavi etmek için tıbbi bitkilerin kullanımında önemli bir artış görülmektedir [16]. Bu ürünler toplumda, sentetik ilaçlara göre yan etkisi daha düşük veya tedavi edici özelliği daha yüksek ve daha güvenli alternatifler olarak algılanabilmektedir [16,17]. Fakat doğru ürün, ancak doğru zamanda, uygun dozda ve uygun formülasyonda kullanıldığı takdirde konvansiyonel tedaviyi destekleyici bir rol üstlenebilmektedir.

Anksiyete semptomlarını tedavi etmek için tıbbi bitkilerin kullanımına dair belgelenmiş geleneksel kayıtlar olsa da modern araştırmalardan elde edilen etkililik kanıtı yeteri düzeyde değildir. Bu nedenle bu ürünlerin anksiyete belirtilerini azaltmadaki etkinliklerini belirlemek için daha fazla araştırmaya ihtiyaç duyulmaktadır.

Uyku Bozuklukları

Uyku, bilincin tamamen veya kısmen kapandığı, karar verme becerisinin geri dönüştürülebilir bir biçimde kaybolduğu, fiziksel ve zihinsel dinlenmeye geçtiği, vücut fonksiyonlarının düzenlenmesi ve yaşam kalitesi için gerekli fizyolojik restorasyonun gerçekleştiği, vücudumuzun her yaşta ihtiyaç duyduğu evrensel ve doğal bir süreçtir [18,19].

"Gece boyunca uykuya dalmakta veya uyku durumunu sürdürmekte güçlük" olarak tanımlanan uykusuzluk önemli ve uzun vadeli sağlık sorunlarına yol açan yaygın bir hastalıktır [20]. Çeşitli toplumlarda yaygınlığının %10-40 olduğu bildirilmektedir [21]. Uykusuzluk genellikle diğer psikiyatrik ve fiziksel bozukluklarla birlikte görülmektedir [21-23]. Uyku bozuklukları hem uyku kalitesi hem de genel sağlıkla ilgili sorunlara neden olabilmektedir [20-23].

Amerikan Uyku Tıbbı Akademisi (American Academy of Sleep Medicine=AASM) tarafından yapılan Uyku Bozukluklarının Uluslararası Sınıflandırmasında (International Classification of Sleep Disorders=ICSD-3); bu sorunlar uykusuzluk, uyku ile ilişkili solunum bozuklukları, merkezi hipersomnolans bozuklukları, sirkadiyen ritim uyku-uyanıklık bozuklukları, uyku ile ilgili hareket, parasomniler ve diğer uyku bozuklukları olarak başlıca 7 kategoride sınıflandırılmaktadır [24-26].

Dünyada toplumun yaklaşık %30-50'sini etkileyen kısa süreli uykusuzluk 3 aydan daha kısa bir süre içinde kendini göstermektedir [27]. Fakat bu durum en az 3 ay boyunca haftada en az 3 kez meydana geldiğinde kronik olarak kabul edilmektedir [27,28]. Bu tür kronik uykusuzluğun dünya nüfusunun %5-10'unu etkilediği tahmin edilmektedir ve günlük hayattaki işlevlerin aksaması, sağlığın ve yaşam kalitesinin olumsuz etkilenmesi ile ilişkilidir [28].

Kronik uykusuzluğun tedavisi genellikle benzodiazepinler ve hipnotikler gibi reçeteli ilaçları içermektedir [27]. Ancak bu ilaçların bağımlılık, baş ağrısı, kâbus görme, gündüz yorgunluğu, mide bulantısı, konfüzyon ve denge kaybı gibi birçok yan etkisi bulunmaktadır [27,28]. Antipsikotikler ve antidepresanlar gibi diğer farmakolojik tedavilerin de benzodiazepinler ve hipnotikler gibi benzer yan etkileri vardır [27,28]. Buna rağmen, özellikle ileriki yaşlarda kronik uykusuzluk durumu yaşayanlar için bu ilaçlar genellikle "endikasyon dışı" olarak reçete edilmektedir [27].

Uykusuzluk durumlarında ilaç tedavisine ek olarak, tamamlayıcı tedaviler ve bilişsel-davranışçı terapi (davranış terapisi, gevşeme tepki eğitimi, farkındalık meditasyon eğitimi) başta olmak üzere çeşitli yaklaşımlar yaygındır [27-29]. Farmakoterapi ve psikolojik müdahaleler uykusuzluk ve uyku bozukluklarının ana tedavileri olsa da, tıbbi bitkiler antik çağlardan beri farklı toplumlar tarafından bu bozuklukları tedavi etmek için kullanılmıştır [27]. Tıbbi bitkilerin uykusuzluk durumunu yaşayan bireylerde gece uykusunu başlatmak ve sürdürmek için takviye olarak kullanılabileceğine dair önemli kanıtlar mevcuttur [26,27]. Genellikle Gama aminobütirik asit (GABA) sistemi üzerinde etkilere sahip olan bu bitkilerin, dopaminerjik yol dahil olmak üzere uyku ile ilgili diğer önemli mekanizmalarda da rol oynayarak sedatif etki gösterdiği bilinmektedir [27-29].

Anksiyete ve uyku bozukluğunda sıklıkla kullanılan tıbbi bitkiler Tablo 1'de belirtilmiştir.

Tablo 1. Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkiler [27,30-41]

Bitki adı	**Yaygın adı**	**Familyası**
Abies pindrow Royle	Batı Himalaya köknarı	Pinaceae
Achillea millefolium L.	Civanperçemi	Asteraceae
Adiantum capillus-veneris L.	Venüssaçı	Pteridaceae
Agrimonia eupatoria L.	Koyunotu	Rosaceae
Albizia adianthifolia (Schum.) W.Wight	Düz taç	Fabaceae

Tablo 1 (*devamı*). Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkiler [27,30-41]

Bitki adı	**Yaygın adı**	**Familyası**
Albizia julibrissin Wild. Durazz.	Gülibrişim	Fabaceae
Albizzia lebbeck (L.) Benth.	Lebbek ağacı	Fabaceae
Aloysia citrodora Paláu	Limon otu	Verbenaceae
Aloysia polystachya (Griseb.) Moldenke	Herhangi bir kayıt bulunamamıştır.	Verbenaceae
Angelica archangelica L.	Melek otu	Apiaceae
Angelica sylvestris L.	Melek otu	Apiaceae
Angelica sinensis Oliv. Diels	Melek otu	Apiaceae
Aniba riparia (Nees) Mez.	Herhangi bir kayıt bulunamamıştır.	Lauraceae
Annona cherimola Mill.	Çerimoya	Annonaceae
Anthemis arvensis L.	Tarla papatyası	Asteraceae
Apocynum venetum L.	Pembekız	Apocynaceae
Artemisia absinthium L.	Pelin otu	Asteraceae
Artemisia arborescens L.	Pelin otu	Asteraceae
Artemisia vulgaris L.	Pelin otu/Bayağı yavşan	Asteraceae
Arum italicum Mill.	Yılan yastığı	Araceae
Arum pictum L.f.	Yılan yastığı	Araceae
Asparagus racemosus L.	Hint kuşkonmazı	Asparagaceae
Avena fatua L.	Yabani yulaf	Poaceae
Azadirachta indica A. Juss.	Nim	Meliaceae
Bacopa monniera (L.) Wettst.	Brahmi/Bakopa	Plantaginaceae
Ballota nigra L.	Köpekotu	Lamiaceae
Blumea lacera (Burm.f.) DC.	Herhangi bir kayıt bulunamamıştır.	Asteracea
Boophone disticha L.f.	Yüzyıl bitkisi	Amaryllidaceae
Borago officinalis L.	Hodan	Boraginaceae
Caesalpinia bonducella (Roxb)	Herhangi bir kayıt bulunamamıştır.	Caesalpiniaceae
Calendula officinalis L.	Aynısefa	Asteraceae
Camellia sinensis (L.) Kuntz	Çay	Theaceae
Cannabis sativa L.	Hint keneviri	Cannabaceae
Caralluma adscendens var. *fimbriata* (Wall.) Gravely & Mayur	Herhangi bir kayıt bulunamamıştır.	Apocynaceae
Casimiroa edulis La Llave	Beyaz sapot	Rutaceae
Catha paniculata Scheidw.	Herhangi bir kayıt bulunamamıştır.	Celastraceae
Cecropia glaziovii Snethl.	Herhangi bir kayıt bulunamamıştır.	Urticaceae
Centella asciatica (L.) Urban	Gotu kola	Apiaceae
Centranthus ruber (L.) DC.	Kırmızı kediotu	Valerianaceae
Chamaemelum nobilis All.	Romen sarı papatyası	Asteraceae
Cirsium rivulare (Jacq.) All.	Deve dikeni	Asteraceae
Citrus aurantium L.	Turunç	Rutaceae
Citrus limon (L.) Osbeck	Limon	Rutaceae
Citrus sinensis (L.) Osbeck	Portakal	Rutaceae
Clinopodium nepeta (L.) Kuntze	Kedi fesleğeni	Lamiaceae
Clitoria ternatea L.	Mavi kelebek sarmaşığı	Fabaceae
Cocos nucifera L.	Hindistan cevizi palmiyesi	Arecaceae

Tablo 1 (*devamı*). Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkiler [27,30-41]

Bitki adı	**Yaygın adı**	**Familyası**
Conium maculatum L.	Baldıran	Apiaceae
Coptis chinensis Franch	Çin altın ipliği	Ranunculaceae
Coriandrum sativum L.	Kişniş	Apiaceae
Corydalis cava (L.) Schweigg. & Körte	Çayır kazgagası/Kırım kazgagası	Papaveraceae
Crataegus aronia L.	Müzmüldek	Rosaceae
Crataegus laevigata (Poir.) DC.	İngiliz alıcı/Bahçe alıcı	Rosaceae
Crataegus monogyna Jacq.	Adi alıç/Yemişen	Rosaceae
Crataegus rhipidophylla Gand.	Kırmızı çiçekli yemişen	Rosaceae
Crinum giganteum Andrews	Herhangi bir kayıt bulunamamıştır.	Amaryllidaceae
Crocus sativus L.	Safran	Iridaceae
Cydonia oblonga Mill.	Ayva	Rosaceae
Cymbopogan citratus DC.	Limonotu	Poaceae
Cynodon dactylon (L.) Pers.	Ayrık otu	Poaceae
Davilla rugosa Poir.	Herhangi bir kayıt bulunamamıştır.	Dilleniaceae
Dianthus seguieri Vill.	Çayır karanfili	Caryophyllaceae
Drymaria cordata (L.) Willd. ex Schult.	Tropikal kuş otu	Caryophyllaceae
Ecballium elaterium (L.) A. Rich	Eşek hıyarı	Cucurbitaceae
Echium amoenum L.	Engerek otu	Boraginaceae
Erythrina mulungu Benth.	Mulungu/Mercan ağacı	Fabaceae
Erythrina variegata L.	Hint Mercan ağacı	Fabaceae
Erythrina velutina Willd.	Mulungu	Fabaceae
Eschzolzia california Cham.	Acem lalesi	Papaveraceae
Eucalyptus globulus Labill.	Mavi okaliptüs	Myrtaceae
Euphorbia hirta L.	Astım otu	Euphorbiaceae
Euphoria longana Lam.	Ejderha gözü	Euphorbiaceae
Eurycoma longifolia Jack.	Tongkat Ali	Simaroubaceae
Euphorbia neriifolia L.	Hint sütleğen ağacı	Euphorbiaceae
Foeniculum vulgare Mill.	Rezene	Apiaceae
Galphimia glauca Cav.	Altın yağmuru	Malpighiaceae
Gastrodia elata Blume	Herhangi bir kayıt bulunamamıştır.	Orchidaceae
Gingko biloba L.	Ginkgo/Mabet ağacı	Ginkgoaceae
Glycine max (L.) Merr.	Soya	Leguminosae
Glycyrrhiza glabra L.	Meyan	Fabaceae
Hallea ciliate (Aubrév. & Pellegr.) J. F.Leroy	Herhangi bir kayıt bulunamamıştır.	Rubiaceae
Hibiscus rosa sinensis L.	Japon gülü	Malvaceae
Humulus lupulus L.	Şerbetçiotu	Cannabaceae
Hyoscyamus niger L.	Kara banotu	Solanaceae
Hypericum perforatum L.	Sarı kantaron	Hypericaceae
Ilex aquifolium L.	Çobanpüskülü	Aquifoliaceae
Justicia hyssopifolia L.	Herhangi bir kayıt bulunamamıştır.	Acanthaceae
Jacobaea delphiniifolia (Vahl) Pelser &Veldkamp	Herhangi bir kayıt bulunamamıştır.	Asteraceae
Kielmeyera coriacea Mart. ex Saddi	Bal bitkisi	Calophyllaceae
Lactuca sativa L.	Marul	Compositae

Tablo 1 (*devamı*). Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkiler [27,30-41]

Bitki adı	**Yaygın adı**	**Familyası**
Lactuca virosa L.	Acı marul	Asteraceae
Laurus nobilis L.	Akdeniz defnesi	Lauraceae
Lavandula angustifolia Mill.	Lavanta	Lamiaceae
Lavandula stoechas L.	Karabaş otu	Lamiaceae
Leucanthemum alpinum Lam.	Alpler ay papatyası	Asteraceae
Litsea cubeba (Lour.) Persoon	May chang	Lauraceae
Lolium multiflorum Lam.	İtalyan çimi/Süt otu	Poaceae
Lolium perenne L.	İngiliz çimi	Poaceae
Lotus corniculatus L.	Gazelboynuzu	Fabaceae
Magnolia dealbata Zucc.	Bulutormanı manolyası	Magnoliaceae
Magnolia obovata Thunb.	Japon büyük yapraklı manolya	Magnoliaceae
Malva cretica Cav.	Gülhatmi	Malvaceae
Malva neglecta Wallr.	Küçük ebegümeci/Çoban çöreği	Malvaceae
Malva sylvestris L.	Büyük ebegümeci	Malvaceae
Mangifera indica L.	Mango	Anacardiaceae
Matricaria chamomilla L.	Mayıs papatyası	Asteraceae
Matricaria discoidea DC.	Ananas otu	Asteraceae
Melilotus officinalis Pall.	Kokulu yonca	Fabaceae
Melissa officinalis L.	Melisa	Lamiaceae
Melittis melissophyllum L.	Melez oğulotu	Lamiaceae
Mentha aquatica L.	Su nanesi	Lamiaceae
Mentha piperita L.	Bahçe nanesi	Lamiaceae
Mentha spicata L.	Kıvırcık nane	Lamiaceae
Mentha suaveolens Ehrh. subsp. *suaveolens*	Elma nanesi	Lamiaceae
Mikania glomerata Spreng.	Guaco	Asteraceae
Mitragyna speciosa (Korth.) Havil.	Kratom	Rubiaceae
Momordica charantia L.	Kudret narı	Cucurbitaceae
Montanoa frutescens Mairet ex DC.	Herhangi bir kayıt bulunamamıştır.	Asteraceae
Morus alba L.	Beyaz dut	Moraceae
Murraya koenigi (L.) Spreng.	Köri ağacı	Rutaceae
Myrtus communis L.	Mersin bitkisi	Myrtaceae
Nardostachys jatamansi (D.Don) DC.	Hint sümbülü	Valerianaceae
Nectandra grandiflora Nees & Mart.	Herhangi bir kayıt bulunamamıştır.	Lauraceae
Nelumbo nucifera Gaertn.	Hint lotusu	Nelumbonaceae
Nepeta cataria L.	Kedi Nanesi	Lamiaceae
Nepeta menthoides Boiss. & Buhse	Herhangi bir kayıt bulunamamıştır.	Lamiaceae
Nepeta persica Boiss.	Herhangi bir kayıt bulunamamıştır.	Lamiaceae
Nerium oleander L.	Zakkum	Apocynaceae
Ocimum basilicum L.	Fesleğen	Lamiaceae
Olea europaea L.	Zeytin	Oleaceae
Opuntia ficus-indica (L.) Mill.	Hint inciri	Cactaceae
Origanum majorana L.	Mercanköşk	Lamiaceae
Paeonia mascula (L.) Mill.	Balkan şakayığı	Paeoniaceae
Paeonia moutan Sims.	Ağaç şakayık	Paeoniaceae

Tablo 1 (*devamı*). Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkiler [27,30-41]

Bitki adı	**Yaygın adı**	**Familyası**
Pachyrrhizus erosus L.	Meksika turpu	Fabaceae
Panax ginseng Meyer	Kore Ginsengi	Araliaceae
Papaver rhoeas L.	Gelincik	Papaveraceae
Papaver setigerum DC.	Truva haşhaşı	Papaveraceae
Papaver somniferum L.	Haşhaş	Papaveraceae
Passiflora caerulea L.	Çarkıfelek	Passifloraceae
Passiflora edulis Sims.	Çarkıfelek	Passifloraceae
Passiflora foetida L.	Çarkıfelek	Passifloraceae
Passiflora incarnata L.	Çarkıfelek	Passifloraceae
Paullinia cupana Kunth	Guarana	Sapindaceae
Pimpinella anisum L.	Anason	Apiaceae
Piper amalago L.	Herhangi bir kayıt bulunamamıştır.	Piperaceae
Piper methysticum G. Forst.	Kava kava	Piperaceae
Piper tuberculatum Jacq.	Herhangi bir kayıt bulunamamıştır.	Piperaceae
Polypodium vulgare L.	Benli eğrelti/Besbaye	Polypodiaceae
Primula veris L.	Yabani çuha	Primulaceae
Primula vulgaris Huds.	Mart çiçeği	Primulaceae
Protium heptaphyllum (Aubl.) Marchand	Herhangi bir kayıt bulunamamıştır.	Burseraceae
Prunus dulcis Koehne	Badem ağacı	Rosaceae
Prunus persica (L.) Batsch	Şeftali	Rosaceae
Psidium guajava L.	Elma guava/Bayağı guava	Myrtaceae
Rauvolfia serpentina (L.) Benth. ex Kurz	Hint yılan kökü	Apocynaceae
Rhodiola rosea L.	Altın kök	Crassulaceae
Robinia pseudacacia L.	Beyaz çiçekli yalancı akasya	Fabaceae
Rosmarinus officinalis L.	Biberiye	Lamiaceae
Rubus brasiliensis Martius	Herhangi bir kayıt bulunamamıştır.	Roseceae
Ruta chalepensis L.	Saçaklı sedef otu	Rutaceae
Salix alba L.	Ak söğüt	Salicaceae
Salvia officinalis L.	Adaçayı	Lamiaceae
Salvia reuterana Boiss.	Adaçayı/Fatmanaotu	Lamiaceae
Santalum album L.	Sandal ağacı	Santalaceae
Santolina insularis (Gennari ex Fiori) Arrigoni	Herhangi bir kayıt bulunamamıştır.	Asteraceae
Sceletium tortuosum (L.) N.E. Brown	Kanna	Aizoaceae
Scutellaria baicalensis Georgi	Çin takkesi	Lamiaceae
Scutellaria lateriflora L.	Mavi kaside otu	Lamiaceae
Securidaca longepedunculata Fresen.	Menekşe ağacı	Polygalaceae
Senecio delphinifolius Vahl.	Herhangi bir kayıt bulunamamıştır.	Asteraceae
Sesbania grandiflora (L.) Poiret.	Sinekkuşu/Batı Hint bezelyesi	Fabaceae
Solanum nigrum L.	İtüzümü	Solanaceae
Sonchus oleraceus (L.) L.	Eşek marulu	Asteraceae
Souroubea sympetala Gilg	Herhangi bir kayıt bulunamamıştır.	Marcgraviaceae
Stachys recta L.	Kara kurbağaotu	Lamiaceae
Tanacetum balsamita L.	Marsıvan otu	Asteraceae
Tanacetum parthenium (L.) Schultz-Bip	Gümüş düğme	Asreraceae

Tablo 1 (*devamı*). Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkiler [27,30-41]

Bitki adı	Yaygın adı	Familyası
Thalictrum aquilegiifolium L.	Çayırsedefi	Ranunculaceae
Thymbra capitata (L.) Cav.	Acı kekik	Lamiaceae
Thymus serpyllum L.	Yabani kekik	Lamiaceae
Terminalia chebula Retz.	Kara/Siyah halile	Combretaceae
Tilia americana L.	Amerika ıhlamuru	Malvaceae
Tilia cordata Mill.	Kış ıhlamuru/Küçük yapraklı ıhlamur	Malvaceae
Tilia platyphyllus Scop.	Büyük yapraklı ıhlamur	Malvaceae
Tragia involucrata L.	Hint ısırgan otu	Euphorbiaceae
Turnera aphrodisiaca Ward	Damiana	Turneraceae
Tussilago farfara L.	Öksürük otu	Asteraceae
Uncaria rhynchophylla (Miq.) Jacks	Kedi pençesi otu	Rubiaceae
Urtica dioica L.	Isırgan otu	Urticaceae
Valeriana montana L.	Kediotu	Valerianaceae
Valeriana officinalis L.	Kediotu	Valerianaceae
Valeriana tripteris L.	Kediotu	Valerianaceae
Verbena officinalis L.	Mineçiçeği	Verbenaceae
Viola odorata L.	Kokulu menekşe	Violaceae
Vitex agnus castus L.	Hayıt	Lamiaceae
Withania somnifera L. (Dunal)	Ashwagandha/Gelinfeneri	Solanaceae
Zea mays L.	Mısır	Poaceae
Zingiber officinale Roscoe	Zencefil	Zingiberaceae
Zizyphus mucronata Willd.	Manda dikeni	Rhamnaceae
Zizyphus jujuba Mill.	Hünnap	Rhamnaceae

Valeriana officinalis L.

Ülkemizde "Kediotu" adıyla bilinen *Valeriana officinalis,* cins adı "Sağlık veya esenlik" anlamına gelen Latince "Valere" kelimesinden köken alan, Valerianaceae familyasına ait çiçekli bir bitkidir. Kuzey Amerika, Asya, Avrupa ve ülkemizde doğal yayılış göstermektedir. Bazı Avrupa ülkelerinde kültürü de yapılmaktadır. Bitkinin kök ve rizomları (Valerianae radix) drog olarak kullanılmaktadır. Bununla birlikte drog, tentür ve ekstre halinde asabi uykusuzluklarda sedatif etki göstermektedir [15,27].

*V. officinalis'*in yapısındaki iridoitler, flavonoitler, monoterpenler ve seskiterpenler sayesinde birçok klinik etkisi mevcuttur. Yaklaşık 2000 yıldır antispazmodik, diüretik, antipiretik, afrodizyak, emanagog, antihelmentik ve antiromatizmal etkileri nedeniyle tıbbi olarak kullanılan *V. officinalis* 16. yüzyılın sonlarına doğru sinir sistemi hastalıklarında serebral bir uyarıcı, analjezik ve yatıştırıcı olarak kullanılmaya başlanmıştır. Sedatif-hipnotik bir ajan olarak kullanılan *V. officinalis'*in potansiyel etki mekanizması tam olarak belirlenememiştir. Fakat bitkinin yapısındaki γ-aminobütirik asitin (GABA), serotonin reseptörü 5 HT aracılığıyla serotonerjik etkiyi arttırdığı ve anksiyolitik etki gösterdiği düşünülmektedir. Özellikle son yıllarda yapılan çalışmalar bitkinin, adenozin reseptörleri ve melatonerjik yolakları üzerinde de etkileri olduğunu kanıtlamıştır. *V. officinalis*'in anksiyolitik etkilerinin yanı sıra hafif psikofizyolojik uykusuzluk üzerinde de olumlu etkileri olduğu bilinmektedir. Yapılan klinik çalışmalar hem uyku yapısı hem de uyku algısı üzerindeki olumlu etkileri olduğunu göstermiştir. Bu durum anksiyete ve uyku bozukluğu olan bireylerde *V. officinalis*'e karşı olan ilgiyi arttırmıştır. Avrupa İlaç Kurumu (European Medicines Agency=EMA) kediotu kökünden "Hafif zihinsel stres semptomlarının giderilmesi ve uyumaya yardımcı olması" için tıbbi bir monograf

geliştirilmesi gerektiğini belirtip, temellerini oluşturmuştur. *V. officinalis*'in güvenli bir bitki olduğunun bildirilmesinin yanı sıra yüksek dozlarda kullanımının kuvvetli sedasyona neden olabileceği göz ardı edilmemelidir. Yapılan çalışmalarda başka herhangi bir yan etki ve kullanım dozu bildirilmemiştir [15,27,28,42-44].

***Passiflora incarnata* L.**

Antik çağlardan beri kaygı ve uykusuzluğu gidermek amacıyla halk ilacı olarak kullanılan *Passiflora incarnata* diğer bir adıyla "Çarkıfelek", Passifloraceae familyasına ait Arjantin, Brezilya ve ABD'de yayılış gösteren ve birçok ülkede kültürü yapılan tırmanıcı bir bitkidir. Topraküstü kısımları (Passiflorae herba) kullanılan *P. incarnata*, flavonoitler (apigenin, luteolin, kersetin, kemferol ve krisin) ve alkoloitler (harmin, harmol, harmalin, harmalol ve harman) açısından zengin fitokimyasal içeriğe sahiptir. Yapısındaki bu bileşenlerin anksiyolitik etkileri olduğu, vücutta GABA salınımını tetiklediği ve benzodiazepin reseptörü kısmi agonisti olduğu bilinmektedir. Ayrıca uyku bozukluğu olan bireylerde yatmadan önce geleneksel çay formunda *P. incarnata'*nın kullanımının uyku kalitesini iyileştirdiği belirtilmiştir. Alman E Komisyonu Monografları (German Comission E) ve İngiliz Bitkisel İlaç Derneği (British Herbal Medicine Association) tarafından anksiyolitik bir ajan olarak kullanımı onaylanmıştır. Bu nedenle günümüzde başta tentür formunda olmak üzere bitkisel ilaçların bileşiminde sıklıkla kullanılan bir bitkidir. Güvenlik ve toksisite verileri mevcut değildir. Fakat literatürdeki çalışmalar *P. incarnata'*nın yan etkilerinin genellikle tolere edilebildiğini ve ruhsal semptomlar üzerinde herhangi bir olumsuz etki oluşturmadığını bildirmiştir. Ayrıca uzun süreli kullanım (> 4 hafta), hamilelik ve emzirme döneminde veya 12 yaşın altındaki çocuklarda/adölesanlarda kullanımı önerilmemektedir [42,45-47].

***Piper methysticum* G. Forst.**

"Kava-kava" olarak da bilinen *Piper methysticum*, Piperaceae familyasına ait bir bitkidir. Yaklaşık 7 m'ye kadar boylanabilen çalı formunda bir bitki olup, Yeni Zellanda ve Hawai gibi tropikal ülkelerde doğal yayılış göstermekte ve kültürü yapılmaktadır. 1900'lü yıllardan beri Pasifik Adalarında sakinleştirici etkisinden dolayı törensel bir kabile içeceği olarak yaygın bir şekilde kullanılmıştır. Bitkinin farmakolojik aktivite gösteren rizomları (Piperis methystici rhizoma), kurutulmuş droglar halinde fitoterapötik amaçla kullanılmaktadır. Kava kavanın yapısındaki kava laktonları olarak bilinen kavain, dihidrokavain, kavapiron, metistisin ve yonganin sayesinde hafif kaygı durumlarında ve uykusuzlukta potansiyel olumlu etkileri olduğu günümüzde yapılan çalışmalarla desteklenmiştir. Kavapironun GABA reseptörleri ile etkileşime girerek beta-adrenerjik etki gösterdiği ve MAO inhibisyonunu sağladığı belirlenmiştir. Bu özelliği nedeniyle benzodiazepinlere benzer bir etki gösterdiği, daha az sedasyona ve kognitif bozulmaya yol açtığı bildirilmiştir. Anksiyete ve uyku bozukluğu üzerindeki potansiyel olumlu etkilerinin yanı sıra uzun süreli kullanımının hepatotoksisiteye neden olabileceği belirtilmiştir. Bu nedenle Kanada, Birleşik Krallık ve bazı Avrupa Birliği ülkelerinde kullanımı yasaklanmıştır. ABD Gıda ve İlaç İdaresi (FDA), kava kavanın potansiyel hepatotoksisitesi konusunda uyarılarda bulunmuş olup güvenliği konusunda daha fazla çalışmaya ihtiyaç duyulduğunu belirtmiştir [27,43].

***Withania somnifera* L. (Dunal)**

Dünyada "Ashwagandha", ülkemizde ise Gelinfeneri olarak bilinen *Withania somnifera*, Hindistan Geleneksel Tıbbında (Ayurveda) zihinsel ve fiziksel performansı arttırmak, hastalıklardan korunmak amacıyla kullanılan ve Akdeniz'den Güneydoğu Asya'ya kadar geniş bir coğrafyada yayılış gösteren, Solanaceae familyasına ait bir bitkidir. Ülkemizde Güney Anadolu'da yayılış gösteren, çalı formundaki bitkinin geleneksel olarak özellikle Batı ülkelerinde sarkopeni, artrit, astım, guatr ve ülserlerin yanı sıra anksiyete, uykusuzluk ve nörolojik bozukluklarda sıklıkla kullanıldığı bilinmektedir. Yapılan çalışmalarla bitkinin özellikle kök ve yaprağında bulunan vitanolitler sayesinde anksiyolitik olarak kullanılabileceği, uykusuzluğu tedavi ettiği ve uyku kalitesini iyileştirdiği desteklenmiştir. *W. somnifera* içeren ürünler, FDA tarafından "Bitkisel diyet takviyeleri" kategorisinde değerlendirilmiştir. Dünyada son yıllarda giderek daha da popülerleşen bu ürünler hakkında literatürdeki çalışmalar, genel kullanım için güvenli olduğunu fakat dozaj önerilerini belirlemek için ek araştırmalara ihtiyaç olduğunu bildirmiştir [48-54].

***Melissa officinalis* L.**

Melissa officinalis, Lamiaceae familyasına ait ve "Melisa veya Oğulotu" olarak bilinen, limon kokulu bir bitkidir. *M. officinalis*, Akdeniz bitkisi olup, ülkemizde de yayılış göstermektedir. Bitkinin tıbbi amaçlarla kullanılan kısmı yapraklarıdır (Melissae folia). Tıbbi çaylarda veya kaba veya ince toz haline getirilmiş bitkisel drog, etanol ekstresi, tentür veya uçucu yağ (Melissae aetheroleum) kullanılmaktadır. *M. officinalis'*in tedavi amacıyla kullanımı çok eski tarihlere dayanmaktadır. MÖ 50-80'li yıllarda "Materia Medica" ile başlayan, Orta Çağda Paracelsus'un tavsiyesi ile Avrupa'da tıbbi bir ajan olarak kullanılan *M. officinalis*'in günümüzde de antienflamatuar, antinosiseptif, antibakteriyel antianjiyogenez, hipolipidemik ve hipoglisemik amaçlarla kullanıldığı kayıtlıdır. Bununla birlikte özellikle son yıllarda yapılan çalışmalar; İbn-i Sina'nın, 1000 yıl önce canlandırıcı bir ilaç olarak kullandığı *M. officinalis'in,* merkezi sinir sistemi üzerinde nöroprotektif etkileri olduğunu, hafif ve orta şiddetteki kaygı ve stresi azalttığını, uyku bozukluğuna olumlu etkilerinin gözlendiğini destekleyici sonuçlar ortaya koymuştur. *M. officinalis'in* anksiyolitik ve antidepresan etkilerini, içeriğindeki flavonoitler, fenolik asit ve tanenler gibi önemli biyoaktif bileşenler sayesinde MAO inhibisyonu sağlayıp, norepinefrin nörotransmisyonlarını arttırarak gerçekleştirdiği bilinmektedir. Fakat günümüzde halen primer biyoaktif bileşenleri belirlenmeye devam edilen *M. officinalis'*in daha birçok potansiyel etkisi olabileceği düşünülmektedir. Çok sayıda bitkide olduğu gibi, *M. officinalis* de optimum şekilde kullanılmadığı takdirde toksisite gösterebilmektedir. Güvenliği göz önüne alındığında, genellikle iyi tolere edildiği ve önemli bir yan etkisinin gözlenmediği, yalnızca baş ağrısı, mide bulantısı, kusma ve karın ağrısı yapabileceği bildirilmiştir [28,55-57].

***Lavandula angustifolia* Mill.**

Lavandula angustifolia (Lavanta), Lamiaceae familyasına ait başlıca Akdeniz'de yayılış gösteren, ülkemizde doğal yayılışı bulunmayan, kültürü yapılan aromatik bir bitkidir. Uykusuzluk, duygudurum bozuklukları, huzursuz bağırsak sendromu ve depresyon üzerindeki olumlu etkileri kanıtlanan *L. angustifolia*, özellikle aromaterapi ve fitoterapide sıklıkla kullanılmaktadır. Lavanta çiçeklerinden (Lavandulae flores) elde edilen uçucu yağın anksiyolitik aktivitesi olduğunu gösteren klinik çalışmalar mevcuttur. Bu klinik etkiler *L. angustifolia*'nın bileşimindeki linalool ve linalil asetata atfedilmektedir. *L. angustifolia'nın* İbn-i Sina ile başlayan anksiyolitik amaçlı kullanımını günümüzde destekleyecek klinik kanıtlar sınırlı sayıdadır. Bu nedenle *L. angustifolia'nın* anksiyete ve uyku bozukluğundaki mekanizmasını tam olarak belirleyebilmek adına daha fazla çalışmaya ihtiyaç duyulmaktadır [57-59].

***Hypericum perforatum* L.**

Hypericum perforatum, diğer bir adıyla "Sarı kantaron" Hypericaceae familyasının Avrupa, Kuzey Afrika ve Asya'ya özgü, çiçekli bir bitkisidir. *H. perforatum*, yüzyıllardır geleneksel tıpta birçok hastalığı tedavi etmek için kullanılmaktadır. Hiperisin ve hiperforin başta olmak üzere flavonoitler, biflavonoitler, floroglusinoller, naftodiantronlar, ksantonlar, proantosiyanidinler, fenolik asitler gibi birçok önemli biyoaktif bileşene sahiptir. Yapısındaki bu biyoaktif bileşenler sayesinde kaygı, hafifve orta şiddetteki depresyon, stres ve uyku üzerine olumlu etkileri olduğu kanıtlanmıştır. *H. perforatum'un* anksiyete üzerindeki etkisi konusunda birçok hipotez bulunmasına rağmen en fazla kanıtlanan mekanizmaların serotonin, dopamin, noradrenalin (norepinefrin) ve GABA salınımının arttırılması olduğu bildirilmiştir. ABD ve birçok Avrupa ülkesinde *H. perforatum* bu etkilerinden dolayı en çok başvurulan doğal takviyelerden biridir. Fakat *H. perforatum'*un yüksek dozlarda ve sık kullanımının gastrointestinal rahatsızlık, baş dönmesi, uyku bozuklukları ve baş ağrısı gibi hafif ve orta şiddette yan etkilere neden olabileceği bildirilmiştir. Ayrıca yapısındaki hiperisin fototoksiktir ve güneş yanığı benzeri semptomlara neden olabilmektedir. Bu nedenle, *H. perforatum* müstahzarları kullanılıyorsa güneşe maruz kalmaktan veya kozmetik lazer tedavilerinden kaçınılmalıdır. Yetersiz klinik veri nedeniyle, hamilelik veya emzirme döneminde kullanılmaması önerilmektedir. *H. perforatum'un* anksiyete ve uyku bozuklukları üzerindeki etkinliğini ve olumlu/olumsuz etkilerini belirleyebilmek adına daha fazla klinik çalışmaya ihtiyaç duyulmaktadır [14,28, 44,62-65].

***Matricaria chamomilla* L.**

Genellikle ülkemizde "Mayıs papatyası" olarak anılan *Matricaria chamomilla,* "Tıbbi türler arasında yıldız" olarak adlandırılan Asteraceae familyasına ait, Güney ve Doğu Avrupa ile Kuzey ve Batı Asya'ya özgü bir bitkidir. *M. chamomilla* geleneksel olarak eski Mısır, Yunanistan ve Roma'da binlerce yıldır özellikle çay formunda sedatif özelliği nedeniyle kullanılmıştır. Günümüzde de özellikle gastrointestinal sistem bozuklukları, soğuk algınlığı, karaciğer rahatsızlıkları, nöropsikiyatrik ve solunum problemleri dahil olmak üzere birçok hastalığın tedavisinde tamamlayıcı unsur olarak kullanılmaya devam edilmektedir. *M. chamomilla'*nın drogları (çiçek, yapraklar, gövde ve tüm bitki) ve hazırlama yöntemlerine (infüzyon, dekoksiyon, buhar inhalasyonu, banyo ve kompres) ilişkin birçok bilgi 26 ülkenin farmakopesinde yer almaktadır. Bu durum aslında *M. chamomilla'*nın yaygın olarak kullanılan tıbbi bitkilerden biri olduğunu destekler niteliktedir. Fakat yine de etkileri ve bileşimi hakkında yeterli düzeyde çalışma bulunmamaktadır. Literatürdeki çalışmalar değerlendirildiğinde; yapısında seskiterpenler, flavonoitler, kumarinler ve poliasetilenlerin bitkinin başlıca bileşenleri olduğu ve bu bileşenlerin bitkinin anksiyolitik etkilerinden sorumlu olduğu bildirilmektedir [68-73].

***Camellia sinensis* L.**

Camellia sinensis, (Çay) Theaceae familyasına ait, anavatanı Çin ve Japonya olan ve ülkemizde de kültürü yapılan önemli bir bitkidir. *C. sinensis*, polifenoller, ksantinler, teanin ve inorganik tuzlar gibi 200'den fazla bileşen içerir. Polifenoller kuru yaprağın kütlece %30'unu oluşturur ve çayın ana bileşenleridir. Kateşinler, özellikle flavan-3-ol ve flavan-3-gallatlar, çay yapraklarındaki en büyük polifenol grubunu temsil eder. Özgün tadı ve yararları nedeniyle hem sosyal hem de tıbbi bir içecek olarak tüketilmektedir. Tarihsel süreçte vücuttaki ağrıların azaltılması, bağışıklık sisteminin güçlendirilmesi, detoksifikasyon, enerji verme ve sakinleştirici özelliklerinden dolayı kullanılmıştır. Günümüzde ise sağlık üzerine olumlu etkilerinin birçoğu kanıtlanmış durumdadır. Bu potansiyel olumlu etkilerin kafein, teobromin ve teofilin başta olmak üzere içerdiği önemli bileşiklerinden kaynaklandığı bilinmektedir. Bu bileşikler antialerjik, antioksidan, antimutajenik, antiaterosklerotik, antibakteriyel gibi pek çok etki göstermektedir. Bu ortak etkilerin yanı sıra her çay tipinin kendine has özellikleri vardır: Yeşil çay, dişleri ve kemikleri güçlendirmeye ve diş çürümesini azaltmaya yardımcı olabilecek yüksek miktarda florür içermektedir. Ayrıca cilt, yemek borusu, mide ve kolon kanseriyle savaşma potansiyeline sahiptir. Bunun yanında kesik ve sıyrıklardan kaynaklanan kanamayı durdurmak veya yavaşlatmak, böcek ısırıklarını hafifletmek ve kan şekeri ve insülin seviyelerini dengelemek amacıyla kullanıldığı bilinmektedir. Oolong çayının ise kolesterol düşürücü olduğu, kanın pıhtılaşma eğilimini azaltarak kan basıncını ve arter hastalığını azaltabileceği bildirilmiştir. Özellikle ülkemizde sık tüketilen siyah çay ise, sindirim sistemini rahatlatmaya yardımcı olabilen tanenler açısından zengindir. Bununla birlikte haricen gözlerdeki kaşıntı ve kızarıklığı, böcek ısırıklarını rahatlatmak amacıyla kullanımı mevcuttur. *C. sinensis'*in sağlık üzerine olan potansiyel olumlu etkilerinin yanı sıra günümüzdeki yanlış kullanımları da göz ardı edilmemesi gereken bir konudur. Özellikle anksiyete ve uyku bozukluğu olan bireylerde *C. sinensis* türlerinin rahatlatıcı olduğu ve uykuyu iyileştirdiği yanılgısı, bu hastalığa sahip bireylerde hastalığın seyrinin kötüleşmesine neden olabilmektedir. Bu nedenle literatürdeki çalışmalarda anksiyete bozukluğu olan kişilerin günlük *C. sinensis* tüketimlerini 1 veya 2 fincan ile sınırlandırması ve son fincanın yatmadan en az 3 saat önce tüketilmesi gerektiği belirtilmektedir [74-76].

***Foeniculum vulgare* Mill.**

Birçok ülkede yaygın olan ve ülkemizde "Rezene" adıyla bilinen Apiaceae familyasının bir üyesi olan *Foeniculum vulgare*, ekonomik önemi ve önemli ilaç endüstrisi uygulamaları nedeniyle dünyanın en önemli tıbbi bitkilerinden biri olarak kabul edilen, en eski baharat bitkilerindendir. Özellikle Asya, Kuzey Amerika ve Avrupa'da doğal yayılış göstermekte olup, ülkemiz de dahil olmak üzere birçok ülkede kültürü yapılmaktadır. Bitkinin tüm kısımları aromatiktir ve bu özelliği nedeniyle birçok ülkenin mutfak kültüründe *F. vulgare*'yi görmek mümkündür. Bu özelliğinin yanı sıra aromaterapi ve farmakoterapötik özellikleri nedeniyle farklı formlarda sıklıkla kullanılan *F. vulgare*, antiaging, antialerjik, antikolitik, antienflamatuar, antimikrobiyal, antiviral, antimutajenik, antinosiseptif,

antipiretik, antispazmodik, antistres, antitrombotik, anksiyolitik, apoptotik, kemomodülatör, antitümör, sitotoksik, diüretik, östrojenik, ekspektoran, galaktojenik, hepatoprotektif, sitokrom P450 3A4 inhibitörü, hipoglisemik, hipolipidemik, nootropik ve okülohipotansif aktivitelere sahiptir. Birçok *in vitro* ve *in vivo* farmakolojik çalışma, *F. vulgare*'nin anksiyolitik ve sedatif etki gösterdiğini ortaya koymuştur. Bu etkilerinden içerdiği fenolik bileşiklerin sorumlu olduğu bildirilmektedir [77-79].

***Humulus lupulus* L.**

Humulus lupulus, bira endüstrisinin hammaddesi olarak dünya çapında tanınan ve ülkemizde "Şerbetçiotu" adıyla bilinen bir bitkidir. Yaygın olarak Kuzey ve Güney Amerika, Güney Afrika, Avustralya'da yetişen Cannabaceae familyası üyesi *H. lupulus'*un, hastalıkları tedavi etmek için tıbbi bir ilaç olarak kullanımı uzun bir geçmişe sahiptir. Geçmişte geleneksel olarak uykusuzluk ve sinirlilik halinin tedavisinde hafif bir sakinleştirici olarak kullanılmıştır. Eski çağlarda şerbetçiotu toplayıcılarının bu bitkiden reçine toplamaları esnasındaki durumu değerlendirildiğinde; reçineli ellerini ağızlarına değdiren toplayıcılarda uyku hali ve yorgunluğun ortaya çıktığı görülmüştür. Bununla birlikte *H. lupulus'*un iştahı ve sindirimi iyileştirmek, diş ağrısı, kulak ağrısı ve nevraljiyi gidermek için kullanıldığına dair kanıtlar bulunmaktadır. Günümüzde ise yapısındaki terpenler, kateşinler ve flavonol glikozitleri nedeniyle diüretik, antispazmodik ve anafrodizyak etkiler gösterdiği klinik çalışmalarla kanıtlanmıştır. *H. lupulus'un* merkezi sinir sistemi ve özellikle uyku bozuklukları üzerindeki etkinliği *in vitro* çalışmalarda incelenmiştir, ancak çalışmaların birçoğu çelişkili sonuçlar ortaya koymuştur. Ayrıca, *H. lupulus'*un anksiyolitik ve sedatif olarak kullanımını destekleyen klinik çalışmaların sayısı oldukça sınırlıdır; bu nedenle uyku bozukluklarının tedavisinde etkinliği hala tartışmalıdır. EMA Bitkisel Tıbbi Ürünler Komitesi (HMPC=Committee on Herbal Medicinal Products), hafif zihinsel stres ve uykusuzluk semptomlarının giderilmesi için *H. lupulus* çiçeklerinin geleneksel kullanımını rapor etmektedir. Alman E Komisyonu (Commission E) ve Avrupa Fitoterapi Bilimsel Kooperatifi (ESCOP=European Scientific Cooperative on Phytotherapy) ise, *H. lupulus*'u huzursuzluk, kaygı ve uyku bozuklukları için kullanılabilecek tamamlayıcı tedavi ajanı olarak onaylamıştır [27,29,45,80,81].

***Mentha x piperita* L.**

Ülkemizde "Nane" adıyla bilinen *Mentha* cinsi, Lamiaceae familyasının çoğunlukla Kuzey Amerika, Avrupa, Afrika, Avustralya ve Asya'da yayılış gösteren bir üyesidir.

"İngiliz nanesi" adıyla bilinen *M.* x *piperita, Mentha aquatica* ile *Mentha spicata'*nın melezi olup, sağlık üzerindeki olumlu etkileri ile terapötik ve ekonomik açıdan önemli aromatik bir bitkidir. *Mentha* türleri geleneksel olarak antik çağlardan beri tıpta aroma maddesi olarak, kozmetik ve farmasötik müstahzarlar için kullanılmıştır. *M.* × *piperita*'nın Geleneksel Çin Tıbbında hastalıkları tedavi edici olarak kullanıldığı, Mısır'da ise kurutulmuş yapraklarının Mısır piramitlerinde bulunduğu bilinmektedir. Geleneksel İran Tıbbında ise karminatif, tonik, antiviral ve antifungal ajan olarak kullanılmaktadır. *Mentha* cinsinin uçucu yağında bulunan aromatik bileşikler ve terpenoitler gibi metabolitlerin varlığından dolayı antienflamatuar, antimikrobiyal, antihipertansif, antioksidan, antialerjik ve antiviral etkileri olduğu bildirilmiştir. *M.* × *piperita* yaprakları ve uçucu yağı, Batı ve Doğu geleneksel ilaçlarında antispazmodik, antiseptik, aromatik olarak ve ayrıca soğuk algınlığı, mide bulantısı, boğaz ağrısı, diş ağrısı, kramp ve hazımsızlık tedavisinde kullanılmış ve günümüzde de birçok ülkede kullanılmaya devam edilmektedir. Özellikle polifenolik içeriğinden dolayı en sık kullanılan kısımlarının toprak üstü kısımları ve yaprakları (Menthae piperitae folia) olduğu bilinmektedir. Son yıllarda yapılan çalışmalarda *M.* × *piperita*'nın anksiyolitik etkileri olduğu, vücutta GABA salınımını indüklediği ve uykuyu iyileştirdiği belirlenmiştir. Fakat yine de *M.* × *piperita*'nın potansiyel etki mekanizması, doz miktarı ve yan etkilerinin belirlenmesi adına daha fazla çalışmaya ihtiyaç duyulmaktadır [82,83].

SONUÇ VE TARTIŞMA

Günümüzde birçok kişi anksiyete ve uyku bozukluğu sorunu yaşamaktadır. Bu psikiyatrik hastalıklar kişinin fizyolojik sağlığını ve günlük yaşantısını olumsuz etkilemekte, bu durum toplum için

hem sosyal hem de ekonomik problemlere neden olabilmektedir. Bu nedenle bu hastalıkların tedavisi üzerinde durulması büyük önem taşımaktadır.

Anksiyete ve uyku bozukluğunun esas tedavisi antidepresanlar, anksiyolitikler, benzodiazepinler ve hipnotik ilaçlar ile sağlanmaktadır. Fakat bu rahatsızlıkları olan bireyler esas tedavilerine ek olarak hem bu ilaç gruplarının uzun süreli kullanımına bağlı olarak oluşabilecek fiziksel ve psikolojik bağımlılığı yaşamamak adına hem de "daha güvenli/daha etkili" buldukları, başta tıbbi bitkiler olmak üzere farklı tamamlayıcı tedavilere yönelmektedir.

Oysa farklı kaynaklardan temin edilen birçok ürünün söz konusu hastalıklar üzerindeki etkileri ve etki mekanizmaları tam olarak kanıtlanmış değildir. Bu nedenle Sağlık Bakanlığı tarafından onaylı standardize ürünlerin uygun formülasyonda, uygun dozda, uygun süreyle ve hekim/eczacı kontrolünde kullanımı hassasiyetle üzerinde durulması gereken bir konudur. Ayrıca günümüz medyasının hatalı yaklaşımları, yönetmeliklerdeki ve denetimlerdeki eksiklikler, aktar ve baharatçılarda ya da medya üzerinden bu ürünlerin satışı gibi etkenler tüketicileri yanlış yönlendirebilmektedir. Bu ürünlerin tek başına ortaya çıkarabileceği olumsuz etkilerin yanı sıra kronik hastalıklarla, besinlerle, ilaçlarla ve kendi aralarında etkileşime girebileceği de dikkate alınmalıdır. Bu nedenle tıbbi bitkilerin bilinçsiz kullanımının, hastalığın seyrinin kötüleşmesine dahi yol açabileceği göz ardı edilmemelidir.

Anksiyete ve uyku bozukluğunda kullanılan başlıca tıbbi bitkilerin incelendiği çalışmalara baktığımızda; bazı tıbbi bitkilerin potansiyel etki mekanizmaları ve olumlu/olumsuz etkilerinin hala tam olarak belirlenemediği görülmektedir. Bu amaçla kullanılan birçok tıbbi bitki/bitkisel drog/bitkisel ürün ile ilgili aydınlığa kavuşturulması gereken pek çok nokta bulunmaktadır.

İlgili literatür incelendiğinde; anksiyete ve uyku bozukluğunda kullanılan tıbbi bitkileri geniş bir derleme şeklinde ele alan çalışmaların kısıtlı olduğu görülmektedir. Bu derlemeyi yaparken temel amacımız; literatürde yer alan ve günümüzde bu amaçla kullanılan bitkileri biraraya toplamak ve bunlardan en yaygın kullanılanları hakkında genel bilgi vermektir. Bu nedenle çalışmamız anksiyete ve uyku bozukluğunda tıbbi bitkilerin kullanımı ile ilgili bir kaynak oluşturacaktır.

YAZAR KATKILARI

Kavram: E.B., G.E.C.; Tasarım: E.B., G.E.C.; Denetim: E.B., G.E.C.; Kaynaklar: E.B.; Malzemeler: E.B.; Veri Toplama ve/veya İşleme: E.B., G.E.C.; Analiz ve/veya Yorumlama: E.B., G.E.C.; Literatür Taraması: E.B.; Makalenin Yazılması: E.B., G.E.C.; Kritik İnceleme: E.B., G.E.C.; 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., 47(3): 1101-1118, 2023
Doi: 10.33483/jfpau.1214245

REVIEW ARTICLE / DERLEME MAKALE

TRADITIONAL USAGES OF SOME MEDICINAL PLANTS FOR PEDIATRIC DISEASES

ÇOCUK HASTALIKLARINDA GELENEKSEL OLARAK KULLANILAN BAZI BİTKİLER

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ABSTRACT

Objective: *Different types of pediatric diseases negatively affect the lives of many people, physically. Here, we aimed to document some medicinal plants used as traditional folk medicine in pediatrics treatment.*
Result and Discussion: *117 taxa and 53 families have been identified as traditional herbal medicines used in defined pediatric diseases. The most frequently used medicinal plant species according to the number of citations Foeniculum vulgare Mill., Juglans regia L., Dryopteris filix-mas (L.) Schott, Rosa canina L., Mentha x piperita L., Matricaria chamomilla L. All findings are expected to form the basis for new pharmaceutical products and become a handbook for healthcare professionals.*
Keywords: *Ethnobotany, medicinal plants, pediatric diseases, traditional medicine, Turkey*

ÖZ

Amaç: *Çocuk hastalıklarının farklı türleri birçok insanın yaşamını fiziksel olarak olumsuz etkilemektedir. Burada geleneksel halk hekimliği olarak pediatri tedavisinde kullanılan bazı şifalı bitkileri belgelemeyi amaçladık.*
Sonuç ve Tartışma: *Tanımlanmış çocuk hastalıklarında kullanılan geleneksel bitkisel ilaçlar olarak 117 takson ve 53 familya tespit edilmiştir. Atıf sayılarına göre en sık kullanılan tıbbi bitki türleri Foeniculum vulgare Mill., Juglans regia L., Dryopteris filix-mas (L.) Schott, Rosa canina L., Mentha x piperita L., Matricaria chamomilla L. Tüm bulguların yeni farmasötik ürünler için temel oluşturması ve sağlık profesyonelleri için bir el kitabı olması bekleniyor.*
Anahtar Kelimeler: *Çocuk hastalıkları, etnobotanik, geleneksel tıp, tıbbi bitkiler, Türkiye*

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Submitted / Gönderilme : 04.12.2022
Accepted / Kabul : 09.06.2023
Published / Yayınlanma : 20.09.2023

INTRODUCTION

Usage of plants for treatment begins with history of humanity. Thousands of years ago, humans had found out power of plants for treatment and have derived benefit from it to sustain healthy way of living. Today, the method of using plants is still an important way in the treatment of various diseases. The records of the information about these plants are made with ethnobotanical studies. Medicinal plant lore or herbal medicine is a major component of traditional medicine [1]. The use of medicinal plants can be considered an important element in the maintenance of local knowledge and culture. This practice also represents an affordable therapeutic option, in communities where poverty and a lack of access to modern medicine are prominent factors. Just like other medicines, medicinal plants may have side effects however, and administration to children should be performed with careful attention.

Globally, childhood diseases represent a significant health problem and remain a significant health burden [2]. 12 million children under the age of 5 die each year in developing countries and that approximately 70% of deaths are due to communicable diseases and malnutrition. More than 5 million children died in 2020 before reaching their fifth birthday. Nearly half of these deaths, 2.4 million of them, occurred among newborns. According to the report, more than 50 countries will not be able to reach 2030, a country that cannot reach, and 60 newborn deaths will not occur [2-4].

Throughout human history medicinal plants have been used in health recovery, and have evolved from simple household preparations to complex artificial forms [5]. Despite the growing development of public health policies around the globe, data from the World Health Organization [6] estimates that between 1.3 and 2.1 billion people live without access to essential medicines, and that in many developing countries traditional medicine represents a key option for primary care for 70 to 95% of the population [7,8].

Childhood diseases represent a sizeable proportion of mortality burden that could potentially be alleviated when effectively managed. Information regarding plants used for childhood diseases were obtained from different scientific databases and ethnobotanical books. African countries come first among the countries where these diseases are most common. In Africa, it is estimated that about 75% of the commonly occurring diseases such as pneumonia, diarrhoea, malaria, measles and otitis media affect children [2-4]. This has a severe effect on the quality of life for the children and the future of Africa [9]. Infant mortality is on the rise due to inadequate healthcare services and restricted access to basic needs such as clean and safe water, particularly in rural areas. The inequality between rural and urban districts have also widened over-time [10] leaving many people in remote areas to resort to traditional health practitioners (THPs) and medicinal plants as alternative to orthodox medicine. Approximately 80% of the global population, particularly those of rural settlements, depend on medicinal plants in various forms as medicines for the maintenance of their health [11]. In the Western countries with highly developed health care systems, phytotherapy is considered as a part of complementary and Alternative Medicine (CAM) and it has significantly increased in recent years [12]. Globally, the use of medicinal plants for childcare and general well-being has long been recognized. Ethnobotanical surveys have been reported in countries such as Romania [34] and Brazil [8]. Generally, ethnobotanical surveys are one of the primary steps in the identification and development of drugs from medicinal plants [13,14]. However, there are limited studies reporting on medicinal plants used for treating child- hood diseases when compared to the other diseases [15].

According to the report prepared by IGME, given current trends, 60 million children will die before the age of five between 2017 and 2030, and half of these children will be newborns. The majority of newborn deaths occurred in two regions: South Asia (39 percent) and sub-Saharan Africa (38 percent). Half of newborn deaths occurred in five countries: India (24 percent), Pakistan (10 percent), Nigeria (9 percent), the Democratic Republic of the Congo (4 percent) and Ethiopia (3 percent). Pneumonia and diarrhea are among the leading infectious diseases that cause death in children under the age of five, globally. In these deaths, the rate of pneumonia is 16 percent and diarrhea is 8 percent. Premature birth, pregnancy, childbirth, and postpartum complications accounted for 30 percent of neonatal deaths in 2016. In addition to 5.6 million under-5 deaths, 2.6 million babies are stillborn each year, the majority of which are preventable [16]. The aim of our study is to define some medicinal plants traditionally used for the treatment of pediatric diesases in Earth. A literature search was conducted on

medicinal plants used for pediatric in Eart by referencing studies published in journals, reports and books from 1903 to 2022. Detailed information about taxa such as botanical, family and local names, used parts, preparation methods and ailments treated/therapeutic effects were given in Table 1. Based on the data, the most frequently used plant families and taxa for each stated disease are presented in charts. The scientific names of plants and plant families were verified using The International Plant Names Index.

RESULT AND DISCUSSION

In this research, a total of 117 taxa belonging and 53 families were determined as being traditionally used for pediatric diseases on Earth. These medicinal plants are arranged in alphabetical order of their taxa and presented in Table 1 with the relevant information. Studies have shown that plants used for therapeutic purposes among the public in pediatric diseases are mostly used for skin diseases (22 taxa), nutritional regulation (21 taxa) and Diarrhea (20 taxa), respectively. Digestive system diseaese (18 taxa), Jaundice (11 taxa), Abdominal pain (11 taxa), Enuresis (9 taxa), Cough (8 taxa) follow this order (Figure 1). The first plant families with the highest number of plants were found as Apiaceae, Rosaceae, Asteraceae, Lamiaceae, Juglandaceae, Dryopteridaceae (Figure 2).

People uses from various plant parts such as L, F, Aer, Fl, Ro, Br, B, S and stem. It was found that the leaves are the most commonly used part of the plant (25%), for the treatment of pediatric diseases. Aer are the second most commonly used part of the plant, accounting for 18%. As a result of review, different forms of preparation like Inf (39%), Dec (23%), Cr (%16), no data (%58) any were detected. These preparations are applied more Ext (53%) than Int (47%) (Figures 3-5).

Table 1. Plants traditionally used in pediatric diseases

Botanical name	Family Name	Local Names	Used Parts	Preparation / Administration	Ailments treated	Reported Literature Uses
Areca catechu L.	Malvaceae	Cateşu	Aer	-/-	Ear pain	80
Artemisia absinthium L.	Asteraceae	Pelinotu	Aer	Int	Nutritional	80
Achillea biebersteinii Afan.	Asteraceae	Bovijan	Aer	Inf	Anxiety disorder	81
Achillea millefolium L.	Asteraceae	Civanperçemi	L	-/-	Abdominal Pain	82
Allium porrum L.	Amaryllidaceae	Pırasa	Stem	Cr	Toothache	83
Allium sativum L.	Amaryllidaceae	Sarımsak	Aer	İncense, -/-	Evil Eye, Flatulence	83,80
Allium cepa L.	Amaryllidaceae	Soğan	Aer	-/-	Digestive system	80
Ananas comosus (L.) Merr.	Bromeliaceae	Ananas	Aer	-/-	sprue	80
Anethum graveolens L.	Apiaceae	Mazı, Dere Otu	Br, L, F	Inf, Int	Digestive system Abdominal Pain	83 84
Anthemis cotula L.	Asteraceae	Papatya	Fl	-/-	Chıldren's Hearts	84
Anthemis chia L.	Asteraceae	Papatya	Fl	Inf	Antipyretic	85
Anthemis tinctoria L.	Asteraceae	Papatya	Fl	Dec	Abdominal pain	1
Arnica montana L.	Asteraceae	Arnica	L	-/-	Anxiety disorder, Trauma	54,55
Artemisia absinthium L.	Asteraceae	Pelinotu	L	Cr	Epilepsy, Athrepsia	57,58,54,55
Artemisia scoparia Waldst. Kitam	Solanaceae	Malang	L, F	-/-	Jaundice, Poisoning	86,87
Astragalus adscendens Boiss. Hausskn	Fabaceae	Geven	Ro	-/-	Bloating	88
Aristolochia bodamae Dingler	Aristolochiaceae	Kaynana Kokusu	Ro	Inf	Enuresis	89
Bellis perennis L .	Asteraceae	Papatya	Fl	Dec	Stomachache	85
Berberis vulgaris L.	Berberidaceae	Berberis	F	-/-	Jaundice	90

Table 1 (*continue*). Plants traditionally used in pediatric diseases

Botanical name	Family Name	Local Names	Used Parts	Preparation / Administration	Ailments treated	Reported Literature Uses
Betula pendula Roth	Betulaceae	Huş	Aer	Dec	Nutritional, Athrepsia	78,54
Brassica oleracea L.	Brassicaceae	Hardal	L	-/-	Wounds, İmpetigo, Measles	57, 55
Calendula officinalis L.	Asteraceae	Aynısefa	Fl	-/-	Insomnia	54
Carica papaya L.	Caricaceae	Papaya	Tree	-/-	Digestive system	80
Cannabis sativa L.	Cannabaceae	Kenevir	S	-/-	Infection, İmpetigo	58
Carum carvi L.	Apiaceae	Kimyon	S	-/-	Flatulence, Cramps, Colic, Diarrhea	57,58
Citrus limon (L.)	Rutaceae	Limon	Pericarp	Ext	Diarrhea	90
Centella asiatica (L.) Urb.	Apiaceae	Kleinkattekruid [A]	L	Paste	Diarrhea , Ear pain	92
Cotoneaster persicus	Rosaceae	Cotoneaster	F	-/-	Jaundice	94.
Cocos nucifera L.	Areaceae	Hindistan Cevizi	Tree	-/-	Flatulence, Sore throat	80
Curcuma longa L.	Zingiberaceae	Zerdeçal	Ro	-/-	Diarrhea	80
Cupressus sempervirens	Cupressaceae	Selvi	Cone	Cr	Enuresis	96
Chenopodium album L.	Chenopodiaceae	Imbikicane	L	Boi	Vitamin C and Iron deficiency	97
Chelidonium majus L.	Papaveraceae	Kırlangıç Otu	Ro	-/-	General pain	54
Cichorium intybus L.	Asteraceae	Beyaz Hindiba	Ro	-/-	Epilepsy	55
Conium maculatum L.	Apiaceae	Baldıran	L	-/-	Paralysis, Skin diseases	54
Cornus mas L.	Cornaceae	Kızılcık	F	-/-	Diarrhea, Agitation, Fever, Nutritional	57,54,55,98,58
Corylus avellana L.	Betulaceae	Fındık	L	-/-	Nutritional	54,58
Cucurbita pepo L.	Cucurbitaceae	Balkabağı	Pulp	-/-	Endocrin diseases, Metabolic, Nutritional	54
Cupressus sempervirens L	Cupressaceae	Selvi	Cones	Dec	Tonic	85
Cynodon dactylon (L.) Pers.	Poaceaea	Ayrık Otu	Ro	-/-	Jaundice	99
Cydonia oblonga Mill.	Rosaceae	Ayva	L	Dec	İnfluenza, Cold	1
Cyperus longus L.	Cyperaceae	Şanfır	Aer	Cr	Digestive system, Toothache	100
Cymbopogon citratus (DC) Stapf.	Poaceaea	Limon Otu	Aer	-/-	Cough	102
Daucus carota L.	Apiaceae	Havuç	Ro	Cr	Musculoskeletal, Digestive system, Wounnds, İmpetigo	57,54, 55
Dryopteris filix- mas (L.) Schott	Dryopteridaceae	Erkek Eğrelti Otu	Rhizome	Ext	Digestive system, Wounnds, Epilepsy, Endocrin diseases, İnsomnia, Musculoskeletal, Blood and Lymph Diseases, Nutritional, Metabolic	57,54
Equisetum arvense L.	Equisetaceae	Kırkkilit	Aer	-/-, Inf	Diarrhea, Abdominal pain, Enuresis	54,92,105
Euphorbia seguieriana Neck.	Euphorbiaceae	Sütleğen	La	Int	Malaria	106
Ficus carica L.	Moraceae	İncir	F	Dec	Blood and Lymph Diseases	54
Ferula assa-foetida L.	Apiaceae	İncir	Aer	-/-	Constipation	107
Foeniculum vulgare Mill.	Apiaceae	Rezene	F	Inf	Cramps	57,54,55
Foeniculum vulgare Mill.	Apiaceae	Rezene	F	Inf	Flatulence	57,54,55

Table 1 (*continue*). Plants traditionally used in pediatric diseases

Botanical name	Family Name	Local Names	Used Parts	Preparation / Administration	Ailments treated	Reported Literature Uses
Foeniculum vulgare Mill.	Apiaceae	Rezene	S	Inf	Colic	93,11,23
Foeniculum vulgare Mill.	Apiaceae	Rezene	S	Inf	Laxative, Swollen stomach, Swollen stomach, Cold Digestive system, Cough, Stress removal, Diarrhea, Antifungal activity, İndigestion, Abdominal pain, Digestive system	93,95,108,109, 110
Hibiscus rosa-sinensis L.	Malvaceae	Ebegümeci	Fl	-/-	Fever	80
Hordeum murinum L. subsp. Glaucum	Poaceaea	Tilki Kuyruğu	Aer	Inf	Urinary Tract İnfections	111
Humulus lupulus L.	Cannabaceae	Şerbetçi Otu	Fl	Ext	Infections, Wounds, Nutritional, Nutritional	57,54,55
Hypericum perforatum L.	Hypericaceae	Sarı Kantaron	Aer	Inf	Hiperactiviticy disorder	110
Juglans regia L.	Juglandaceae	Ceviz	S	Int	Skin diseases, Atopic dermatit, Nutritional, Diarrhea, Eczema, Wounds, Infections, Vomiting, Intestinal pain, Digestive system, Blood and Lymph Diseases	78,54,55,57
Lamium purpureum L. var. purpureum	Lamiaceae	Arıotu	Fl	Int	Children aspirate its nectar	112,113
Ligustrum vulgare L.	Oleaceae	Kurtbağrı	B	Ext	Scabies	54
Linum usitatissimum L.	Linaceae	Keten	S	Dec	Retained placenta	114
Malus sylvestris L.	Rosaceae	Elma	L,Fl	-/-	Enuresis, Respriatory diseases, Diphtheric tonsillitis, Impetigo	112,113, 54,55
Mamordica charantia L.	Cucurbitaceae	Kudret Narı	Aer	-/-	Swelling	80
Manihot utilissima Pohl.	Euphorbiaceae	Manihot	Aer	-/-	Flatulence	80
Manihot esculenta Crantz.	Euphorbiaceae	Manihot	Aer	Int	Diarrhea	115
Matricaria chamomilla L.	Asteraceae	Tıbbi Papatya	Fl	Int, Ext	Abdominal Pain, Cramps, Digestive system, Wounds, Impetigo, General pain, Cough, Epilepsy	54,55,78,57
Matricaria aurea [Loefl.] Sch.Bip.	Asteraceae	Beybunıc	Fl	Ext	Throat and Back pains	116
Melissa officinalis L.	Lamiaceae	Melisa	L	Dec	Epilepsy	55
Mentha x piperita L.	Lamiaceae	Nane	L	Dec, Inf, Ext, Int	Antispasmodic, Cold, Flu, Diarrhea, Cramps, Wounds, Impetigo	117, 57,54,55,70,71
Morus nigra L.	Moraceae	Dut	F	Boi	Respriatory diseases	54, 55
Myrtus communis L. subsp. communis	Rosaceae	Mersin	L	Inf	Skin redness in children	118
Nasturtium officinale R.Br.	Tropaeolaceae	Su Teresi	Aer	-/-	Jaundice	134
Nigella sativa L.	Apiaceae	Çörek Otu	S	-/-	Seizure	88
Ocimum basilicum L.	Apiaceae	Fesleğen	L	-/-	Diarrhea	148
Olea europaea L.	Oleaceae	Zeytin	O	-/-	Tyroid failure	135
Oryza sativa L.	Poaceaea	Pirinç	S	Inf	Boils, Smallpox	80

Table 1 (*continue*). Plants traditionally used in pediatric diseases

Botanical name	Family Name	Local Names	Used Parts	Preparation / Administration	Ailments treated	Reported Literature Uses
Paliurus spina-cristi L.	Rhamnaceae	Karaçalı	F/S	-/-,Int	Cough, Evil eye	18,29,69,134
Papaver somniferum L.	Papaveraceae	Gelincik	Wh	Int, Cr	Colic, Digestive system, General pain, Cough, Insomnia	57,78,54,133
Papaver rhoeas L.	Papaveraceae	Boynuzlu Gelincik	Aer	-/-	Red spots on body	134
Piper ningrum L.	Piperaceae	Karabiber	Aer	-/-	Catch a cold	80
Piper betle L.	Piperaceae	Biber	F	Dec	Ear infections	80
Pimpinella anisum L.	Apiaceae	Anason	F	Dec, -/-	Cramps, Abdominal pain	78,54,1
Pinus sylvestris L.	Pinaceae	Sarıçam	L	-/-	Musculoskeletal	54
Pistacia atlatica Desf.	Anacardiaceae	Atlantik Fıstığı	F	-/-	Jaundice	86
Plantago major L.	Plantaginaceae	Sinir Otu	L	-/-	Respriatory diseases, Cough, Urological diseases, Digestive system, Nutritional, Musculoskeletal	54,55
Polypodium vulgare L.	Polypodiaceae	Feriguita	Rhizome	Inf	Digestive system	54
Populus alba L.	Salicaceae	Kavak	Stem	Inf	Nutritional, Bone Development	54
Populus nigra L.	Salicaceae	Kavak	Stem	Cr	Nutritional	54
Portulaca oleracea L.	Portulacaceae	Porpine	Aer	Dec	Iron deficiency, Child development	131
Prunus persica (L.) Batsch.	Rosaceae	Şeftali	L	-/-	Alergy	130
Prunus spinosa L.	Rosaceae	Çakal Eriği	F	-/-	Cough, Dysentery, Diarrhea	55
Pulmonnaria officinalis L.	Boraginaceae	Çakal Eriği	Fl	-/-	Nutritional	54,55
Punica granatum L.	Punicaceae	Nar	F	-/-	Diarrhea, Digestive system	80
Pyrus communis L.	Rosaceae	Armut	L	-/-	Athrepsia, Cashexia, Diarrhea	57,58
Quercus robur L.	Fabaceae	Armut	Co	-/-	Diarrhea	57,58
Ricinus communis Linn.	Euphorbiaceae	Hint Yağı	Aer F	Inf	Nutritional, Fever	80
Rosa canina L.	Rosaceae	Kuşburnu	F	Inf	Diarrhea, Constipation, Abdominal pain, Cold, Endocrin diseases, Cachexia, Colic, Lack of appetite	54 55,123,124
Rumex dentatus L.	Polygonaceae	Kıvırtak	L	-/-	Nutritional	125
Ruscus aculeatus L.	Liliaceae	Tavşan Memesi	F	-/-	Enuresis	80
Ruscus aculeatus L. var. *aculeatus*	Liliaceae	Enir	F	Ea	Enuresis	85
Salix alba L.	Salicaceae	Söğüt	L	-/-	Epilepsy, Fright	55,126
Salvia officinalis L	Lamiaceae	Adaçayı	L	-/-	Nutritional, Respriatory diseases	54 55
Sambucus ebulus L.	Adoxaceae	Lor	L	Dec, Ext, Int	Nutritional, Cramps, Parasites, Enuresis	58,54
Sambucus nigra L.	Adoxaceae	Yigidinotu	L	Int, -/-	Rash in children, Enuresis	112,113,128
Sanguisorba officinalis L.	Rosaceae	Çayırdüğmesi	Aer	-/-	Diarrhea, Dysentery, Colic, Nutritional	54
Satureja hortensis L.	Lamiaceae	Tavuk Otu,	Aer	Inf	Appetizing	128

Table 1 (*continue*). Plants traditionally used in pediatric diseases

Botanical name	Family Name	Local Names	Used Parts	Preparation / Administration	Ailments treated	Reported Literature Uses
Scutellaria orientalis L. subsp. *virens* (Boiss. Kotschy.) Edmondson	Lamiaceae	Kesel Mahmut	Aer	-/-	Digestive system	128
Scoparia dulcis L.	Scrophulariaceae	Chini Gura	L	-/-	Diarrhea, Dysentery	129
Solanum nigrum L.	Solanaceae	İt Üzümü	Aer	-/-	Cough	88
Tamarindus indica L.	Fagaceae	Tamarind	Tr	-/-	Sore throat, Fever	80
Teucrium polium L.	Lamiaceae	Kefen Otu	Br/ Aer	Inf/ Dec	Enuresis, Diabetes	120,118
Tilia platyphyllos Scop.	Tiliaceae	Ihlamur	Fl	Dec, Boi	Cold, Influenza	1
Torilis arvensis [Huds.] Link.	Apiaceae	Şeytan Havucu	Aer	-/-	Abdominal pain	118
Trifolium arvense L.	Fabaceae	Yonca	Aer	-/-, Cr	Insomnia, Irritability	54
Triticum aestivum L.	Poaceaea	Buğday	S	Inf	Abdominal pain	119
Thymus vulgaris L.	Lamiaceae	Kekik	L	-/-	Digestive system	54.
Valeriana officinalis L.	Caprifoliaceae	Kedi Otu	Ro	-/-	Diarrhea, Endocrin diseases, Insomnia, Skin diseases	57,55
Verbena officinalis L.	Verbenaceae	Verbina	Aer	-/-	Nutritional, Digestive system, Skin diseases	55
Vitis vinifera L.	Vitaceae	Üzüm	F	-/-	Fever	120
Zea mays L.	Poaceaea	Mısır	F	Inf, Ext	Skin diseases, Scabies, Impetigo	55
Ziziphus jujuba L.	Amaranthaceae	Hünnap	L	Ext	Jaundice, Roupe	86
Ziziphus mauritiana Lam.	Rhamnaceae	Hünnap	L	Cr	Retained placenta	122

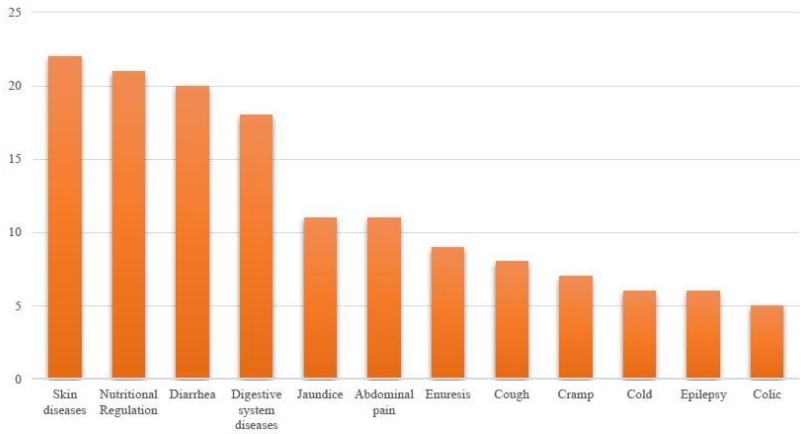

Figure 1. Most common diseases in pediatrics

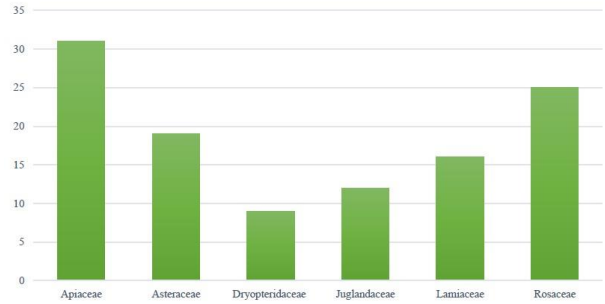

Figure 2. Most common used families in pediatric diseases

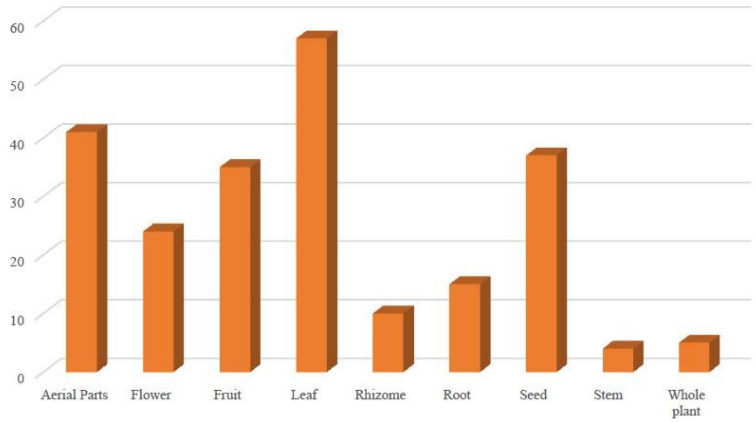

Figure 3. Plants parts used to treat pediatric diseases

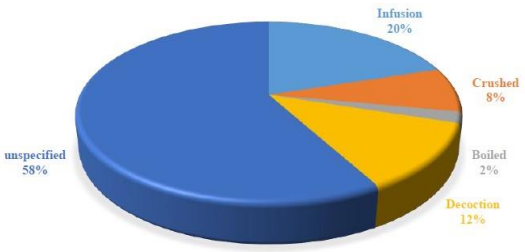

Figure 4. Preparation methods of the plants used to treat pediatric diseases

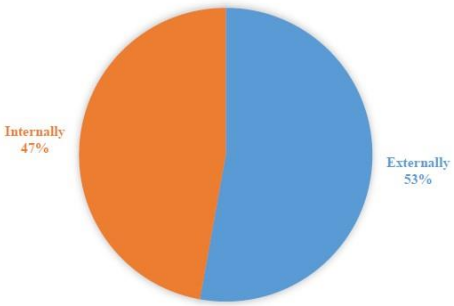

Figure 5. Adminstration methods of plants used to treat pediatric diseases

The 6 plant species with the highest usage value were described morphologically and examined in terms of their phytochemical and pharmacological properties.
- *Foeniculum vulgare* Mill.
- *Juglans regia* L.
- *Dryopteris filix- mas* (L.) Schott
- *Rosa canina* L.
- *Mentha x piperita* L.
- *Matricaria chamomilla* L.

***Foeniculum vulgare* Mill.**

Foeniculum vulgare Mill. is a biennial medicinal and aromatic plant belonging to the family Apiaceae (Umbelliferae) [17]. It is a hardy, perennial-umbelliferous herb with yellow Fl and feathery leaves. It grows to a height of up to 2.5 m withhollow stems. The leaves grow up to 40 cm long; they are finely dissected with the ultimate segments filiform (thread like) of about 0.5 mm wide. The Fl are produced in terminal compound umbels. The F is a dry S 4-10 mm long. It is generally considered

indigenous to the shores of Mediterranean Sea but has become widely naturalised in many parts of the world especially on dry soils near the sea coast and on the river banks. Some authors distinguish two sub-species of fennel, piperitum and vulgare: Sub-species piperitum has bitter Ss, while sub-species vulgare has sweet Ss which are used as flavouring agents in baked goods, meat and fish dishes, ice creams, alcoholic beverages, etc due to their characteristic anise odour [18].

It is a highly aromatic and flavourful herb with culinary and medicinal uses. Fennel Ss are anise like in aroma and are used as flavourings in baked goods, meat and fish dishes, ice cream, alcoholic beverages and herb mixtures [19]. Fennel and its herbal drug preparations are used for dyspeptic complaints such as mild, spasmodicgastric intestinalcomplaints, bloatingandflatulence. It is alsoused for the catarrh of the upper respiratory tract [103]. It is also used to flavor foods, liqueurs and in the perfumery industry [20].

The safety of medicinal and spice plants and of their preparations deserves increased scientific attention. One of the main conditions for use of herbal preparations in medicinal conditions is the absence of such risks as mutagenicity, carcinogenicity, and teratogenicity. In general, such products need to have minimal toxicity and side effects [21]. *Foeniculum vulgare* is used to eliminate gas and regulate intestinal function in children, may cause premature thelarche, and thus, the use of such preparations should be limited [22]. Our systematic review showed beneficial effect of *Foeniculum vulgare* (Fennel) on redaction of infantile colic and also led to significant increase on prolactin levels in lactating mothers [23].

***Juglans regia* L.**

The genus *Juglans* (family Juglandaceae) comprises several species and is widely distributed throughout the world. Green walnuts, shells, kernels and Ss, B, and leaves are used in the pharmaceutical and cosmetic industries [24,25]. Leaves are easily available in abundant amounts. Walnut leaves are considered to be a source of healthcare compounds and have been intensively used in traditional medicine for the treatment of venous insufficiency, hemorrhoids, hypoglycemia, diarrhea, and fungal or microbial infections.

Walnut (*Juglans regia* L.) is the most widespread tree nut in the world Walnut has been used in human nutrition since ancient times. The walnut tree is native to central Asia, the western Himalayan chain and Kyrgyzstan and was cultivated in Europe as early as 1000 BC^2. Since then, it has spread and become well adapted to many regions with Mediterranean- type ecosystems throughout the world [26]. At present, walnut is cultivated commercially throughout southern Europe, northern Africa, eastern Asia, the USA and western South America, Walnuts, the Ss of *Juglans regia* L. (Juglandaceae), are a highly nutritious food. They are also used as a traditional remedy for treating cough, stomach ache, and cancer in Asia and Europe [27].

Green walnuts, shells, ker- nels and Ss, B and leaves have been used in the phar- maceutical and cosmetic industries [24]. Leaves are easily available and in abundant amounts, while tree B is scarce and its collection compromise the plant life. Walnut leaves are considered a source of healthcare compounds, and have been intensively used in traditional medicine for treatment of venous insufficiency and haemorrhoidal symptomatology, and for its antidiarrheic, antihelmintic, depurative and astringent properties [28-30]. Keratolytic, antifungal, hypoglycaemic, hypotensive, antiscrofulous and sedative activities have also been described [31,32]. In Portugal, as in some other European countries, especially in rural areas, dry walnut leaves are frequently used as an Inf. The conclusion of the study was that the extract is safe and effective. There is some scientific evidence regarding the traditional use of *J. regia* in eczema, skin infections, scro- phulosis, intestinal parasites, but not in the pediatric population. The therapeutic efficacy in adults of various Juglans regia extracts was proved for certain skin dis- eases, such as eczema [33] and atopic dermatitis [35]. The plant also showed *in vitro* or *in vivo* anti-mycobacterial (Cruz-Vega) and antiparasitic activity [34,36,37].

***Dryopteris filix- mas* (L.) Schott.**

Dryopteris filix mas (L.) Schott from the family is Dryopteridaceae, a plant that has been used as an anthelmintic since the Middle Ages. Previously, its rhizome was used in the form of powdered drug in folk medicine, later the ethereal extract form was preferred [38].

Dryopteris filix-mas (Dryopteridaceae), commonly known as male fern, dryopteris or water loving fern, is an evergreen plant growing up to 60-150 cm. It is found in stream, moist environments, open grounds, stone and brick walls [39]. Its L Dec is popularly used by traditional healers in various parts of Edo and Delta States, Nigeria as a therapy for inflammation, rheumatoid arthritis, ulcers and wounds (Personal communication). Its reported pharmacological activities include antioxidant and cytotoxic [40], antimicrobial [41], antihelmintic [42], antidiarrheal [43] and tocolytic [44] activities. Considering the traditional benefits of *D. filix-mas* L in curbing inflammatory disorders among Southern Nigerian populace, this study evaluated its antiinflammatory properties and also characterized its bioactive anti- inflammatory component using bioassay- guided purification and isolation approaches. Dryopteris filix-mas' biological activities are not yet scientifically studied, except for its antiinflammatory potential (one animal study) [45]. *Dryopteris filix- mas* is more digestive: Intestinal worms, Skin: Wounds; Neurological: Epilepsy; Endocrine, metabolic and nutritional: Delayed growth; Psychological (ext): Insomnia; Musculo skeletal: Rickets, Bone diseases, Bone deformities; Blood and lymph nodes: Scrophulosis is used for the purpose [34].

***Rosa canina* L.**

Rosa canina L., a member of the Rosaceae family, is a shrub widespread in Europe. *Rosa canina* [Rosaceae], commonly known as kuşburnu, itburnu, kopek golo, has been used as both food and folk remedy in Anatolia. The genus Rosa contains over 100 species that are widely distributed mostly in Europe, Asia, the Middle East and North America [46] *Rosa canina* (dog rose) is an erect shrub of up to 3.5 meters height, sometimes climbing; its Bres are often curved or arched. Petals are white to pale pink, rarely deep pink and F ripens late [47].

Rosa canina is a medicinal plant largely used in traditional folk medicine. The use of *Rosa canina* as medicinal remedy dates back to the time of Hippocrates; the role of this plant peaked during the World War II when the syrup of rose hips, extremely rich in vitamin C, was introduced in the diet to overcome the lack of fresh citrus Fs and then to prevent the scurvy [48]. Rose hip extracts are nowadays used in traditional European folk medicine as diuretic, laxative, for kidney and lower urinary tract disorders, arthritis, gout, fever, colds and for vitamin C deficiency [49,50].

Rose Fs have long been used in Turkey for food, medicinal, and many other purposes and for several special traditional products such as rose hip F juice, rose hip jam, rose hip marmalade, rose hip pestil and rose hip syrup [51]. Additionally, rose hip tea is made with both their Fs and Ros [52].

In the German Commission E Monographs, Fs (rose-hips, with Ss) of *R. canina* are reported to possess prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis, rheumatism, gout, sciatica, for diseases with fever; for colds and infectious diseases including influenza, against gastrointestinal disorders, to aid digestion, prevention of inflammation of the gastric mucosa and gastric ulcer, for gallstones, biliary complaints, as a laxative, for disorders of the kidney and the lower urinary tract, as a diuretic, for dropsy and as an astringent.

In addition to the effects of the Fs described above, the F is known as the most effective remedy against hemorrhoids and diabetes mellitus in Turkish folk medicine. Besides, the Ros and leaves of the plant have also been used against bronchitis. To date, reports on the antioxidant, antiinflammatory, antiulcer, antimicrobial, antimutagenic effects and inflammatory cytokines inhibitory activity of *R. canina* Fs are available [53].

In pediatric diseases, the Fs of the Rosa canina plant are prepared by Inf and used for Diarrhea, Constipation, Abdominal pain [54-56] cold, while Dec is made for Endocrin diseases, Cachexia, Colic, Lack of Appetite [57,58].

***Mentha piperita* L.**

Mentha piperita, of the Labiatae or Lamiaceae family is a well-known plant that is used in numerous forms [ie, oil, L, L extract, and L water][59].

It is widely grown in temperate areas of the world, particularly in Europe, North America and North Africa but nowadays cultivated throughout all regions of the world. The medicinal parts are the essential oil extracted from the Aer of the Fling plant, the dried leaves, the fresh Fling plant he dried leaves, the fresh Fling plant and the whole plant. *M. piperita* is a perennial 50-90 cm high, normally

quadrangular and a prototypical member of the mint family [60]. The usually Bred stems are often purplish or tinged violet but sometimes they are gray-tomentose. The dark or light green leaves are short-petioled, oblong-ovate and serrate their margins finely toothed. The Fl are purple or pinkish having false spikes with numerous inconspicuous bracts and rarely bear Ss [61].

The plant is generally sterile and spreads by means of runners. The plant grows in a sunny side and prefers acid, neutral and basic, light, medium soils but can also grow in heavy clay soil [15].

In Eastern and Western traditional medicine peppermint and its oil have been used as an antispasmodic, aromatic, antiseptic and also in the treatment of cancers, colds, cramps, indigestion, nausea, sore throat and toothaches [60]. Peppermint oil possesses antibacterial activity *in vitro*. Different commercial preparations exhibit various activities [63]. Peppermint is also found to possess antiviral and fungicidal activities [64]. In clinical trials peppermint oil's role in irritable bowel syndrome affirms its effectiveness compared with a placebo with no serious constipation or diarrhea [65]. In this paper, the antibacterial effects of leaves extracts and essential oil against different bacterial strains, antioxidant activities and phytochemical screening of *M. piperita* are presented. Its L is used as a remedy for common cold, inflammation of the mouth, pharynx, liver, as well as disorders in the gastrointestinal tract such as nausea, vomiting, diarrhea, cramps, flatulence and dyspepsia. It is also used as antioxidant, antimicrobial, antiviral, antiinflammatory, and anticarcinogenic [66].

The list of purported benefits and uses of peppermint as a folk remedy or in complementary and alternative medical therapy include: biliary disorders, dyspepsia, enteritis, flatulence, gastritis, intestinal colic, and spasms of the bile duct, gallbladder and gastrointestinal (GI) tract [67].

In pediatric diseases, the leaves of the *Mentha piperita* plant are prepared by Inf and used for Diarrhea, Cramp [54,68,69] while Dec is made for Antispasmodic, Cold, Flu [69]; it is also used Ext in impetigo and wounds [68,70,71].

***Matricaria chamomilla* L.**

Matricaria chamomilla L. is a well-known medicinal plant species from the Asteraceae family often referred to as the "star among medicinal species. Nowadays it is a highly favored and much used medicinal plant in folk and traditional medicine. Its multitherapeutic, cosmetic, and nutritional values have been established through years of traditional and scientific use and research.

Chamomile has been used in herbal remedies for thousands of years, known in ancient Egypt, Greece, and Rome [72]. This herb has been believed by Anglo-Saxons as 1 of 9 sacred herbs given to humans by the lord. The chamomile drug is included in the pharmacopoeia of 26 countries [73].

It is an ingredient of several traditional, unani, and homeopathy medicinal preparations [74].

As a drug, it finds use in flatulence, colic, hysteria, and intermittent fever [75]. Chamomile is used mainly as an antiinflammatory and antiseptic, also antispasmodic and mildly sudorific [76]. It is used Int mainly as a tisane (infuse 1 table-spoonful of the drug in 1l of cold water and do not heat) for disturbance of the stomach associated with pain, for sluggish digestion, for diarrhea and nausea; more rarely and very effectively for inflammation of the urinary tract and for painful menstruation. Ext, the drug in powder form may be applied to wounds slow to heal, for skin eruptions, and infections, such as shingles and boils, also for hemorrhoids and for inflammation of the mouth, throat, and the eyes [77]. Tabulated products from chamomile Fl extracts are marketed in Europe and used for various ailments. Chamomile tea eye washing can induce allergic conjunctivitis. Pollen of *M. chamomilla* contained in these Infs are the allergens responsible for these reactions [76].

In pediatric diseases, the Fl of the *Matricaria chamomilla* L. plant are prepared by Inf and used for Abdomınal pain, cramps, Gas pain, Epilepsy, Thorat and Back pain [116], while Ext is made for Wounds, İmpetigo [54,57,78,79].

In concluding, even though debate continues as to whether plants are sufficiently effective in the treatment of pediatric diseases, herbal therapies are still in demand. Their popularity has increased even more in recent years due to belief that medicinal plants are cheaper and safer than allopathic medicines. There is an immense amount of information on herbal therapies which can help researchers, pharmacists and doctors. However, the most important problems encountered in herbal treatment are the lack of standardization of the active substance in the herbal preparations in terms of concentration and purity

and the inability to control their side effects. Therefore, the pediatrist who wants to use herbal treatment in practice should know the effects and side effects of the plant.

We have compiled the some medicinal plants traditionally used in the treatment of pediatric diseases and determined of them which are most frequently used. Despite the fact that there have been several pharmacological and phytochemical studies proving the efficacy of the plants in the treatment, more studies are needed for some species. In conclusion, findings reinforce the importance of the ethnobotanical literature as a potential source of pharmaceutical raw materials. It can be hoped that it will shed a light that can give life to these souls and guide them with health.

AUTHOR CONTRIBUTIONS

Concept: F.S., S.K., M.K.; Design: F.S., S.K., M.K.; Control: F.S., S.K., M.K.; Sources: F.S., S.K., M.K.; Materials: F.S., S.K., M.K.; Data Collection and/or Processing: F.S., S.K., M.K.; Analysis and/or Interpretation: F.S., S.K., M.K.; Literature Review: F.S., S.K., M.K.; Manuscript Writing: F.S., S.K., M.K.; Critical Review: F.S., S.K., M.K.; 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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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 1119-1131, 2023
Doi: 10.33483/jfpau.1253376

REVIEW ARTICLE / DERLEME MAKALE

PROPERTIES OF IDEAL WOUND DRESSING

İDEAL YARA ÖRTÜSÜNÜN ÖZELLİKLERİ

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ABSTRACT

Objective: *Many substances have been used for wound-burn treatment to date. Recent studies have focused not only on covering the wound but also on the production of dressings that will provide patient comfort at the highest level. This review mentions the types of dressings and the expected features of an ideal wound dressing. An ideal wound dressing should close the wound and protect against external factors such as microorganisms. It must have an appropriate absorption capacity for the exudate level in terms of not drying the wound. It should mimic healthy skin with mechanical strength and flexibility. Wound dressing is expected to accelerate healing by supporting cell proliferation and migration.*
Result and Discussion: *One or more of these features come to the fore when the patient's age and chronic diseases, wound depth, degree, and stage are considered. As a result, since many factors are influential in wound dressing selection, it is crucial to examine the properties of materials. Wound dressings should be developed by considering tissue debridement, infection control, moisture balance, and epithelization.*
Keywords: *Hydrogels, nanofibers, wound, wound dressing, wound healing*

ÖZ

Amaç: *Yara ve yanık tedavisinde günümüze kadar birçok madde kullanılmıştır. Son yıllardaki çalışmalar yaranın yalnızca kapatılmasına değil hasta konforunu en üst düzeyde tutacak malzemelerin üretilmesine de odaklanmaktadır. Bu derleme makalesinde yara örtüsü türlerinden ve ideal bir yara örtüsünden beklenen özelliklerden bahsedilmektedir. İdeal bir yara örtüsü yarayı kapatmalı, yarayı dış faktörlerden ve mikroorganizmalardan korumalıdır. Yarayı kurutmaması açısından eksuda düzeyine uygun emilim kapasitesine sahip olmalıdır. Mekanik mukavemet ve esneklik konusunda sağlıklı cildi taklit etmelidir. Yara örtüsünün hücre çoğalmasını ve göçünü destekleyerek iyileşme sürecini hızlandırması beklenmektedir.*
Sonuç ve Tartışma: *Hastanın yaşı ve kronik hastalıkları, yaranın derinliği, derecesi ve evresi dikkate alındığında bu özelliklerden bir veya birkaçı ön plana çıkmaktadır. Sonuç olarak yara örtüsü*

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Submitted / Gönderilme : 19.02.2023
Accepted / Kabul : 04.07.2023
Published / Yayınlanma : 20.09.2023

seçiminde pek çok faktör etkili olduğu için malzemelerin özelliklerinin incelenmesi büyük önem taşımaktadır. Yara pansumanları doku debridmanı, enfeksiyon kontrolü, nem dengesi ve epitelizasyon dikkate alınarak geliştirilmelidir.
Anahtar Kelimeler: *Hidrojeller, nano lifler, yara, yara iyileşmesi, yara örtüsü*

INTRODUCTION

The skin is composed of three layers: the epidermis, dermis, and subcutaneous layers. The skin accounts for approximately 15% of an adult's body weight. The epidermis, which contains 95% keratinocyte cells, is the outermost layer of the skin [1]. There are collagen fibers, elastic tissue, fibroblasts, plasma cells, lymphocytes, dendritic cells and blood vessels in the dermis layer. The dermis layer contains free nerve endings and nerves that are responsible for touch and pressure sensations. The subcutaneous layer is the innermost layer of the skin and consists of lipocytes. Around 80% of the body's fat is stored in the subcutaneous layer [2].

A wound is defined as the deterioration of skin and tissue integrity as a result of physical or thermal factors [3]. Wounds are classified as acute or chronic based on their natural healing time and depth. Wounds that heal spontaneously within 8-12 weeks are called acute wounds, while those that do not heal normally and require surgical operation are called chronic wounds [4]. Wound healing is a complex process that involves a series of signal transduction and cellular activities in the body, starting with injury. It occurs in four phases: hemostasis, inflammation, proliferation and remodeling. The necessary condition for wound healing is the correct timing and succession of these processes as soon as possible. Factors such as the patient's age, chronic conditions, the wound's stage, and the wound's depth should be considered when selecting the appropriate dressing [5].

In the hemostasis phase, the coagulation process begins with the activation of platelets and the release of clotting factors at the injury site. The hemostasis and inflammation phases begin within a few seconds at the time of injury. The inflammation phase involves purifying the injured area from pathogens and dead cells. Inflammation occurs due to the response of the immune system. The prolonged duration of this phase or high degree of infection can cause painful processes that seriously reduce patient comfort [6]. Cellular activities taking place at the inflammatory stage prepare the wound bed for new tissue formation. Neutrophils are the first blood cells that migrate into the wound to destroy bacteria and remove dead tissue. Neutrophils decrease in number after about 2-3 days, leaving their place to macrophages. Macrophages attract other immune system cells to the area by secreting growth factors and proteins, thus accelerating tissue repair [5]. The proliferation phase begins with the formation of new tissue in the cleaned areas. Granulation tissue is formed during the accumulation of new cells in the wound bed. This granulation tissue can provide the filling of the wound with connective tissue and the formation of new blood vessels. The epithelial tissue around the wound is stretched simultaneously because the cells are pulled toward the center of the wound. This phase continues for 4-20 days until the wound bed is completely covered. The new tissue formed when the remodeling phase begins is weak in terms of mechanical strength and flexibility compared to healthy tissue. Collagen fibers differentiate, and the process of gaining elasticity and strength of the newly formed tissue continues for a long time. This stage can continue for years depending on the patient and the condition of the wound. As a result, scar tissue with a strength of approximately 80% of healthy tissue is formed [6,7].

The literature contains numerous studies on wound dressings, each of which generally focuses on a specific feature. For instance, one study may emphasize the physicomechanical properties of wound dressings [8], while another may examine their antimicrobial effectiveness [9]. In a brief comment, Carta et al. [10] outlined the characteristics of an ideal burn cover. In this review, the properties of an ideal dressing have been explained for the first time in the literature. This review aims to draw attention to the selection of the ideal wound dressing by providing information about modern dressing types. The different types of dressings are explained under the title of "Wound Dressing," followed by a discussion of the general characteristics of an ideal wound dressing under various headings.

Wound Dressings

Wound therapy has come a long way in recent years, with modern wound dressings playing a key

role in current approaches to wound management [11]. Modern wound dressings offer a range of benefits, including improved healing rates, reduced risk of infection, and enhanced patient comfort. In this context, it is essential to understand the applications of these dressings in wound therapy. This topic is of great importance for healthcare professionals, patients, and their families, as it can provide insights into the latest trends and developments in wound care. Wound dressings have been used for centuries to protect wounds from infection and promote healing. However, modern wound dressings have become increasingly sophisticated and effective with advancements in technology and materials science. Today, a wide variety of wound dressings are available to meet the specific needs of different types of wounds and patients. These modern wound dressings have revolutionized the approach to wound therapy, providing improved outcomes, reduced healing times, and greater patient comfort. In this context, it is essential to understand the various applications of modern wound dressings and how they are used in current wound therapy approaches [12].

Wound dressings can be produced from a variety of materials including starch, dextran, chitosan, alginate, polyurethane, cellulose derivatives, hyaluronic acid, polysaccharide derivatives, collagen and gelatin [13,14]. Based on the production method and materials used, modern wound dressings can be classified into several categories: films, foams, nonwovens (including electrospun and blow spun), 3D printed dressings, and hydrogels [15].

Film Dressings

Film dressings are typically made by creating a thin film through the process of solvent evaporation, using a natural or synthetic polymer or a combination of both [16]. One of the notable characteristics of film dressings is their mechanical properties, such as strength, tensile strength, softness, flexibility, and elasticity [17]. Additionally, film dressings possess a transparency feature that allows for the monitoring of the wound without having to remove the dressing [18]. Paramylon (β-1,3-glucan), a natural compound synthesized by Euglena gracilis, is used in the production of film dressings. Paramylon film dressings, similar to bacterial cellulose, keep the wound moist and support the continuity of cellular activities. Furthermore, it has been observed that paramylon films promote a greater closure of the wound compared to cellulose films [19].

Foam Dressings

Foam dressings, made of polyurethane foams, are produced by reacting organic compounds (polyols) containing multiple hydroxyl groups with diisocyanate [20]. Polyurethane foams have various advantages, including softness, flexibility, air permeability, and high exudate absorption (about 1500%). Research has indicated that the absorption capacity and resistance to pressure of foam dressings increase when natural polyols such as alginate and hydroxypropyl methylcellulose are used in foam synthesis [21]. However, a disadvantage of foam dressings is that they can dry out wounds with very little exudate, making them more suitable for use in highly exuding wounds [22].

Nonwoven Membrane Dressings

Nonwoven materials are composed of fibers ranging in thickness from a few microns to several hundred nanometers. While various methods can be used to produce nanofibers, the electrospinning technique is preferred due to its simplicity and cost-effectiveness. This technique involves delivering a polymeric melt or solution under high electrical power to a collector at a specific flow rate as fibers [23]. By modifying the basic electrospinning device or adjusting conditions, fibers with a wide range of properties can be produced [24]. In a study by Yang et al. [25], natural and synthetic polymers were used to produce Ag nanoparticles and ciprofloxacin-loaded nanofibers using a side-by-side spinneret without dissolving them in the same solvent. An alternative method for nanofiber production is the blow-spinning process, which uses a compressed air unit instead of high voltage. Unlike electrospinning, fibers can be deposited on any surface without the requirement of being conductive. It has been reported that blow spinning can be used to cover intra-body wounds during surgery [26,27].

Nonwoven wound dressings also possess several characteristic properties that make them unique and well-suited for wound care: absorbency, breathability, conformability, comfort, biocompatibility, and cost-effectiveness. Nonwoven wound dressings are designed to absorb and retain exudate from the

wound bed, which helps to keep the wound moist and promote healing. Nonwoven dressings are typically made from porous materials that allow for proper ventilation and oxygenation of the wound site [28]. They can conform to the contours of the wound bed, ensuring that the dressing stays in place and providing protection against further injury or infection. They are typically lightweight and comfortable to wear, reducing discomfort for the patient. Nonwoven dressings are made from materials that are biocompatible and non-toxic, which minimizes the risk of allergic reactions or other adverse events. They are often less expensive than other types of wound dressings, making them a cost-effective option for wound care. Overall, nonwoven wound dressings are a reliable and effective option for wound care, with a range of properties that promote healing, protect the wound from further damage or infection, and provide comfort for the patient [29].

3D Printed Dressings

The use of 3D printer technology enables the production of wound dressings with controlled micro-architecture and geometry. In recent years, 3D printing has emerged as an alternative method to produce biocompatible materials for wound care using polymers and bioactive materials. These materials are designed to support cell adhesion, migration and tissue regeneration [30]. The most important features expected from 3D materials are their ability to mimic skin, promote cell growth, enhance blood circulation, and deliver nutrients to tissues [31]. Long et al. [32] produced a dressing material based on chitosan-pectin (CS-PEC) using the 3D printing method. The CS-PEC hydrogel formulations were shaped into a 3D structure with a cubic lattice made of polylactic acid (PLA). The CS-PEC dressing materials are biodegradable, temperature-sensitive, absorbent, flexible and can be integrated into the tissue, according to the authors [32].

Three-dimensional (3D) wound dressings possess several characteristic properties that make them unique from traditional flat dressings. One of the key features of 3D dressings is their ability to create a three-dimensional structure that conforms to the contours of the wound bed. This allows for improved contact between the dressing and the wound, which can enhance the delivery of therapeutic agents and promote faster healing. In addition, 3D dressings can also provide increased mechanical support to the wound bed, which can help to protect the wound from further damage and promote tissue regeneration. The porous nature of many 3D dressings also allows for better ventilation and moisture management, which can help to prevent infection and improve wound healing. Overall, the special characteristic properties of 3D wound dressings make them a promising option for wound care, offering improved outcomes and greater patient comfort compared to traditional flat dressings [33].

Hydrogels

Hydrogels are commonly used in the biomedical field as drug delivery systems, for cell adhesion support, as a barrier, contact lens, absorbent, and scaffold. Their three-dimensional polymeric network structures allow them to absorb high amounts of exudate and other body fluids. Additionally, hydrogels cool the wound surface and reduce pain. Bacterial cellulose is one of the natural polymers used in hydrogel production, and although it does not possess antimicrobial properties, its cross-linked fibrous structure allows it to carry antimicrobial agents. PVA hydrogels have the advantage of absorbing exudate, but their low elasticity and mechanical strength require support from carbon-based materials such as graphene or graphene oxide (GO) [34-37].

The dressing produced with calcium alginate hydrogel is an affordable option that provides a moist healing environment. However, its mechanical properties weaken after swelling due to the absorption of wound fluid. Additionally, it does not have antimicrobial properties and does not contain sites suitable for cell adhesion. Therefore, it is often used in combination with antibiotics, nanoparticles, or natural antibacterial molecules [38]. Chitosan is a natural polymer that has wound healing properties and can inhibit the growth of microorganisms. PVA/chitosan hydrogels can be produced through a simple freeze-thawing process without the need for aldehyde-based crosslinkers. The material has strong intramolecular and intermolecular hydrogen bonds and is non-toxic [39].

Hydrogel wound dressings have several unique characteristic properties that make them suitable for wound care. Some of these properties include moisture retention, cooling effect, biocompatibility, non-adhesive, non-occlusive, and long-lasting. Hydrogel dressings are highly absorbent and can retain

large amounts of fluid. This helps to maintain a moist wound environment, which is important for wound healing [40]. The dressings have a cooling effect when applied to the wound, which can help to reduce pain and inflammation. They are biocompatible, meaning they are not toxic to living tissue. This makes them safe to use on all types of wounds, including those that are sensitive or fragile. Hydrogel dressings are non-adhesive and do not stick to the wound bed. This makes them easy to remove without causing additional trauma to the wound. They are permeable to gases, which allows for the exchange of oxygen and carbon dioxide. This helps to maintain a healthy wound environment and promotes healing. They can be left in place for extended periods, which reduces the need for frequent dressing changes and minimizes the risk of infection. Overall, hydrogel wound dressings provide a gentle and effective way to manage a wide range of wounds, making them a popular choice for healthcare professionals and patients alike [41].

Properties of Ideal Wound Dressing

When selecting a wound dressing, several factors are considered, including the patient's age and chronic disease history, as well as the depth, stage, infection level, and exudate amount of the wound. Although the definition of an ideal dressing may vary depending on the patient and wound type, there are common desirable features. An ideal wound dressing should protect the wound from the environment and microorganisms while also being comfortable, biocompatible, and durable. It should not irritate the wound and should be non-adherent, although it may have a frame that adheres to the surrounding tissue. Additionally, it should allow for gas exchange and maintain a moist wound bed [42,43]. The European Wound Management Association (EWMA) has established a wound care strategy that includes four key points: tissue debridement, infection control, moisture balance, and epithelialization [44,45].

An ideal wound dressing should promote and accelerate the natural healing process of the wound while protecting it from external factors. It should maintain a moist environment to prevent the wound from drying out, but at the same time, it should not create excessive moisture that can lead to maceration or bacterial growth. The dressing should be able to absorb excess exudate while preventing leakage to the surrounding healthy skin. It should also be easy to apply, remove, and dispose of. An ideal dressing should have antimicrobial properties to prevent or manage infection in the wound area. The dressing material should be biocompatible, non-toxic, and non-allergenic, without causing any adverse reactions in the body. Finally, the ideal dressing should be cost-effective and readily available in different forms suitable for various wound types and stages. The combination of these properties can help promote healing, reduce pain, and improve the quality of life of patients with wounds [46].

The properties of the ideal dressing are explained under the headings of mechanical properties, infection control, moisture balance, epithelialization, gas exchange, and cost-effectiveness.

Mechanical Properties

Since the primary purpose of wound dressings is to cover and protect the wound, their mechanical strength is an important consideration. To determine the mechanical properties of dressing material, factors such as elasticity modulus (Young's modulus), tensile strength, and stability (thermal and chemical) are investigated [47,48]. The elasticity modulus (stress/strain) refers to a material's resistance to deformation when subjected to applied stress. When the applied tensile force exceeds the elastic limit, the material becomes irreversibly deformed. Tensile strength (force/area) is the maximum tensile force a material can withstand before breaking [49]. In a study [50], the effects of adding graphene oxide (GO) to alginate/PVA nanocomposite sponges on the sponges' tensile strength and elasticity modulus were investigated. The results showed that the addition of GO at a rate of 2% significantly increased the tensile strength and elasticity modulus of the nanocomposite sponges. It was suggested that GO improves the mechanical properties of alginate and PVA by increasing the crosslink density [50].

Flexibility is an important feature sought in wound dressings to provide ease of movement to patients and to allow for adaptation to various joints and folding areas. Flexible materials are characterized by high elasticity modulus and tensile strength [51]. Silk fibroin dressing materials [52] are reinforced with dextrose to increase flexibility and mechanical strength. While the elongation of silk fibroins at rupture was $3.2 \pm 0.7\%$, the addition of 15% dextrose increased this ratio to $40.1 \pm 2.5\%$ [41]. One flexible dressing on the market is Foam Lite™, which is a foam dressing designed for use on low-

exudate wounds. It has been noted that this dressing can adapt perfectly to a patient's body movements and posture positions [53]. Biatain® is another flexible dressing produced in suitable forms for different parts of the body [54]. Acticoat® Flex 3 and 7 are made from a single braided polyester material that promises to minimize discomfort and local trauma for the patient [55,56].

Infection Control

Patients with burns that cover more than 40% of the total body surface have a high risk of dying from infection, estimated to be 75%. Orthopedic traumas and surgical wounds are also considered high risk for infection [57]. During the initial stages of wound healing, gram-positive bacteria such as S. aureus and *S. pyogenes* are the main microorganisms that cause infection, while in the chronic phase, gram-negative species such as *E. coli* and *P. aeruginosa* predominate. When infection occurs in a healthy individual, the immune system becomes active, and macrophages migrate to the wound site. In the later stages of infection, T lymphocytes secrete interferon-γ and CD40 ligands, which help in fighting the infection. However, if the immune system fails to destroy the pathogen, infection occurs, and extracellular matrix components (collagen, elastin, and fibrin), growth factors, and granulation tissue are disrupted, which can delay healing [58]. Therefore, it is crucial to develop a dressing that prevents microorganisms from entering the wound or inhibits microbial growth. Antimicrobial agents commonly used in dressings include antibiotics, nanoparticles, natural compounds, honey, essential oils, and chitosan [59]. Over the years, many antibiotics such as ciprofloxacin [60], streptomycin [61], vancomycin [62], and gentamicin [63] have been loaded into wound dressing materials. Infection Defense® is an adhesive bandage containing neomycin sulfate, bacitracin zinc, and polymyxin B antibiotics. This marketed antibiotic-loaded wound dressing is intended for large and minor wounds to prevent infection and promote healing [64].

The study investigated the antimicrobial activity of crosslinked films made of gelatin, chitosan, and cinnamaldehyde against various bacterial species, including gram-positive *S. aureus* and gram-negative *P. aeruginosa*, *Salmonella*, and *E. coli* [65]. Cinnamaldehyde is the main component of cinnamon oil and is known for its antimicrobial properties. While gelatin does not have antimicrobial properties, chitosan has been shown to have a broad range of activity against microorganisms. The addition of cinnamaldehyde to the films resulted in increased inhibition of bacterial growth, likely due to the interaction between the aromatic aldehyde groups in cinnamaldehyde and the amine groups in the peptidoglycan layer of the bacterial cell wall [65]. Aside from plant-derived compounds, extracts obtained from various parts of plants, such as leaves, flowers, roots, and stems, are also used as antimicrobial agents. For instance, the root extract of *Isatis tinctoria*, also known as woad or isatis, loaded onto polyvinylpyrrolidone (PVP), has been shown to inhibit the growth of many bacterial species [66]. Honey is another natural product with antimicrobial activity, which is attributed to its multiple components, including bee-derived defensin-1, an antimicrobial peptide, and polyphenols. Honey inhibits the growth of microorganisms through its high osmotic pressure and low pH [67]. A study has shown that incorporating Manuka honey, a type of honey produced by bees from *L. scoparium* flowers in New Zealand, into cellulose acetate nanofibers resulted in films with 2.74 ± 0.24 and 3.6 ± 0.35 mm inhibition zones against *S. aureus* and *E. coli*, respectively [68]. Roosin® is a wound dressing that contains Manuka honey and is designed for the treatment of second-degree burns, foot ulcers, and pressure ulcers [69]. Another example of commercial dressings with antibacterial properties is Mepilex® Ag, which contains silver nanoparticles. This dressing can inactivate wound-related pathogens within 30 minutes and maintain its effectiveness for up to 7 days. It is used for the treatment of low or moderate exudate wounds, such as leg and foot ulcers and partial burns [70].

Moisture Balance

The use of wound dressings can help maintain moisture balance, reduce pain and infection, and decrease wound care costs by shortening the healing period. It is important to create and maintain a moist wound environment but avoid excessive fluid accumulation. During the inflammatory phase, wounds produce a high amount of exudate which can cause surrounding tissues to soften, become unstable, and encourage microbial growth. Dressings should therefore absorb and remove excess fluid, without drying out the wound. Additionally, the dressing should be able to maintain its structure and

remain in place for an extended period [71]. Hydrogels produced with hyaluronic acid and chitosan were found to have a swelling capacity of 4500%, making them "superabsorbents" [72]. Adding citric acid as a crosslinker to super absorbent dressing materials produced with carboxymethyl cellulose increased the swelling rate to 5000% at its highest concentration. Increasing the crosslinker concentration in hydrogels can improve their swelling capacity. Crosslink density is also important, as it allows hydrogels to absorb liquid while resisting high amounts of fluid [73].

Although nanofibers have a lower swelling capacity than hydrogels, they have potential use in wounds with little exudate. In a study by Mutlu et al. [74], swelling ratios were increased from 50% to 332% by adding curcumin to Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) nanofibers. In PVA/chitosan nanofibers [75], it has been observed that chitosan reduces the swelling rate. It has been reported that the addition of chitosan makes the structure more difficult to dissolve and firmer. Nanofibers were stabilized using methanol, glutaraldehyde, crosslinkers, and heating methods. The stabilized PVA and PVA/chitosan showed 800% and 300% swelling rates, respectively [75]. One synthetic polymer used to improve the swelling property of chitosan is PVP. The addition of 50% PVP increased the swelling rate of chitosan hydrogels from 400% to 1210% [76].

Cutimed® is an absorbent wound dressing made of sodium (80%) and calcium (20%) alginate that is derived from seaweed. When exposed to exudate, alginate transforms into a moist gel due to a chemical reaction between sodium ions in the exudate and calcium ions in the dressing. This dressing is particularly effective for highly exuding wounds as it absorbs exudate, which helps to keep the wound bed clean [77]. Mextra® is a super-absorbent dressing that is highly effective in managing wounds with high exudate. Its high fluid absorption rate can shorten treatment time and reduce costs. To ensure patient comfort, the edges of the dressing are made from soft materials. Additionally, the dressing is easily removable from the wound because it retains its structural integrity after absorbing exudate [78,79].

Epithelization

Epithelialization is the natural process of wound closure by the formation of new epithelial tissue. However, this process can be exceptionally prolonged in chronic wounds. An ideal wound dressing is expected to expedite wound healing by either accelerating epithelial tissue formation or indirectly promoting healing through antimicrobial and swelling properties. This is because cleaning the wound of microorganisms and absorbing exudate creates a conducive environment for healthy cell growth. While antimicrobial agents and substances that promote cell migration are effective in wound healing, their effectiveness is limited in duration. In addition, some antimicrobial agents, such as silver sulfadiazine, can cause damage to healthy skin and blood cells [80]. Peripheral neuropathy can increase skin damage, making the patient more vulnerable to microorganisms. A seemingly minor wound in a patient at high risk of infection can lead to organ loss or even death. Abnormal blood sugar levels can slow down the arrival of blood cells to the wound area. This is because peripheral neuropathy can damage signal transduction pathways, leading to a delay in cell migration signals and consequently, delayed arrival of blood cells. In the treatment of diabetes wounds, it is crucial to use effective wound care products and to maintain balanced blood sugar levels [81].

In a study conducted by Eğri et al. [82], a bilayer membrane was produced using a modified electrospinning process. The membrane consists of a PEG/PCL polymeric layer and a layer containing PEG-encapsulated *H. perforatum* (St. John's wort) oil. Cytotoxicity studies were performed on mouse fibroblast cells using the MTT assay, and wound closure was evaluated on mice with second-degree burns. The study concluded that wounds treated with *H. perforatum* oil-loaded membranes showed a high degree of epithelialization and closed quickly [82]. HydroClean® is an antibacterial product composed of polyacrylic acid (PAA) and cellulose fibers that accelerate wound healing. This product releases the drug in the wound for three days and inactivates matrix metalloproteinase (MMP) [83]. It has been reported that an increase in MMP levels can cause tissue destruction and slow healing in chronic wounds [84].

Gas Exchange

Gas exchange is an important property of wound dressings that allows for the exchange of oxygen and carbon dioxide between the wound and the surrounding environment. This exchange is important

for promoting wound healing, as oxygen is required for many of the metabolic processes involved in tissue repair. Wound dressings that allow for gas exchange typically have a permeable membrane or mesh that allows for the passage of gases. These dressings may also have a hydrophilic coating or other surface modifications that help to facilitate gas exchange. In addition to promoting wound healing, dressings that allow for gas exchange can also help to prevent the accumulation of excess moisture around the wound, which can lead to maceration and delayed healing. By allowing for the exchange of gases, these dressings can also help to prevent the buildup of harmful gases, such as carbon dioxide, around the wound [85].

Tan et al. [86] developed electrospun wound dressings made of cellulose acetate and loaded with Pramipexole. The study reported that the wound dressing exhibited a water vapor permeation rate of around 256.18 ± 3.26 mg/cm^2/h. The researchers also noted that the porous structure of the nanofibers allowed for wound breathability. DuoDERM®, a semi-permeable film dressing, that allows the gas exchange of air and water vapor, is impermeable for bacteria and fluids, very flexible and conformable [87].

Cost-effectiveness

The cost of wound dressings can vary widely depending on the type of dressing, the size and severity of the wound, and the frequency of dressing changes required. Some wound dressings are more expensive than others but may be necessary for certain types of wounds or patients with specific healthcare needs. Some factors that can impact the cost of wound dressings include the type of dressing, frequency of dressing changes, size and severity of the wound, healthcare setting, and insurance coverage. Different types of wound dressings have different costs associated with them. For example, advanced wound dressings, such as hydrogels and collagen dressings, may be more expensive than traditional dressings like gauze or adhesive bandages. Dressings that need to be changed frequently may require more frequent purchases and can increase the overall cost of wound care. Larger and more severe wounds may require more dressing material, which can increase the overall cost of wound care. The cost of wound dressings may vary depending on the healthcare setting, such as inpatient versus outpatient care. The cost of wound dressings may be covered by insurance, which can help to reduce out-of-pocket costs for patients [88].

In addition to the cost of the dressing itself, there may be additional costs associated with wound care, such as healthcare provider time, medications, and other supplies. Patients and healthcare providers need to consider all of these factors when assessing the overall cost of wound care. In summary, the cost of wound dressings can vary widely depending on a variety of factors. While cost is an important consideration, it's also important to balance the cost of the dressing against its benefits and select the dressing that is most appropriate for the patient's wound and healthcare needs [88].

Li et al. [89] have designed MXene@polydopamine-decorated (PDA) chitosan nanofibers as a cost-effective wound dressing. The wound dressing demonstrated superior wound healing performance compared to Tegaderm™ film in a full-thickness skin defect model. It has been reported that this provides further evidence that the MXene@PDA material can promote the reformation of fibrinogen during the initial stages of the wound healing process [89].

RESULT AND DISCUSSION

There are thousands of commercially available wound dressings that can manage all aspects of wound care. However, there is no single superior product that provides complete healing for chronic ulcers, such as third-degree burns. Current research is focused on producing dressing materials that address the main intervention factors of the normal healing process, which can greatly assist patients and wound care professionals. Although it is not possible to identify an ideal dressing, the basic features expected from one can be explained. For example, hydrogels are superior in maintaining moisture balance, while nanofibers can mimic the components of the extracellular matrix and support epithelialization. Nanoparticles are taken into consideration for infection control with the development of nanotechnology. Natural-origin antimicrobial agents have become preferred due to their biocompatibility. Combining all of these features can be disadvantageous in terms of cost. Therefore,

when selecting a wound dressing, factors such as the depth, type, stage of the wound, the age of the patient, and chronic diseases should be carefully analyzed, and the necessary features should be provided first.

ACKNOWLEDGEMENTS

We appreciate the Council of Higher Education (YÖK) for the 100/2000 Ph.D. scholarship and the Scientific and Technological Research Council of Turkey (TÜBİTAK) for the BİDEB 2211-C scholarship.

AUTHOR CONTRIBUTIONS

Concept: S.K., S.D.; Design: S.K., S.D.; Control: S.D.; Sources: S.D.; Materials: S.K., S.D.; Data Collection and/or Processing: S.K.; Analysis and/or Interpretation: S.K., S.D.; Literature Review: S.K., S.D.; Manuscript Writing: S.K.; Critical Review: S.K., S.D.; 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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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 1132-1140, 2023
Doi: 10.33483/jfpau.1261673

REVIEW ARTICLE / DERLEME MAKALE

VACCINATION AGAINST COVID 19 INFECTION DURING PREGNANCY AND LACTATION: A BRIEF REVIEW

GEBELİK VE LAKTASYON DÖNEMİNDE COVID 19 ENFEKSİYONUNA KARŞI AŞILAMA: KISA BİR DEĞERLENDİRME

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ABSTRACT

Objective: *Vaccination plays a crucial role in the protection against the Covid 19 infection. However, pregnant and lactating women are excluded from clinical trials of vaccines due to the unknown effects of the vaccine on the expectant mother, fetus, and infant. Pregnancy and the following lactation periods are long processes with unique physiological, psychological, and pathological characteristics, in which many practices are discussed for the mother and the baby. Based on the limited data available on the mechanisms of action of vaccine types, Covid 19 vaccines cannot possibly cause any risk to pregnant women and nursing mothers. On the other hand, there is currently insufficient data on the safety of Covid 19 vaccines in pregnant and lactating women.*
Result and Discussion: *Evidence-based and personalized information about vaccines is needed to support pregnant and breastfeeding women's decision-making about vaccines. Vaccination should be recommended to all pregnant and lactating women after they have been sufficiently informed about the advantage and risks of Covid 19 vaccines and their consent has been obtained. This brief review was conducted to discuss vaccination against Covid 19 infection during pregnancy and the lactation period based on scientific data and literature.*
Keywords: *Covid 19 infection, lactation, pregnancy, vaccination*

ÖZ

Amaç: *Aşılama, Covid 19 enfeksiyonuna karşı korunmada hayati rol oynamaktadır. Bununla*

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Submitted / Gönderilme : 12.03.2023
Accepted / Kabul : 10.07.2023
Published / Yayınlanma : 20.09.2023

birlikte, gebe ve emziren kadınlar aşının anne adayı, fetüs ve bebek üzerindeki bilinmeyen etkileri nedeniyle aşıların klinik denemelerinin dışında tutulmaktadır. Gebelik ve onu takip eden laktasyon dönemleri, anne ve bebek için pek çok uygulamanın tartışıldığı, kendine has fizyolojik, psikolojik ve patolojik özellikleri olan uzun bir süreçtir. Aşı türlerinin etki mekanizmalarına ilişkin mevcut sınırlı verilere dayanarak, Covid 19 aşılarının hamile kadınlar ve emziren anneler için herhangi bir risk oluşturması muhtemel değildir. Öte yandan, şu anda hamile ve emziren kadınlarda Covid 19 aşılarının güvenliğine ilişkin yeterli veri bulunmamaktadır.

Sonuç ve Tartışma: *Hamile ve emziren kadınların aşılar hakkında karar vermesini desteklemek için aşılar hakkında kanıta dayalı ve kişiselleştirilmiş bilgilere ihtiyaç vardır. Tüm gebe ve emziren kadınlara Covid 19 aşılarının yararları ve riskleri hakkında yeterince bilgi verildikten ve onamları alındıktan sonra aşı önerilmelidir. Bu bağlamda bu kısa derleme gebelik ve laktasyon döneminde Covid 19 enfeksiyonuna karşı aşılamanın bilimsel verilere ve literatüre dayalı olarak tartışılması amacıyla yapılmıştır.*

Anahtar Kelimeler: *Aşılama, Covid 19 enfeksiyonu, gebelik, laktasyon*

INTRODUCTION

The World Health Organization (WHO) China Country Office reported cases of pneumonia of unknown etiology in Wuhan, Hubei Province, China, on 31 December 2019. On January 7, 2020, the agent was determined to be a new coronavirus strain never before identified in humans [1,2,3]. Covid 19, which significantly affects the respiratory system and spreads rapidly, quickly swept the world and was declared a pandemic by WHO on March 11, 2020 [4]. Fever and dry cough are the most common findings of Covid 19, but also severe malaise, nasal congestion, myalgia, headache, sore throat, dyspnea, and taste and smell disorders. Recently, gastrointestinal symptoms such as diarrhea have been reported [5]. It has been observed that comorbidities such as cardiovascular disease, chronic lung disease, asthma, diabetes, and hypertension, together with Covid 19, may enhance the risk of the disease occurring and exacerbate the clinical status [6].

This study, which aims to provide comprehensive information on vaccination against Covid 19 infection during pregnancy and lactation is a brief review. In such studies, the information in the relevant literature is researched, classified analyzed, and compared. The primary outcome is to examine the effect on mortality. The review was based on a literature search in the following databases for publications up to January 2023, Pub Med; Web of Science, ScienceDirect, Google Scholar, and ResearchGate. Keywords used for the search were; Covid 19 infection, lactation, pregnancy, and vaccination. All results of the search were manually scanned for relevant information and their references were also searched for additional publications that might be relevant. Relevant national and international publications, recommendations, and guides published in the specified date range are also included in the study.

Covid 19 Infection During Pregnancy

Pregnancy, while not being comorbidity, is a long process with its own physiological, psychological and pathological characteristics. Hormonal changes at this time increase susceptibility to infection in pregnant women compared with non-pregnant women. The high mortality rates from SARS-CoV and MERS-CoV viruses in pregnant women were reported in the past [7]. Therefore, the risk of Covid 19 may be also higher during pregnancy. However, the Turkish Maternal-Fetal Medicine and Perinatology Society stated that no raised sensitivity for Covid 19 was reported in pregnant women compared to others [8]. Due to insufficient data, guidelines were quickly issued to ensure the management of the process for pregnant women who belong to the risk groups. These guides were revised according to the progression of cases and in light of new findings [9]. This phase seeks to develop strategies for the impact of Covid 19 on the expectant mother and fetus. Treatment planning in pregnant women, possible vaccine options, and vaccination protocol concerning the measures taken for pregnant women in the previous SARS and MERS epidemics [10].

CDC (Centers for Disease Control and Prevention) and ACIP (Advisory Committee on Immunization Practices), in cooperation with ACOG (American College of Obstetricians and Gynecologists) and AAP (American Academy of Pediatrics), have published a guideline that pregnant

women should be included in Covid 19 vaccinations in December 2020 [11]. As stated in this guideline, Covid 19 carries the risk of causing severe health problems for the mother and fetus during pregnancy. The mortality rate due to Covid 19 in pregnant women ranges from 0.4 to 2%. This rate is 70% higher than in non-pregnant women. On the other hand, pregnant women hospitalized with Covid 19 are more likely to stay in the intensive care unit (ICU) than women who are not pregnant but have infections [12]. According to research by Wong et al., about 50% of pregnant women with SARS were followed in the ICU, 33% required mechanical ventilation, and the mortality rate was up to 25% [13]. Pregnant women with Covid 19 are at higher risk for preterm delivery than those without Covid 19. They are also considered at higher risk for other adverse pregnancy events. The incidence of preterm birth in the third trimester is increased approximately threefold, possibly (likely) due to the possibility of vertical transmission and medical interventions for maternal conditions [14].

The approach to disease prevention, assessment, diagnosis, and treatment is similar to that of non-pregnant side effectsiduals in pregnant women with suspected Covid 19. It is offered that pregnant women with Covid 19 disease or in a suspicious condition be cared for in an isolated negative pressure room and continue at home if the illness is mild or if the clinic is not appropriate [15]. The option of untreated follow-up in pregnant women with uncomplicated Covid 19 infection is primarily evaluated. Treatment is considered when risk factors or a severe prognosis are present in pregnant women with a possible diagnosis. Regarding the recommended treatment options for antiviral treatment for Covid 19 in pregnant women, it is foreseen to decide according to the patient's condition, drug's pregnancy category, and risk-benefit ratio. For example, while lopinavir and ritonavir, which are drugs used in the first days of the pandemic but not currently used in the treatment of Covid, are recommended during pregnancy, favipiravir is contraindicated [16].

Because of the presence of ACE 2 (Angiotensin-converting enzyme) receptors in the placenta, it is hypothesized that there is a theoretical possibility of infection of the placenta with Covid 19 and, thus, the transmission of the disease to the fetus. On the other hand, no virus was found in amniotic fluid, cord blood, placental tissues, and throat swabs taken from newborns in studies, and based on these available data, it has been reported that there is no possibility of transplacental transmission of Covid 19 disease in the advanced gestational week or that it is practically negligible [17]. The literature does not know whether and how the infection affects the embryo and the fetus.

Covid 19 Infection During Lactation

The lactation period is defined as the period that begins with the secretion of milk after birth. The health of the nursing mother is essential for the health and development of the baby during the lactation period, which is a sensitive period for the health of both mother and child. UNICEF recommends that even HIV (Human Immunodeficiency Virus) positive mothers continue to breastfeed their babies for up to two years [18].

According to the ABM (Academy of Breastfeeding Medicine) statement and WHO recommendations, the general trend in coronavirus infection is to continue breastfeeding even if the mother is infected [19]. Since the intake of breast milk has been shown to have no adverse effect on the course of the disease, breastfeeding is recommended even in SARS and MERS infections that have already been experienced. It has been reported as a precaution that the risk of infection in the baby is reduced due to the mother paying attention to hand-chest hygiene and breastfeeding with a medical mask [20].

The treatment options recommended in the guidelines for Covid 19 in nursing mothers should be decided based on the risk-benefit ratio according to the patient's condition. While lopinavir and ritonavir are recommended, favipiravir is contraindicated, as in pregnancy [16]. According to WHO, the sick mother should start breastfeeding as soon as she feels well. If the baby is younger than six months, the mother and baby should not be separated, skin-to-skin contact should be ensured as soon as possible, and only breast milk should be given for the first six months. Breastfeeding should continue with safe and healthy complementary feeding when the baby is older than six months [21]. The active Covid 19 disease virus has not been detected to be transmitted to the infant through breast milk and breastfeeding [22].

RESULT AND DISCUSSION

Vaccines Against Covid 19 Infection

-Inactivated Vaccines: Inactivated vaccines contain the entire pathogen killed by chemical or physical methods. Therefore, the agent can't replicate and cause infection [23]. The immune system is stimulated by decomposing and neutralizing the virus and then delivering it to the body. They are considered safer in the first stage since they include the killed/ inactivated virus, but their production is more complicated and slower than other vaccine types [24]. Sinopharm, Sinovac's Coronavac vaccine, which contains inactivated SARS-CoV-2 and an alum adjuvant, and Türkiye's Turkovac vaccine was developed using this method.

-Viral Vector (Adenovirus) Vaccines: They cannot cause disease in humans because they are weakened while the microorganisms in the vaccines are alive; the attenuated virus (vector) carries the desired antigen (S protein) gene in its genome. When the virus infects the cells, it carries the foreign gene into the cell. After transcription and translation in the cell, the gene produces the desired antigen, lifting it to the cell surface and stimulating the immune system. It is quickly produced in cell cultures and can be easily purified without destroying the structure of the antigen [23,24]. Sputnik-V, Johnson & Johnson's Janssen, and Oxford/AstraZeneca vaccines fall into this group. The Janssen adenovirus vaccine has been modified to prevent replication. Therefore, viremia during pregnancy is not expected, and the vaccine is not expected to reach the fetus [25].

-Messenger RNA (mRNA) Vaccines: The vaccine contains highly purified single-stranded mRNA obtained by amplifying the relevant RNA sequence encoding the spike (S) protein of SARS-CoV-2 in vitro in a cell-free medium. Once in the cell, the RNA functions as an mRNA and reprograms the cell to produce the foreign protein usually produced by pathogens (e.g., viruses) or cancer cells. These protein molecules then activate the body's adaptive immune response so that the body learns to destroy pathogens or cancer cells inside the protein [23,24,26]. This group includes BioNTech/Pfizer and Moderna vaccines. Because of the short half-life of the mRNA vaccine, it is unlikely to pass to the fetus during pregnancy or to the baby during breastfeeding. If the mRNA vaccine is excreted into breast milk, it will not be functionally absorbed due to its degradation during digestion. The protein expressed by the mRNA remains in the body for several days, similar to conventional protein-based vaccines. Because the expressed protein probably remains associated with the cell, it is unlikely to pass via breast milk to the fetus during pregnancy or lactation [27]. Table 1 lists Covid 19 vaccines by their mechanism of action, the number of doses administered and dates of emergency use.

Table 1. Covid 19 vaccines by the mechanism of effect, number of doses administered, and date of emergency use approval

Country	Vaccine	Number of doses	Mechanism of effect	Emergency use approval by FDA	Emergency use approval by WHO
Sinovac, China	Coronavac	2	Inactivated Vaccine	---	1 June 2021
Pfizer/BioNTech, USA, Germany	BNT162b2	2	Messenger RNA (mRNA) Vaccine	11 December 2020	31 December 2020
Moderna, USA	mRNA-1273	2	Messenger RNA (mRNA) Vaccine	18 December 2020	30 April 2021
Johnson&Jonhson, USA	Ad26COVs1	1	Viral Vector (Adenovirus) Vaccine	27 February 2021	12 March 2021
AstraZeneca, UK	ChAdOx1	2	Viral Vector (Adenovirus) Vaccine	---	16 February 2020
Gamaleya Res Inst, Russia	Sputnik V	2	Viral Vector (Adenovirus) Vaccine	---	---
Turkovac, Turkey	ERUCOV-VAC	2	Inactivated Vaccine	---	---

CDC notes that the only absolute contraindication to vaccination is having an allergy to vaccine components [28]. Table 2 lists Covid 19 vaccines by their mechanism of action and contraindication status.

Table 2. Covid 19 vaccines by the mechanism of action and their contraindication status

Vaccine name	Type	Contraindication
Pfizer/BioNTech	Messenger RNA (mRNA) Vaccine	Polyethylene glycol allergy
Moderna	Messenger RNA (mRNA) Vaccine	Polyethylene glycol allergy
Johnson&Jonhson	Viral Vector (Adenovirus) Vaccine	Polysorbate allergy
AstraZeneca	Viral Vector (Adenovirus) Vaccine	Polysorbate allergy

Vaccines Against Covid 19 Infection in Pregnancy and Lactation

The main target of any disincentive prenatal intervention is to minimize risk to the first mother and then the fetus while optimizing health benefits. Thus, the primary indication for administering Covid 19 vaccination is for maternal protection. The purpose of immunization during pregnancy is to protect both the pregnant woman and the fetus and newborn from infection. Since the maternal antibodies are transferred from mother to child during pregnancy by the transplacental route and after birth with the mother's milk, there is protection against infection in the early phase of the child's life [29]. Influenza vaccine administered in the third trimester has been reported to reduce febrile respiratory infections by 36% in the first six months of infant life and a very high 63% reduction in the incidence of laboratory-confirmed influenza in infants born to vaccinated mothers [30].

ACIP states that inactive viral/bacterial and toxoid vaccines do not pose a risk to the fetus during pregnancy. Still, caution should be used when administering live attenuated viral and bacterial vaccines during pregnancy [23]. A woman receiving a live vaccine is not recommended to become pregnant within the next four weeks. Most vaccine manufacturers list this period as three months on the product's brief product information sheet. If a live vaccine is inadvertently given to a pregnant woman or if pregnancy occurs within four weeks of vaccination, the person should be counseled about the vaccine's possible effects on the fetus. Still, abortion is not recommended in such a case [31].

The mother, who has been sufficiently immunized during pregnancy, protects herself and the fetus in the womb with antibodies of the type IG G (immunoglobulin G) passed on to the baby via the placenta. Again, the antibodies delivered from breast milk to the baby after birth via breastfeeding are critical for immunity to disease in the neonatal period. In the 2008 study by Gottfredsson et al., the mortality rate for pregnant women in 1918 cases with influenza was 37%, while this rate was 2-6% for others [32].

Although many studies have been conducted and many ongoing studies of emergency-approved vaccines developed for Covid 19, pregnant and lactating women are often excepted from studies for many reasons, including associated physiologic changes, fetal/infant protection, diagnosis, and treatment limitations. However, it should be noted that no vaccine falls into category A, which is considered safe for use during pregnancy. The bivalent HPV vaccine is in pregnancy category B, the anthrax vaccine is in pregnancy category D, and all other vaccines (including H1N1 and seasonal influenza) are in pregnancy category C. It is recommended to apply vaccination in the second trimester to prevent congenital disabilities that may occur for other reasons from being attributed to vaccination [33].

Considering the guidelines of CDC, ACIP, and AMP mentioned in the second part and the more dramatic course of Covid 19 infection in pregnant women, vaccination of pregnant women who are healthcare workers in the high-risk pregnancy classification who work in markets, restaurants, shopping malls, who have to work at a distance of fewer than 1.5 meters from other employees, who smoke and who are overweight, have diabetes, heart disease, asthma, and hypertension are recommended [11].

Based on the mechanisms of the Covid 19 vaccine types, they are considered unlikely to cause a risk to pregnant women [34]. However, there is currently too little data on the safety of Covid 19 vaccines in pregnant women and the general population. The studies examining the safety and efficacy of Covid

19 vaccines in pregnant women are currently planned and ongoing. There are preliminary data from animal studies that received the mRNA Covid 19 vaccine before or during pregnancy that there are no safety concerns in pregnant animals or their offspring. In addition, no side effects have been reported after vaccination with mRNA vaccines in pregnant women other than in non-pregnant women [14].

The first study in pregnant women began in February 2021 (ClinicalTrials.gov identifier NCT04754594). This is a continuing phase II/III randomized, placebo-controlled, observer-blind study in 700 healthy pregnant women vaccinated at four weeks. The study assessed the safety, tolerability, and immunogenicity of two doses of the Pfizer/Biotech vaccine administered 21 days apart versus a placebo [35,45].

Table 3 compares the V-Safe project's preliminary found side effects during pregnancy in pregnant women who have and have not been vaccinated against Covid 19.

Table 3. Comparison of pregnant women with and without mRNA Covid 19 vaccine in terms of side effects during pregnancy [27]

Side Effects	Covid-19 Vaccinated Pregnant Women (%)	Non-Covid-19 Vaccinated Pregnant Women (%)
Gestational diabetes	10	7-14
Preeclampsia and gestational hypertension	15	10-15
Eclampsia	0	0.27
Intrauterine growth restriction	4	3-7
Abortus	15	26
Stillbirth	1	0.6
Premature birth	10	0.1
Neonatal death	0	0.38

Considering to the CDC, from January 2020 to July 2021, approximately 102 thousand pregnant women with COVID-19 were in the United States. 448 (0.44%) patients stayed in an ICU, and 114 (0.11%) patients passed away [35].

Additionally, in a study of about 400,000 women with COVID-19, they were possibly admitted to an ICU, received invasive ventilation and extracorporeal membrane oxygenation (ECMO), and had a 70.0% raised risk of death [36]. As of September 2021, more than 160 thousand pregnant women had been vaccinated with the COVID-19 vaccine and were registered with the COVID-19 vaccine safety monitoring system (V-SAFE). However, adverse events have not been identified through V-SAFE. A study conducted in Israel analyzed data collected from approximately 15 thousand vaccinated and unvaccinated pregnant women. This study showed that vaccinated pregnant women have a substantially lower risk of SARS-CoV-2 than those not vaccinated and Pfizer's vaccines were safe [37].

A large registry-based study of births in Sweden and Norway (28506 vaccinated; 129015 unvaccinated) found no significantly increased risk of adverse pregnancy outcomes including preterm birth, stillbirth, small for gestational age, or NICU admission in people vaccinated against SARS-CoV-2 during pregnancy [38].

Breast milk is optimal for babies because it protects against infections, promotes optimal growth and development, decreases morbidity and mortality rates, is economical, and offers many other benefits [39]. The need to feed infants only breast milk for the first six months is emphasized at every opportunity [40]. Breastfeeding individuals were excluded in accelerated clinical trials for COVID-19 vaccines, which can be used under emergency use authorization from health authorities [15].

Professional associations and government health authorities should therefore recommend offering COVID-19 vaccines to breastfeeding women, as the potential benefits of maternal vaccination while breastfeeding outweigh the risks [41].

Statement of Key Findings

There are preliminary data from animal studies that received the mRNA Covid 19 vaccine before or during pregnancy that there are no safety concerns in pregnant animals or their offspring [14].

COVID-19 vaccines are not considered a risk to nursing mothers or their infants because of how vaccines work in the body. For this reason, it is recommended that nursing mothers may also receive the COVID-19 vaccine. Reports have demonstrated that breastfeeding individuals vaccinated with COVID-19 mRNA have antibodies in their breast milk that may help protect their babies [42-44].

Strengths and Limitations

This study is important in terms of summarizing and guiding the results of Covid-19 vaccines in pregnant and lactating mothers. However, the results cannot be generalized because pregnant and lactating women are not included in drug and vaccine studies and the number of studies is limited.
Covid 19 vaccine data on pregnancy and lactation are limited, as pregnant and lactating women are excluded from vaccine trials. This is not because of any particular safety concern but rather a matter of precaution, as in the problems of many other drugs. However, preclinical studies in animals that received the Covid 19 vaccine did not raise any safety concerns [14]. Evidence continues to build showing that Covid 19 vaccination before and during pregnancy is safe, effective, and beneficial to both the pregnant person and the baby.

There is no harm, at least theoretically and within the context of limited clinical data, in administering all vaccine types during pregnancy and lactation [14]. All over the world, health authorities such as ACOG, RCOG (Royal College of Obstetricians and Gynaecologists), EBCOG (European Board & College of Obstetrics and Gynaecology), The Joint Committee on Vaccination and Immunisation (JCVI) and Turkish Ministry of Health recommend access to COVID-19 vaccines, especially for pregnancy at high risk [46,47]. Thus, Covid 19 vaccination is not only suggested but strongly recommended for pregnant and breastfeeding populations to protect mothers and newborns.

AUTHOR CONTRIBUTIONS

Concept: A.E.B.; Design: A.E.B., A.P.; Control: A.T.Ö.; Sources: A.E.B., A.P, A.T.Ö.; Materials: A.E.B., A.P., A.T.Ö.; Data Collection and/or Processing: A.E.B., A.P.; Analysis and/or Interpretation: A.E.B., A.P.; Literature Review: A.E.B., A.P.; Manuscript Writing: A.E.B., A.P.; Critical Review: A.E.B., A.P., A.T.Ö.; 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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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(3): 1141-1154, 2023
Doi: 10.33483/jfpau.1328811

REVIEW ARTICLE / DERLEME MAKALE

THE POSSIBLE RELATIONSHIPS BETWEEN SOME GENE POLYMORPHISMS AND SJOGREN'S SYNDROME

SJÖGREN SENDROMU İLE BAZI GEN POLİMORFİZMLERİ ARASINDAKİ OLASI BAĞLANTILAR

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ABSTRACT

Objective: *Sjögren's syndrome is a complex and widespread autoimmune disease whose pathogenesis is not fully elucidated and environmental and genetic factors affect the development of the disease. In order to reveal the effect of genetic contribution, studies have been conducted on the genes previously shown to play a role in other autoimmune diseases such as systemic lupus erythromatosus. In addition, two GWAS studies were conducted to investigate the role of more genes in the disease by screening the entire genome and the relationship of previously unknown genes with SS was shown.*

Result and Discussion: *Studies are being conducted with spontaneous and genetically modified animal models in order to better reveal the relationship between SS and genes and to reinforce the data obtained from humans. In this study, the relationship between the genes previously studied in other autoimmune diseases and the genes associated with SS in GWAS studies and the possible pathways that may contribute to the pathogenesis of the disease through related genes were investigated.*

Keywords: *Autoimmune disease, gene polymorphisms, genetic toxic effects, genotoxicity, Sjögren's syndrome*

ÖZ

Amaç: *Sjögren sendromu hala patogenezisi tam olarak aydınlatılamamış, hastalık gelişimini çevresel ve genetik faktörlerin etkilediği kompleks ve yaygın bir otoimmün hastalıktır. Genetik katkının etkisini ortaya koymak için daha önce sistemik lupus eritromatozus gibi diğer otoimmün hastalıklarda rolü gösterilen genler üzerinde bu genlerin SS ile ilişkisini ortaya koymak için çalışmalar yapılmıştır. Ayrıca iki GWAS çalışmasıyla da tüm genom taranarak daha fazla genin hastalıkta rolü incelenmiş ve daha önce SS ile ilişkisi bilinmeyen genlerin SS ile ilişkisi gösterilmiştir.*

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Submitted / Gönderilme : 17.07.2023
Accepted / Kabul : 09.08.2023
Published / Yayınlanma : 20.09.2023

Sonuç ve Tartışma: *SS'in genlerle ilişkisini daha iyi ortaya koymak ve insanlardan elde edilen verilerin pekiştirilmesi için spontan ve genetiği modifiye edilmiş hayvan modelleriyle de çalışmalar yürütülmektedir. Bu çalışmada daha önce diğer otoimmün hastalıklarda incelenen genler ile GWAS çalışmalarında ilişkili bulunan genlerin SS ile ilişkisi, ilişkili bulunan genler üzerinden hastalığın patogenezisine katkısı olabilecek olası yolaklar irdelenmiştir.*
Anahtar Kelimeler: *Gen polimorfizmi, genetik toksik etkiler, genotoksisite, otoimmün hastalık, Sjögren sendromu*

INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune condition characterized by lymphocytic infiltration in exocrine glands such as saliva and lacrimal glands. Typical clinical findings in patients with SS due to progressive damage to exocrine glands are dry mouth (crestomyia) and dry eye (keratocongicivitis sicca). In SS patients, in addition to symptoms of dryness, extrraglandular signs such as Raynaud's phenomenon, fatigue, or arthritis are common [1-4]. Another feature of the disease is B cell hyperactivity. Major autoantibodies in SS target the intracellular antigens Ro52/TRIM21, Ro60/TROVE2 and La/SSB antigens, which are ribonuleoprotein-RNA complexes [5,6].

SS predominantly affects premenopausal women, and the incidence is 9: 1 in women compared to men [4,7]. It is known that SS occurs at 0.4-4.0% of the general population [4,8,9]. Modified European and American diagnostic criteria are the most commonly used diagnostic criteria in clinical practice [4,10]. According to this criterion, when it is accompanied by other rheumatoid diseases such as SLE or RA, it is classified as secondary SS (sSS) and SS alone is classified as primary SS (pSS). SS progresses with or without other autoimmune diseases [4,10,11]. Disease severity is determined according to ESSDAI (The EULAR SS disease activity index) [12]. Although mortality in SS is not different from the general population, quality of life in SS patients is significantly affected by reduced morbidity. SS patients are dependent on palliative methods in order to relieve major symptoms of crestomyia along with immunesuppressive methods. However, there are still no effective therapies to restore the SS process or to repair secretion dysfunction [4,13]. The risk of developing Non-Hodgkin lenfs B cell lymphoma is 44 times higher in SS patients compared to healthy individuals [4,14].

The etiopathology of the disease is unknown. It has been suggested that genetic factors, as well as exogenous agents such as Epstein-Barr virus (EBV), Hepatitis C (HCV) and human T-cell leukemia virus-1 (HTLV-1), hormones and microcremism may cause the onset of this disease [15].

SS' Relations with Genes

Genetic predisposition is one of the main features of autoimmune diseases [16,17]. SS is a genetically complex disease and little is known about the contribution of genetic factors to the disease. There are studies on monozygotic twins in other autoimmune diseases to investigate genetic contribution. In these studies, the comorbidity between disease and monozygotic twins was reported to be 25-40% in monozygotic twins [18]. However, there are no studies on monozygotic twins for SS. Case reports were made for case monozygotic and dizygotic SS twins only for SS. However, reliable correlation between twins is not evaluated in these presentations [19-21]. The incidence of other autoimmune diseases in the families of SS patients was reported to be 30-35%. Thyroid diseases, SLE and RA are the most common autoimmune diseases [22,23]. To date, gene studies on SS are included in the study of specific genes that may be genetic risk factors.

Candidate Genes

The first genetic studies of SS were carried out in genes that were previously known in the immune system and which had important functions or were shown to affect other autoimmune diseases such as romataid arthritis and systemic lupus erythromatosis (SLE).

In 1977, HLA genes were shown to be a risk factor for SS [24]. HLA (Human Leukocyte Antigens) complex is located on the short arm of chromosome 6 [25-27]. HLA antigens are expressed on many cell surfaces and have an important role in the recognition of antigenic stimulants, stimulation

of the immune system, and regulation of cellular and humoral immunity [26]. HLA complexes are classified into three classes as Class I, Class II and Class III [27].

HLA Class II proteins have the largest hereditary susceptibility to autoimmune diseases including SS. Reported risk haplotypes differ slightly from phenotype and race. The HLA-DR3 SS relationship was shown primarily in the white race, and the SS relationship of HLA-DR3-DQ haplotypes was shown in different ethnic groups [16,17,28-36] However, a meta-analysis identifies DRB1*0301, DQA1*0501, DQB1*0201, and DRB1*03 alleles as risk factors for SS, while identifying DQA1*0201, DQA1*0301 and DQB1*0501 alleles as preservatives [37]. Recently, a strong association between HLA-DRA, HLA-DQB1 and HLA-DQA1 and SS in 6p21 locus in a large study in Europe was reported [38]. In a study in China, HLA-DRB1/HLA-DQA1 in 6p21.3 locus and two independent signals associated with HLA-DPB1/COL11A2 [39]. Deterioration of autoreactive T cell tolerance through the presence of abnormal antigen demonstrates the key role of HLAs in autoimmune diseases. The disease relationship of HLA-suspected alleles is common in autoimmune diseases and different specific alleles and haplotypes are formed, different alleles direct targeting of specific autoantigens [40]. HLA Class II is associated with autoantibody production in SS, whereas anti-Ro/SSA and anti-La/SSB are significantly higher in HLA-DQ1/HLA-DQ2 heterozygous patients [41] but not related to other clinical features [32]. HLA-DRB1*1501-DRB1*0301 is associated with anti-ACA (anticyclic citrullinated antibodies) [42]. Amino acid variations in the hypervariable region (HVR) region of the HLA complex affect peptide binding and T cell presentation; The association of specific variations in binding wells 7 and 9 of HLA-DRB1 with changes in depth and polarity was shown in the Chinese population [35]. Although HLA Class I and HLA Class III genes were also studied in the following years, studies focused on HLA Class II genes.

On the other hand, polymorphisms in non-HLA genes, which have been shown to be associated with other autoimmune diseases, were also investigated in SS. One of the non-HLA genes is STAT4. STAT4 (signal transducers and activators of transcription-4) is an important transcription factor for the transmission of IL-12, IL-23 and Type 1 interferon-mediated signals involved in Th1 and Th17 differentiation, activation of monocytes and INFγ production [43-45]. STAT4 haplotypes have been shown to be a risk factor for the development of SLE and RA in the caucasians and its relationship with SS [46]. STAT4 polymorphism was investigated in different ethnic groups in different loci such as rs7574865 [47] and rs7582694 [48]. In these three studies, it was determined that rs7582694 polymorphism posed a risk for SS. This polymorphism was found to be poorly correlated with m-RNA levels of various interferon-induced genes in peripheral blood mononuclear cells of SS patients [48].

The distinctive feature of this disease is B cell hyperactivity. B cell hyperactivity was demonstrated by the presence of autoantibodies and hypergammaglobulinemia. The most risky group in development of lymphoma -especially non-Hodgkin's lymphoma- in all autoimmune diseases is pSS [49].

In addition, the association of BAFF (B-cell activating factor) polymorphisms in the development of other autoimmune diseases has been shown previously [49-55]. BAFF also known as B lymphocyte stimulator, is a member of the TNF superfamily that regulates the immune [56-58]. A cytokine facilitates B cell survival and maturation [56,58]. It is expressed as membrane bound (mBAFF) and soluble protein (sBAFF) [3,58-60]. Many cells are produced by antigen-presenting cells (B cells, monocytes/macrophages, dendritic cells (DC), plasmacytoid DC, follicular DC), epithelial cells, active T lymphocytes) [58,59]. In the presence of type 1 interferon (INFγ, LI-10, TLR3, TLR4, TLR9, etc.), BAFF expression increases [57,58,60]. Binding of BAFF to BAFFR triggers NF-κB (non-canonical nuclear factor κB) signaling [58,61]. The relationship of BAFF with overexpression with mature B cell hyperplasia and development of SLE and SS-like symptoms in lymphoid tissues has been previously demonstrated in experimental models [1,58,62].

It is thought that genetic variation of BAFF increases the risk of developing lymphoma [53,63]. It has been reported that various SNP (single nucleotide polymorphism) in various BAFF genes contribute to anti-Ro and/or anti-La positivity or high sBAFF level [64]. In addition, the association of the BAFF receptor with His159Tyr mutation, which causes deregulation of apoptosis by activation of the NF-κB pathway, has also been demonstrated [65]. It is also known that BAFF affects Type I and Type II interferon regulation and thus its contribution to SS development is bi-directional with its

contribution to B cell hyperactivity as well as its contribution to cytokine production. Studies on the effect of BAFF on both B cell and cytokine production showed a relationship between gene polymorphism and serum BAFF level, blood and salivary gland BAFF transcription level [66].

TNFAIP3 interacting protein 1 (TNIP1) encoded by the TNIP1 gene is an important signaling protein in the NF-κB pathway. Together with TNFAIP3 (Tumor necrosis factor alpha inducible protein 3), it acts together with the TNFAIP3 protein to suppress NF-κB activation. The association of TNIP1 gene polymorphism with many autoimmune diseases such as systemic sclerosis, rheumatoid arthritis (RA), psoriasis, SLE [67-76] was determined. In addition, its relationship with SS and anti-Ro/SSA and anti-La/SSB autoantibody seropositivity in SS were also shown [38]. On the other hand, the TNFAIP3 gene has been reported to be associated with diseases such as SLE and RA [66,77-79], and is also associated with pSS in GWAS studies. Allelic variations of the TNFAIP3 gene have been reported to be associated with pSS [80,81].

Thrombospondin-1 (TSP-1) is an adhesion matrix protein encoded by the THBS1 gene, which activates latent TGFβ and some anti-inflammatory cytokines and regulates extracellular and intracellular signaling complexes; expressed in the corneal epithelium, stroma and endothelium. Dry eye is one of the most important symptoms in SS. In this respect, the relationship between THBS1 polymorphism and SS has attracted attention and the relationship between THBS1gene variations and anisotropy and orientation symmetry coefficients of corneal nerve fibers has been shown [82]

None of the candidate genes reported for polymorphism in SS studies have changed the coding sequences of these genes, and only single SNPs have been studied in studies with the candidate gene approach.

GWAS Studies

Genome-wide association studies (GWAS) are a powerful molecular method that scans the entire DNA to determine the relationship between specific disease phenotypes and any loci. It yields SNPs associated with different polymorphic alleles covering the entire genome. GWAS studies are conducted in large patient and control populations, which is important, and allows a good comparison between races and to determine whether the observed relationship is race-bound. In addition, since these studies are clinically studied in a broad spectrum, the selection of participants is better [39].

Unlike other autoimmune diseases, there are only two GWAS studies for SS. One of them is found in the European population by over 10,000 participants. All patients were diagnosed with SS according to European-American Consensus Criteria. In this study, seven genetic regions were identified that could exceed the statistical threshold $p<5\times10^{-8}$, MHC-II loci, IRF5, STAT4, IL12A, BLK, CXCR5 and TNIP1. The strongest association was found in the HLA-II locus, followed by STAT4 and IRF5. The HLA-II locus, STAT4 and IRF5 were previously identified by the candidate gene approach, a stronger statistical value was obtained with this study, more samples were studied. IL12A, BLK and CXCR5 are important genes in immune signaling and their association with SS was demonstrated for the first time. TNIP1 is involved in the NFκB pathway and is a new gene associated with SS in the GWAS study. TNFAIP3, DGKQ, and FCGRN2 were found to be statistically poorly correlated. In this study, genes associated with SS are important genes in immune system functions [83].

The other GWAS study was performed in 1090 healthy and 597 SS cases in the Chinese population and 642,832 SNPs were detected. In this study, the strongest relationship was found in the GTF2I gene, which is a general transcription factor, and the other related genes are MHC-II, STAT4, and TNFAIP3 genes (Table 1) [84].

The results of these extensive studies conducted in two different populations show that there are differences and similarities between the European and Chinese populations. GTF2I polymorphism was observed only in Chinese population, while IRF5 polymorphism and other polymorphisms were not seen in Chinese population. The two important genes involved in the NFκB pathway were found to be related differently in both populations for TNIP1 and TNFAIP3. TNFAIP3 was found to be statistically significant in the Chinese population and weak in the European population. TNIP1 was associated only in the European population. These results indicate different genetic risks for both populations and should be confirmed in further studies.

Table 1. SS Associated Non-HLA genes in GWAS Studies [39]

Alleles with Gene	Function	Determination	References
STAT4	Transcription Factor	2	83
IRF5	Transcription Factor	1	83
IL12A	The cytokine	5	83
BLK	BLK B Cell Kinase	2	83
CXCR5	Chemokine	3	83
TNIP1	NFκB signaling	3	83
GTF2I	Transcription Factor	1	84
TNFAIP3	NFκB signaling	1	84

In these studies, none of the genes associated with SS were lacrimal and salivary glands, proteins associated with nerve conduction in these glands, secretion devices and X-crosomes. Associated genes are those related to immune system functions. Based on these results, it is seen that the immune system and the differences of activity in the immune system are the most important factors in the pathogenesis of SS.

Although the pathogenesis of SS is still unclear, the mechanisms that may contribute to the pathogenesis of the disease have been proposed based on candidate gene approach and GWAS related genes. The most common pathway is increased interferon signaling and cytokine production. The IRF5, STAT4, and IL12A genes contributing to this pathway were associated with SS in candidate gene approach and GWAS studies. The second possible pathway is B cell production, antibody formation and changes in antibody clearance. BLK, CXCR5 and FCGR2 are also related genes involved in these pathways and involved in these pathways. The third pathway is the NFκB pathway and the genes involved in these studies are TNIP1 and TNFAIP3 (Figure 1) [39].

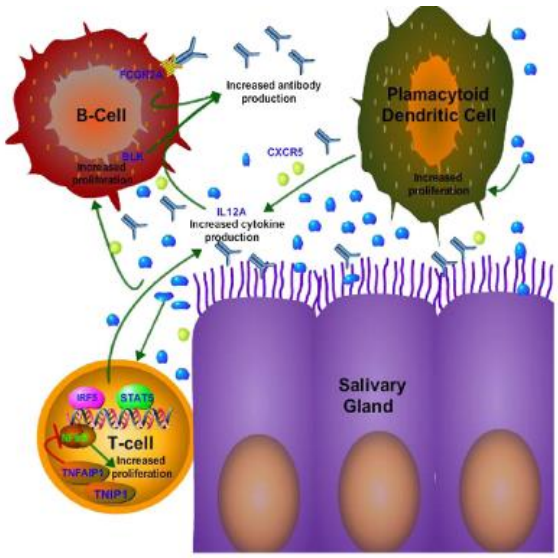

Figure 1. Functional changes in potential paths [39]

Functional Studies

All gene polymorphisms associated with SS were detected in non-coding sequences of the gene of interest. This led to gene studies to evaluate the effect of these polymorphisms on gene expression. In the GWAS study, mRNA expression level of some genes was compared in groups with and without polymorphism. Accordingly, if this polymorphism is found in the transcriptional regulatory region of

the gene, IRF5 and HLA-II gene expression levels are higher in SS patients, and related polymorphisms such as GTF2I have no effect on gene regulation [39].

Depression is a common condition in SS. In a study, the association of platelet serotonin levels with the serotonin transporter gene (5-HTT) polymorphisms in SS patients was investigated and it was reported that platelet serotonin levels were lower in the presence of intronic 5-HTTVNTRin2 (I/s) polymorphism compared to controls [85].

In another study, the relationship between the level of surfactant protein-D (SP-D) and SP-D genotype, which is thought to have an effect on the pathophysiology of the disease, was examined but no relationship was found between them [86].

Protein tyrosine phosphotase non-receptor type 22 (PTPN22) T cells, B cells, natural killer cells, DCs, monocytes and macrophages are expressed in many immune-related cells [87], regulate T cell receptor signaling [88]. PTPN22 in myoloid cells potential TLR-induced Type I interferon (IFN) production [89]. PTPN22 allelic variations are risk factors for many autoimmune diseases such as Type 1 diabetes, RA, SLE and hashimoto trodiditis [87-90]. Many PTPN22W-related diseases have been reported to be associated with impaired adaptive immunity and autoantibody production [88]. The frequency of phenotype PTPN22W* variation in pSS patients with low Type I INF blood levels was reported to be higher than controls and pSS errors with high Type I INF blood levels [91]. Gene variations of the F11R protein, which has many functions such as intracellular signaling, regulation of cellular permeability, stimulation of cell translocation during inflammatory processes, and cytokine production were investigated in SS. In this study, it was reported that some variations were associated with SS and F11R mRNA expression was lower in SS patients compared to healthy controls [92].

Animal Studies

In order to understand SS pathogenesis, human genotyping and genotype and phenotype compatibility studies are conducted concurrently with animal studies. Thus, the results obtained from humans and animals are compared and tried to prove the accuracy of the predicted mechanisms.

Animal models have been developed in order to understand the pathogenesis of SS and studies are being conducted on these models. Animal models are very valuable for elucidating pathogenesis and applying therapeutic approaches. Animal models are important especially in the absence of very clear clinical indicators at the onset of the disease and inability to detect changes in disease onset and help researchers to monitor changes that contribute to pathogenesis at the onset of disease [93]. The animals developed and used for this purpose are genetically similar to animals, such as mice and rats suitable for gene cloning and transgenic modification [94]. However, the data obtained from animal studies are limited in the elucidation of the disease due to factors such as developmental process between humans and mice, differences in adaptive and innate immune response and environmental conditions [95].

Spontaneous animal models allow for an understanding of the tendency or resistance loci of the disease, the time-related profile of disease formation and progression. Many spontaneous animal models used for pSS are derived from non-obese diabetic (NOD) mice. NODs develop not only Type 1 diabetes but SS-like autoimmune exocrinopathy. For this reason, SS is one of the most powerful tools for revealing the pathological mechanism [96]. Infiltration of the salivary and lacrimal glands occurs at 12-16 weeks of age. In addition, autoantibodies such as ANA, anti-SSA/Ro, anti-SSB/La and anti-M3R are also seen in SS patients [97,98].

In addition, genetically modified HTLV-1 tax transgenic (Tg) mouse, IL-6 Tg mouse, IL-10 Tg mouse, IL-12 Tg mouse, IL-14α Tg mouse, B-cell, to investigate the pathophysiology of SS-like diseases activating factor (BAFF) Tg mouse, retinoblastoma associated protein 48 (RbAp48) Tg transgenic mouse species and transforming growth factor beta 1 (TGF-β1) KO (knock out)mouse, inhibitor of differentiation 3 (Id3) -/- KO mouse, aromatase-deficient (such as Ar KO) mouse, phosphoinositide 3-kinase (PI3K) KO mouse and thrombospondin-1 (TSP-1) -deficient conjugate mouse are also used [99].

Some of the results from animal experiments are consistent with previous human studies. For example, IL-12 transgenic mouse model expressing both subgroups of IL-12 showed features similar to human SS such as increased SSB autoantibody production, decreased saliva flow, and lymphocytic

infiltration in glands [95]. In the GWAS study, the IL-12 gene is one of the genes associated with SS [39].

RESULT AND DISCUSSION

Despite the studies, the etiopathology of the disease is still unknown. Genetic factors, as well as exogenous and endogenous factors, have been shown to cause the onset of the disease.

Polymorphisms of the HLA, STAT4, BAFF and TNFAIP3 genes and the expression of these genes in tissues such as blood and salivary glands have been shown in the studies on the association of genes associated with SS in other autoimmune diseases such as romataid arthritis and SLE.

Again, two GWAS studies with larger populations showed the association of HLA, STAT4, IRF5, IL12A, BLK, CXCR5, TNIP1, GTF2I and TNFAIP3 genes and the expression of these genes in SS.

In these studies, mechanisms that could contribute to the pathogenesis of the disease were predicted from related genes. The most common of these is increased interferon signaling and cytokine production pathways. The second possible pathway is B cell production, changes in antibody formation and antibody clearance, and the third pathway is the NFκB pathway. Revealing the mechanisms that may contribute to the pathogenesis is very important in terms of contributing to the development of new drugs for the treatment of the disease.

In addition to human studies, gene polymorphisms which may be related to animal studies and appropriate animal models and the expression of this gene expression in tissues such as blood and salivary gland are compared with human studies and possible mechanisms are explained.

Although studies on suspected genes have shown that many genes may contribute to the pathogenesis of the disease, studies on genes that are not yet studied but that may be related to the disease should also be conducted. So far, gene studies are only SNP studies and there is a need for studies to determine multigenetic factors and to produce more powerful data in larger populations.

AUTHORSHIP CONTRIBUTIONS

Concept: U.T., I.A.; Design: I.A.; Control: I.A.; Sources: U.T., I.A.; Materials: I.A.; Data Collection and/or Processing: U.T., I.A.; Analysis and/or Interpretation: U.T., I.A.; Literature Review: U.T.; Manuscript Writing: U.T., I.A.; Critical Review: I.A.; 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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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 47(3): 1155-1169, 2023
Doi: 10.33483/jfpau.1316067

DERLEME MAKALE / REVIEW ARTICLE

SPORCULAR TARAFINDAN KULLANILAN DOĞAL KAYNAKLI ERGOJENİK DESTEKLER

NATURALLY SOURCED ERGOGENIC SUPPORTS USED BY ATHLETES

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ÖZ

Amaç: *Ergojenik destekler, sporcuların egzersiz öncesi, sırası ve sonrasında fiziksel performanslarını desteklemek veya iyileştirmek için kullandıkları maddeler, uygulamalar veya tekniklerdir. Bu araştırma kapsamında incelenen ergojenik maddeler çeşitli formülasyonlarda ve içeriklerde piyasada bulunabilmekle birlikte, son yıllarda sporcular tarafından en çok tercih edilen tipi, bir kısmı gıda olarak da kullanılan ve "daha zararsız olduğu düşünülen" doğal kaynaklı ergojenik desteklerdir. Doğal kaynaklı ergojenik destekler, doğru bitki, doğru drog, doğru ürün, doğru zaman, uygun doz ve uygun formülasyonda kullanıldığı takdirde sporcunun performansını desteklemektedir. Aksi halde sporcunun kullandığı bu destekten fayda görmesinin yanı sıra sağlığını olumsuz etkileyebilecek sonuçlar ortaya çıkabilmektedir. Her ne kadar sporcu için sportif performans öncelikli olsa da asıl önemli olanın sporcunun sağlığı olduğu literatürdeki çalışmalarda da bildirilmektedir.*

Sonuç ve Tartışma: *Bu derlemede sporcular tarafından son yıllarda tercih edilen doğal kaynaklı ergojenik desteklerin PubMed, Science Direct, Google Akademik, BioMed Central, TÜBİTAK Ulakbim veri tabanlarında detaylı bir literatür taraması ile belirlenmesi, en sık kullanılanlarının ise olumlu ve olumsuz yanlarının güncel veriler ışığında incelenmesi amaçlanmıştır.*

Anahtar Kelimeler: *Doğal kaynaklı ürünler, ergojenik destekler, performans, sporcu*

ABSTRACT

Objective: *Ergogenic aids are substances, practices, or techniques that athletes use to support or improve their physical performance before, during, and after exercise. Although the ergogenic substances examined in this research can be found in the market in various formulations and contents, the most preferred type by athletes in recent years is natural-source ergogenic aids, some of which are also used as food and "considered to be more harmless". Naturally sourced ergogenic aids support the performance of the athlete if the right plant, the right drug, the right product, the right time, the right dose and the appropriate formulation are used. Otherwise, in addition to benefiting from this support used by the athlete, results that may adversely affect his health may*

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Gönderilme / Submitted : 17.06.2023
Kabul / Accepted : 17.08.2023
Yayınlanma / Published : 20.09.2023

ocur. Although sportive performance is a priority for the athlete, it is also reported in the literature that the most important thing is the health of the athlete.
Result and Discussion: *In this review, it is aimed to determine the natural origin ergogenic aids preferred by the athletes in recent years with a detailed literature review in PubMed, Science Direct, Google Scholar, BioMed Central, TUBITAK Ulakbim databases, and to examine the positive and negative aspects of the most frequently used ones in the light of current data.*
Keywords: *Athlete, ergogenic aids, naturally sourced products, performance*

GİRİŞ

Ergojenik kelimesi köken olarak Yunanca "ergon (iş)" ile "genon (üretmek)" kelimelerinin bir araya gelmesiyle oluşmuştur [1]. Sporcular performans kapasitelerini ve çalışma verimlerini arttırma, fizyolojik ve psikolojik olarak daha yüksek bir başarıya ulaşma arzusu barındırırlar [2]. Bu nedenle daha yüksek bir sportif performans avantajı sağlamalarına yardımcı olduğunu düşündükleri ergojenik destekleri kullanmaya yönelirler [2-5].

Sporcuların ergojenik destekleri kullanma fikri antik dönemlere kadar uzanmaktadır (M.Ö. 400) [6]. Antik dönemlerde daha çok deneme-yanılma yoluyla veya bilge kişilerin katkılarıyla hazırlanan reçeteler güç ve performansı arttırmak amacıyla kullanılmıştır [6]. Ancak günümüz modern toplumunda sporcuların performans arayışları farklılaşmış ve bu durum sporcuların farklı ergojenik desteklere yönelmesine neden olmuştur [6]. Böylelikle tarihsel süreçte ergojenik desteklerin çeşitliliği artmış ve piyasada farklı formülasyonlarda (kapsül, tablet, yumuşak jeller, sıvı, toz ve çubuk) ve içeriklerde yer almaya başlamıştır [2,4].

Egzersiz öncesi, sırası ve sonrasında kullanılan ergojenik destekler sportif performansı arttırmasının yanı sıra kas dokusunun ve dayanıklılığının artmasına, yorgunluk hissi oluşumunun gecikmesine, egzersiz sonrası vücutta oluşabilecek hasarın minimuma indirilmesine, bağışıklık sisteminin korunmasına, diyet ile yeteri kadar alınamayan vitamin ve mineralin alınmasına, antrenman ve müsabaka sonrasında vücudun toparlanma süresinin kısaltılmasına, kondisyon ve koordinasyon gibi motor beceri işlevlerinin yerine getirilme yetisinin arttırılmasına yardımcı olmaktadır [1-7].

Ergojenik destekler; fizyolojik, psikolojik, mekanik, biyomekanik destekler, farmakolojik ve besinsel destekler olmak üzere başlıca 5 ana grupta sınıflandırılmaktadır [8]. Kullanım amacı göz önünde bulundurularak yapılan bir diğer sınıflandırma yönteminde ise bu destekler kas geliştirici, ağırlık azaltıcı, performans geliştirici ve genel sağlığı geliştirici olarak 4 ana grupta toplanmıştır [9].

Sporcular tarafından ergojenik destek kullanımı her geçen gün popülerliği artan bir durum haline gelmektedir [6]. Bu durum sporcuların sağlığı ve performansları açısından önem taşıdığından hassasiyetle üzerinde durulması gereken bir konudur [2]. Bu nedenle bazı ergojenik destekler sporcu sağlığını ve spor yaşamını olumsuz etkileyebilecek bileşenleri içerdiğinden spor komiteleri tarafından doping olarak kabul edilerek kullanımı yasaklanmıştır [3].

Takviye edici gıda ve doping kavramı ergojenik destek kullanım amacı ile sıklıkla karıştırılan kavramlardır. Takviye edici gıdalar; sporcuların yarışma öncesi ve sırasında oyuna hazırlanırken, performanslarını arttırmak adına kullandıkları maddelerdir (vitamin, protein, aminoasitler, karnitin, kreatin gibi). Doping ise takviye edici özelliğinin yanı sıra spor ahlakı ve etiği çerçevesine uymayan, fizyolojik ve psikolojik sağlığı olumsuz etkileyebilen maddeler ve diğer olası yöntemlerdir. "Doping" kelimesinin, Güney Afrika ve Hollanda lehçelerinde "doop" kelimesinden köken aldığı ve Güney Afrika'da bir kabile olan Zulu savaşçılarının üzüm tortusundan hazırlanan hem savaşlarda güç kazanmak için hem de dini törenlerinde içtikleri "dope" isimli bir içecek şeklinde tüketildiği bilinmektedir. M.Ö. 3. yüzyılda Phlostratos ve Calenos'un yazılarında, o dönem yapılan olimpiyatlarda sporcuların performanslarını yapay olarak arttırabilmek adına her türlü yönteme başvurduğu görülmektedir. Orta ve Güney Amerika'da koşu ve yürüyüş spor dallarında yarışan sporcuların *E. coca* Lamarck (Koka ağacı) yapraklarını yediklerini, atıcıların ise daha hızlı koşabilmek adına bol miktarda mantar yediğini eldeki kayıtlar göstermektedir. Anlaşılacağı gibi, çok uzun bir tarihsel süreci olan doping maddeleri günümüzde daha da yaygınlaşarak kullanılmaya başlanmış ve bu doğrultuda çeşitliliği artmıştır. 19. yüzyıla gelindiğinde doping önemli spor komitelerince tanımlanmış ve doping kategorisine

giren maddelerin sporcu sağlığını hayati derecede etkileyebileceği görülmüş olup bu maddelerin kullanımının yasaklanması noktasına gidilmiştir [10-12].

Günümüzde Uluslararası Olimpiyat Komitesi (IOC), Dünya Anti-doping Ajansı (WADA) ve Uluslararası Spor Federasyonları (IF) performansı arttırmak amacıyla kullanılan, etkin olan ve olmayan her türlü droğu doping olarak kabul etmekte 1980 yılından beri düzenli olarak her yıl doping listeleri hazırlamakta ve bunları yayınlamaktadır. Yapılan denetimler sonucu sporcuların idrarlarında bu maddelere ya da metabolitlerine rastlanması sporcunun ceza almasına neden olmaktadır [10-12].

Her ne kadar birçok doping maddesi doğal kaynaklardan elde edildiği için zararsız olabileceği düşünüldüğü veya fitokimyasal içerikleri nedeniyle gıda olarak kabul edildiği için doza bakılmaksızın tüketilebileceği düşüncesi yaygın olsa da Paracelsus'un belirttiği gibi "Tüm maddeler zehirdir, ilacı zehirden ayıran dozudur" ifadesi bu noktada önem taşımaktadır. Bu doğrultuda doping maddelerinin yasal sınırları belirlenirken kullanım şekli, dozu ve potansiyel etki mekanizmasını belirlemek oldukça önemlidir. Bu süreçte ulusal veya bölgesel bir otorite tarafından yayınlanan, farmasötik ürünler için standartlar ve kalite spesifikasyonlarını içeren bilimsel ve yasal dayanaklı bağlayıcı referans kaynakları olan "Farmakopelerin" rehberliğine ihtiyaç duyulmaktadır. Aksi takdirde sporcular bu maddelerden ya çok az yarar görebilir ya hiç yarar göremeyebilir ya da zarar görebilirler. Bu çalışma, sporcuların kullandıkları doğal kaynaklı ergojenik desteklerin detaylı bir literatür taraması ile belirlenmesi, en sık kullanılanların ise olumlu ve olumsuz yanlarını güncel veriler ışığında ortaya koymayı amaçlamaktadır [10].

SONUÇ VE TARTIŞMA

Sporcuların ergojenik destek kullanımı ve bu desteklerin performansları üzerindeki etkilerini değerlendirmek amacıyla Google Akademik, PubMed, Science Direct, Web of Science, Scopus ve ULAKBİM veri tabanlarında literatür taraması yapılmıştır. Literatür İngilizce olarak; "ergogenic aids", "ergogenic aids and athlete performance", "natural origin ergogenic aids", "natural origin ergogenic aids and athlete performance"; Türkçe olarak ise "ergojenik destekler", "ergojenik destekler ve sporcu performansı", "doğal kaynaklı ergojenik destekler", "doğal kaynaklı ergojenik destekler ve sporcu performansı" anahtar kelimeleriyle kapsamlı bir şekilde taranmıştır. Konu açısından uygunluğuna karar verilen bilimsel çalışmalar (deneysel çalışmalar, meta-analiz araştırmaları ve sistematik derlemeler) incelenerek konu ile ilgili bilimsel bir bütünlük oluşturulmaya çalışılmıştır.

Google Akademik, PubMed, Science Direct, Web of Science, Scopus ve ULAKBİM veri tabanlarında yapılan kapsamlı literatür taraması sonucunda sporcular tarafından en sık kullanılan doğal kaynaklı ergojenik destekler Tablo 1'de verilmiştir.

Tablo 1. Doğal kaynaklı ergojenik destekler [1,3,4,13-17]

Doğal Kaynaklı Ergojenik Destekler	**Kaynağı**	**Metabolit Türevi**	**Literatür**
Arjinin	Hayvansal	Primer metabolit	[1,4]
***Arthrospira maxima* (Stiz.) Geitl.**	Bitkisel	Sekonder metabolit	[17]
***Arthrospira platensis* Gomont**	Bitkisel	Sekonder metabolit	[17]
***Arnica montana* L.**	Bitkisel	Sekonder metabolit	[17]
***Astragalus membranaceus* (Fisch.) Bunge**	Bitkisel	Sekonder metabolit	[17]
***Avena sativa* L.**	Bitkisel	Sekonder metabolit	[16]
Beta (ß) alanin	Hayvansal	Primer metabolit	[1,4]
***Camellia sinensis* (L.) Kuntz**	Bitkisel	Sekonder metabolit	[1,17]
***Capsicum annuum* L.**	Bitkisel	Sekonder metabolit	[13,17]

Tablo 1 (*devamı*). Doğal kaynaklı ergojenik destekler [1,3,4,13-17]

Doğal Kaynaklı Ergojenik Destekler	**Kaynağı**	**Metabolit Türevi**	**Literatür**
***Citrus aurantium* L.**	Bitkisel	Sekonder metabolit	[14]
***Cola acuminata* (P. Beauv.) Schott & Endl.**	Bitkisel	Sekonder metabolit	[15]
***Cordyceps sinensis* L.**	Bitkisel	Sekonder metabolit	[13,15,17]
***Crocus sativus* L.**	Bitkisel	Sekonder metabolit	[17]
***Curcuma longa* L.**	Bitkisel	Sekonder metabolit	[17]
C*ystoseira canariensis* Sauv.	Bitkisel	Sekonder metabolit	[13]
***Dioscorea villosa* L.**	Bitkisel	Sekonder metabolit	[15]
***Eleutherococcus senticosus* (Rupr. Et Maxim) Maxim.**	Bitkisel	Sekonder metabolit	[15]
***Ephedra sinica* Stapf.**	Bitkisel	Sekonder metabolit	[15-17]
***Eurycoma longifolia* Jack**	Bitkisel	Sekonder metabolit	[16]
***Ginkgo biloba* L.**	Bitkisel	Sekonder metabolit	[13,17]
Glutamin	Bitkisel	Primer metabolit	[1,3,4]
***Hypericum perforatum* L.**	Bitkisel	Sekonder metabolit	[13]
***Ilex paraguayensis* St.-Hil.**	Bitkisel	Sekonder metabolit	[16,17]
Kafein	Bitkisel	Sekonder metabolit	[1,4,14-17]
Karnitin	Hayvansal	Sekonder metabolit	[1,3,4]
Kreatin/Kreatin monohidrat	Hayvansal	Sekonder metabolit	[1,3,4,14]
***Myrtus comminus* L.**	Bitkisel	Sekonder metabolit	[17]
***Oryza sativa* L.**	Bitkisel	Sekonder metabolit	[15]
***Panax ginseng* C.A.Mey.**	Bitkisel	Sekonder metabolit	[1,4,13-17]
***Panax japonicus* (T. Nees) C.A.Mey.**	Bitkisel	Sekonder metabolit	[16]
***Panax quinquefolia* L.**	Bitkisel	Sekonder metabolit	[16]
***Paullinia cupana* Mart.**	Bitkisel	Sekonder metabolit	[17]
***Pausinystalia yohimbe* (K. Schum.)**	Bitkisel	Sekonder metabolit	[15]
***Pfaffia paniculata* (Mart.) Kuntze.**	Bitkisel	Sekonder metabolit	[15]
***Piper methysticum* Forst.**	Bitkisel	Sekonder metabolit	[13]
Ptychopetalum olacoides Benth.	Bitkisel	Sekonder metabolit	[15]
***Rhamnus frangula* L.**	Bitkisel	Sekonder metabolit	[16]
***Rhodiola rosea* L.**	Bitkisel	Sekonder metabolit	[13,15,17]
***Salix alba* L.**	Bitkisel	Sekonder metabolit	[17]
***Schisandra chinesis* (Turcz.) Baill.**	Bitkisel	Sekonder metabolit	[15,16]
***Serenoa repens* (W. Bartram) Small.**	Bitkisel	Sekonder metabolit	[15]
Sitrülin/Sitrülin malat	Bitkisel	Sekonder metabolit	[3,4,14]

Tablo 1 (*devamı*). Doğal kaynaklı ergojenik destekler [1,3,4,13-17]

Doğal Kaynaklı Ergojenik Destekler	Kaynağı	Metabolit Türevi	Literatür
***Smilax officinalis* Kunth**	Bitkisel	Sekonder metabolit	[15]
Taurin	Hayvansal	Primer metabolit	[1]
***Tribulus terrestris* L.**	Bitkisel	Sekonder metabolit	[1,13,15,17]
***Trigonella foenum-graecum* L.**	Bitkisel	Sekonder metabolit	[17]
***Urtica dioica* L.**	Bitkisel	Sekonder metabolit	[16]
***Withania somnifera* Dun.**	Bitkisel	Sekonder metabolit	[14,15]
***Zingiber officinale* Roscoe**	Bitkisel	Sekonder metabolit	[14,17]

Aşağıda sporcular tarafından en sık kullanılan doğal kaynaklı ergojenik destekler hakkında detaylı bilgi verilmiştir:

Kafein

Camellia sinensis L. (çay), *Coffea arabica* L.(kahve), *Centella asiatica* (L.) Urb. (Halik)/ *Gotu kola* (kola meyvesi) ve *Theobroma cacao* L. (kakao) bitkilerinde bulunan kafein (1,3,7-trimetilksantin) teofilin, teobromin ve paraksantin moleküllerinin ksantin ile bir araya gelmesiyle oluşan bir bileşiktir. Oral yolla alınan kafein formunun neredeyse tamamı hızla emilerek yaklaşık 1 saat içerisinde dolaşıma karışmakta ve yüksek plazma konsantrasyonuna ulaşmaktadır. Fakat bu süre bireysel farklılıklardan (yaş, cinsiyet, vücut kompozisyonu, organ fonksiyonu vb.) etkilenebilmektedir [3,18-25]. Kafeinin sağlık üzerindeki olumsuz etkileri minimum düzeydedir ve bu nedenle dünyada en çok tüketilen maddelerden biri olarak kabul görmektedir. Kafein özellikle sporcuların ergojenik destek olarak sıkça kullandığı bir kaynaktır. IOC, 2004 yılında kafeini yasaklı maddeler listesinden çıkarmıştır. Bu nedenle sporcular, kafein formlarını istedikleri miktarda ve formda tüketebilmektedir. Her ne kadar sporcular tarafından sıklıkla tüketilen kafein kaynağı kahve olsa da farklı formlarda ergojenik desteklerin bulunduğu da bilinmektedir. Ayrıca kapsül formunda alınan kafeinin diğer kafein formlarına kıyasla ergojenik etkilerinin daha hızlı olduğu kayıtlıdır [3,18-25].

Kafeinin egzersiz öncesinde tüketiminin, plazmadaki beta (β) endorfin seviyesini arttırarak yorgunluk ve ağrı hissinin azalmasına neden olduğu bildirilmiştir [21,24-29]. Bununla birlikte kafeinin vücuttaki en önemli etki mekanizmasının önemli bir nörotransmitter olan adenozin aracılığıyla gerçekleştiği bilinmektedir. Adenozin, nöron hücrelerindeki adenozin reseptörlerine bağlanarak, santral sinir sisteminden nörotransmitter salınımını engellemekte, böylece vücudun uyarılmasını azaltmaktadır. Kafein, adenozine benzerliğinden dolayı vücutta adenozin gibi davranmakta ve yorgunluğu geciktirmektedir. Fakat yüksek dozlarda tüketilen kafein ilerleyen süreçlerde bağlandığı adenozin reseptörlerinin yaklaşık %50'sini bloke ederek kafeinin egzersiz sırasında ergojenik etkisini azaltabilmektedir [19-24].

Sporcularda kafein tüketimi ve optimal doz arasındaki ilişki, belirsizliğini korumaktadır. Literatürdeki çalışmalar yaklaşık 3-9 mg/kg kafein tüketiminin iyi bir ergojenik destek için yeterli olduğunu, 9 mg/kg veya daha yüksek dozun ise yan etkiler ortaya çıkarabileceğini bildirilmektedir. Ayrıca kafein dozunun, kişinin tüketim sıklığına, egzersiz türüne, kas kasılma biçimine ve tüketilen kafein formuna bağlı olarak değişebileceği vurgulanmaktadır [23-28]. Kafeinin tek seferde yüksek dozlarda tüketilmesi halinde vücutta farklı yan etkiler görülebilmektedir. "Kafeizm" olarak nitelendirilen bu durumda taşikardi, bulantı, kusma, uykusuzluk, hiperaktivite, tedirginlik, huzursuzluk hali ve kas krampları/yıkımları ortaya çıkabilmektedir [23-28].

Yetişkinlerde yaklaşık 5-10 g/gün kafein tüketiminin kafeizme neden olabileceği bildirilmiştir. Bu nedenle tek seferde yüksek dozlarda kafein alımının yerine önerilen dozların egzersiz türüne ve zamanına göre gün içine yayılarak tüketilmesi önerilmektedir [23-28].

Kreatin

Kreatin; metiyonin, arjinin, glisin ve diğer aminoasitler aracılığıyla başta karaciğer olmak üzere böbrek ve pankreasta sentezlenebilen, başlıca et ve balık ürünleri ile alınabilen veya takviye formu ile vücuda sağlanabilen, esansiyel olmayan guanidin türevi bir bileşiktir. Kreatinin büyük çoğunluğu (%95) vücutta iskelet kaslarında depo edilmektedir. Vücutta depo edilen miktarı yaklaşık 120 g olmasına rağmen günlük aktivitelerde kullanılan miktarı 2 g civarındadır. İskelet kaslarındaki kreatin miktarının optimal düzeyde olması yüksek miktarda fosfokreatin depolarına katkı sağlamaktadır. Bu durum yapılan egzersizin şiddeti ile birlikte ATP (Adenozin trifosfat) hidrolizi hızının belirlenmesine katkı sağladığından önem arz etmektedir [1,3,5,20,29-33].

Kreatin, sporcu performansı üzerindeki olumlu etkileri ve IOC tarafından anti-doping listesinde yer almasından dolayı sporcular tarafından sıkça kullanılan ergojenik desteklerden biridir. Kreatin takviyesi, kısa süreli, yüksek yoğunluklu egzersizlerde, güç ve etkinliğin yanı sıra yağsız vücut kütlesini de artırmaktadır. Bu özelliği nedeniyle en yüksek kullanımın güreşçiler tarafından olduğu bilinmektedir [1,20,29-33].

Sporcular tarafından sıklıkla başvurulan kreatin desteği preparat şeklindedir [1]. Kreatin preparatlarının kısa süreli ve güvenli dozlarda kullanımının vücutta olası bir yan etkiye neden olmadığı, egzersiz sonrası vücudun toparlanma sürecini hızlandırdığı ve vücut ısısının düzenlenmesine katkı sağladığı bildirilmiştir [20,30-33]. Karaciğer ve böbrek rahatsızlığı olan kişilerde kreatin preparatlarının uzun süreli kullanımı konusunda dikkatli olunması gerektiği belirtilmiştir. Ayrıca kreatin kullanımının vücut sıvısının ozmotik etkisi ile kas içine geçebileceği ve bu durumun dehidratasyon riskine neden olabileceği saptanmıştır. Bu nedenle kreatin takviyesi kullanan sporcuların bol sıvı tüketmesi önerilmektedir [29-33].

Piyasada çeşitli formlarda ve kombinasyonlarda bulunan kreatinin, WADA, IOC veya Amerikan Kolej Sporları Kurumu (NCAA) tarafından sporcular için kullanımı yasaklanmamıştır. Fakat kısa süreli kreatin kullanımı güvenli ve önemli yan etkiler olmadan kabul edilmektedir. Ancak uzun süreli çalışmaların sayısı sınırlı olduğundan dikkatli olunmalıdır. Ergojenik bir yardımcı olarak kreatin takviyesinin dozu, kullanım sıklığı ve süresine ilişkin öneriler çeşitlilik göstermektedir. Fakat literatürdeki çalışmalar değerlendirildiğinde 0,03-5 g/gün kreatin takviyesinin sporcular üzerinde potansiyel olumlu etki gösterebileceği belirlenmiştir [29-33].

Ephedra sinica Stapf.

Ephedraceae familyasının üyesi olan *Ephedra* (Deniz üzümü) türlerinde bulunan Efedrin, periferal ve santral sinir sistemi üzerine etkileri olan önemli bir alkoloittir. Efedrin başta olmak üzere *Ephedra* türlerine ait diğer alkoloitlerin (norefedrin, psödoefedrin, norpsödoefedrin vb.) de ergojenik özelliklere sahip güçlü farmakolojik ajanlar olduğu kabul edilmektedir [5,19,20,34].

Efedrinin sporcularda kas performansını arttırdığı, egzersiz öncesinde sporcuların ısınma süresini kısalttığı ve daha atletik bir form kazanmalarını kolaylaştırdığı bilinmektedir. Sporcular üzerinde olumlu etkilerinin yanı sıra baş dönmesi, uykusuzluk, halsizlik, taşikardi, iştahsızlık ve anksiyete gibi pek çok olumsuz etkiye de neden olabilmektedir. Bununla birlikte vücuttaki toksik etkilerinden dolayı Amerikan İlaç ve Gıda Dairesi (FDA) tarafından 2004 yılında *Ephedra* içeren ürünlerin kullanımı yasaklanmıştır. Ayrıca WADA tarafından efedrin ve türevleri uyarıcı etkileri nedeniyle doping maddesi olarak kabul edilmekte olup spor müsabakalarında kullanımı yasaklıdır. Fakat *Ephedra*'nın farklı bitkisel ürünlerle kombinasyonları piyasada satışa sunulmaya devam etmektedir [5,19,20,34].

Glutamin

Glutamin, başlıca plazma ve kas dokusunda serbest halde bulunan, vücuttaki en yaygın amino asittir. Vücutta serbest amino asit deposunun yaklaşık %50'sini oluşturmaktadır. İskelet kasları (%75) ve karaciğerde (%25) depo edilmektedir. İskelet kaslarındaki başlıca depo formu bağırsak epitelinin ihtiyacının doğrudan karşılanmasına büyük ölçüde katkı sağladığından ayrıca önem taşımaktadır [1,3,5,35-37].

Glutaminin vücutta pek çok fizyolojik etkisi bulunmaktadır. Vücuttaki başlıca etkisi yorgunluk mekanizması üzerinedir. Glutaminin bu etkisi; Krebs döngüsündeki anaplerotik tepkimeler ve

glukoneogenez, glukogenezin uyarılması, amonyağın vücutta toksik etkilerinin azaltılması, glutatyon sentezinin uyarılması ve antioksidan etki yolakları sayesinde gerçekleşmektedir. Glutaminin yorgunluk mekanizması üzerindeki etkilerinin yanı sıra bağışıklık sistemini güçlendirdiği, yara iyileşme sürecini hızlandırdığı, vücuda enerji sağladığı, protein sentezini düzenlediği ve nitrojenin taşınmasını kolaylaştırdığı bilinmektedir. Bu özellikleri nedeniyle glutamin sporcular tarafından kullanılan ergojenik destekler arasında önemli bir yer tutmaktadır [1,3,5,35-37].

Sporcular, glutamini sıklıkla potansiyel etkileri daha fazla olan L-glutamin formuyla kullanmaktadır. Glutamin, IOC başta olmak üzere çeşitli spor komiteleri tarafından "çok az etkililik kanıtı olan" veya "hiç etkisi olmayan" takviye olarak kabul edilmiştir. Bu nedenle glutamin ve sporcu performansı ile ilgili çalışmaların sayısının arttırılması gerektiği vurgulanmaktadır [1,3,5,35-37].

Arjinin

Arjinin, vücutta sitrülin ve glutamat başta olmak üzere bazı proteinlerin yıkımı sonucunda ortaya çıkan, doğal yolla üretilemeyen, yarı esansiyel bir amino asittir. Katabolizma sonucu ortaya çıkan bir ürün olmasının yanı sıra et ve süt ürünlerinin tüketilmesi ile de vücuda alınabilmektedir. Arjininin vücutta protein sentezi, önemli bir vazodilatör olan nitrik oksit (NO) ve kreatin üretimi, üre sentezi, poliaminler ve sitrülin oluşumu, büyüme hormonunun (GH) salgılanması, bağışıklıkta önemli bir oligopeptit olan tuftsin sentezi ve antidiüretik hormonunun (ADH) yapısına katılması gibi pek çok önemli görevi bulunmaktadır [1,3,5].

Arjinin, ergojenik etkileri nedeniyle sporcular tarafından sıklıkla kullanılmaktadır. Arjinin vücutta NO salınımını arttırarak kaslarda vazodilatasyonu sağlamaktadır. Böylelikle substrat değişim hızını arttırarak kaslara oksijen ve besin maddesi ulaşmasını kolaylaştırmaktadır. Bu özelliği nedeniyle sporcularda arjinin takviyesinin, esansiyel aminoasitler ile birlikte alımının kaslarda protein sentezini arttırdığı bildirilmiştir. Bununla birlikte arjinin takviyesi kaslarda laktat ve amonyak birikiminin azalmasını sağlayarak kas yorgunluğunu azaltmaktadır [1,3,5].

Literatürdeki çalışmaların sonuçları değerlendirildiğinde, 0,075 g/gün veya 6 g/gün arjinin takviyesinin fiziksel performansı ve NO sentezini artırmadığı fakat arjinin için henüz tam olarak belirlenmiş bir doz önerisi olmadığı görülmektedir. Bu nedenle özellikle yüksek dozlarda ve uzun süreli kullanımının tekrar değerlendirilmesi gerekmektedir [1,3,5,38].

Taurin

Taurin, insanlarda hücre içinde en fazla bulunan kükürtlü aminoasittir. Başlıca deniz ürünleri ve et ürünleri olmak üzere hayvansal kaynaklardan sağlanmaktadır. Vücutta B6 vitamini öncülüğünde esas olarak beyin ve karaciğerde sistein ve metiyonin metabolizmasından sentezlenmektedir [1,5].

Taurinin vücutta hücre içinde ozmoregülasyonun sağlanması, merkezi sinir sisteminde kalsiyum (Ca^{+2}) artışının azaltılıp hücrelerin hipoksiden korunması, antioksidan etkisiyle lipit peroksidasyonun önlenmesi, safra akışının ve üretiminin arttırılıp ateroskleroz ve kolestazın önlenmesi, kan basıncının düşürülmesi, retinanın gelişmesi ve işlevini sürdürmesi, endotel fonksiyonun ve membran stabilizasyonunun düzenlenmesi gibi geniş bir görev yelpazesi bulunmaktadır [1,5,39,40].

Taurin vücuttaki önemli etkileri nedeniyle sporcular tarafından sıklıkla başvurulan ergojenik destekler arasındadır. Özellikle iskelet kaslarında serbest radikal hasarını azaltarak oksitadif strese karşı savunma mekanizmasında önemli bir rol oynadığı bilinmektedir. Bununla birlikte taurinin egzersiz sırasında enerji kullanımını azalttığı, antienflamatuar sitokinlerin salınımını arttırarak kas dokusu hasarını minimize ettiği, egzersiz sonrasında kas hasarı onarım sürecini hızlandırarak performans adaptasyonunu kolaylaştırdığı, aeorobik ve anaerobik performansı iyileştirdiği saptanmıştır [1,5,39,40].

Sitrülin/Sitrülin Malat (CM)

Sitrülin ilk kez 1930 yılında WADA tarafından karpuz suyunda (*Citrullus vulgaris* Schrad) bulunan, esansiyel olmayan bir amino asittir. Karpuzun yanı sıra *Cucurbita moschata* Duschesne (balkabağı), *Cucumis sativus* L. (salatalık), *Cucumis melo* L. (kavun), *Ecballium elaterium* (L.) A. Rich. (acı kavun), *Cucurbita pepo* L. (kabak) gibi Cucurbitaceae familyası meyvelerinde bulunmaktadır [5,41,42].

Sitrülin eksojen olarak vücuda alınabileceği gibi vücutta doğal olarak da sentezlenebilmektedir. İnsanda sitrülin başlıca iki mekanizma aracılığıyla üretilmektedir. Bu mekanizmalardan ilkinde karaciğerde üre döngüsünde, ornitin ve karbomoil fosfattan; ikinci olarak enterositlerde, arjinin ve glutaminden sentezlenebilmektedir. Sitrülin, arjininin oksidasyonu sonucu NO'nun yan ürünü olarak ortaya çıkmaktadır. Bu nedenle iyi bir vazoproteksiyon molekülüdür [5,41,42].

Sitrülin vücutta NO üretim miktarını arttırarak besin öğelerinin taşınmasını kolaylaştırmaktadır. Bunun yanı sıra sitrülin, vücutta yorgunluk ile ilişkilendirilen kan laktat düzeylerinin düşürülmesine yardımcı olur. Ayrıca arjinin, sitrülin aracılığıyla NO'ya dönüşerek kan basıncının düşürülmesine katkı sağlar. Bu özellikleri nedeniyle sporcularda sıkça başvurulan ergojenik destekler arasında yer almaktadır [1,5,41,42].

Sitrülinin, malat ile kombinasyonunun da sporcularda potansiyel etkilere sahip olduğu bilinmektedir. Malat önemli bir trikarboksilik asit döngüsü ara maddesi olduğundan, bu durum kas fonksiyonundaki muhtemel olumlu etkileri açıklayabilmektedir. Fakat bu faydaların yalnızca sitrüline atfedilip atfedilemeyeceğini ve sitrülinin aerobik ve anaerobik performansta hangi rolü oynayabileceğini belirleyebilmek adına daha fazla çalışmaya ihtiyaç duyulmaktadır [41,42].

Beta (β) Alanin

Beta alanin vücutta başlıca iskelet kasında bulunan esansiyel olmayan bir amino asittir. Diyetle kırmızı et, tavuk ve balık ile vücuda alınabildiği gibi karaciğerde urasil yıkımının nihai bir metaboliti olarak da ortaya çıkabilmektedir [1,5,20].

Son yıllarda beta alanin sporcular tarafından büyük ilgi görmeye başlamıştır. Fakat beta alaninin vücuttaki ergojenik etkileri tek başına sınırlıdır. Beta alanin ancak histidin ile birleşerek iskelet kasında "karnozin (β-alanil-L-histidin)" adı verilen intramusküler tamponu oluşturduğunda esas olarak ergojenik etkiyi göstermektedir. Karnozinin yüksek yoğunluklu egzersiz performansını iyileştirdiği ve kaslardaki laktik asit düzeylerini düşürerek yorgunluk eşiğini azalttığı bilinmektedir. Bununla birlikte antioksidan etkisi nedeniyle oksitadif stresi azaltarak egzersiz sırası ve sonrasında toparlanmayı kolaylaştırmaktadır [1,5,20,43-46].

Literatürdeki çalışmalarda beta alaninin ergojenik destek olarak 24 haftaya kadar 3,2-6,4 g/gün dozda kullanımının vücutta potansiyel olumlu etkilerini gösterebileceği bildirilmiştir. Ayrıca ciltte kaşıntı veya karıncalanma hissi (parestezi) gibi yan etkilerin gözlemlenebileceği belirtilmiştir. Paresteziden kaçınmak için 3,2-6,4 g/gün dozunun 3-4 saatte bir eşit dozlarda (0,8-1,6 g/kez) alınması önerilmektedir [1,5,20,43-46].

L-Karnitin

İlk olarak 1905 yılında Gulewitcsh ve Krimberg adlı iki Rus bilim insanı tarafından kas dokusundan izole edilen, Latince "carnis (et)" kelimesinden köken alan amino asit türevi bir maddedir. L-karnitin un kurdunda (*Tenebrio molitor*) vitamin gibi davrandığı için önceleri "BT vitamini" olarak adlandırılmıştır. Günümüzde de yaygın olarak BT vitamini olarak bilinmesine rağmen bu adlandırma genel anlamda yanlıştır. Karnitin, Kaneko ve Yoshida tarafından D ve L olarak başlıca iki formda sınıflandırılmıştır. Esansiyel özelliğe sahip olan ve doğada serbest bir formda bulunan aktif formu L olarak adlandırılırken, D formu kimyasal olarak üretilmektedir [1,3,5,47-50].

L-karnitin (3-hidroksi-4-N-trimetilaminobütirat) vücutta farklı dokularda sentezlenebilen yağ asitlerinin mitokondriyal oksidasyonunda yaşamsal bir kofaktör görevi yapan doğal bir amonyum bileşiğidir. L-karnitinin vücutta sentezlenebilmesi için esansiyel amino asit olan lizin ve metiyonine ihtiyaç duyulmaktadır. Bununla birlikte niasin, C vitamini, B6 vitamini, B12 vitamini ve demir yetersizliğinde de L-karnitinin işlevinde bozukluklar ortaya çıktığı belirlenmiştir. L-karnitin vücutta endojen olarak sentezlenebildiği gibi (%25) diyet yolu ile (%75) eksojen olarak da vücuda alınabilmektedir. Başlıca diyet kaynakları; kırmızı et, balık, tavuk ve süt ürünleridir. Bununla birlikte eser miktarda meyve, sebze ve tahıllarda da bulunabilmektedir [1,3,5,47-50].

L-karnitin, uzun zincirli yağ asitlerinin sitoplazmadan mitokondri matriksine geçişini kolaylaştırmada görev almakta, glikoliz inhibisyonunu teşvik etmekte olup bu özelliği nedeniyle β-oksidasyon ve yağ oksidasyonu ile yakından ilişkilidir. Böylece beden kütle indeksini azaltarak kilo

vermeye yardımcı olmaktadır. Ayrıca L-karnitin kan laktat düzeylerini arttırarak kas ağrısı ve yorgunluğunu azaltmakta ve fiziksel performansı iyileştirmektedir. Bununla birlikte hücresel hasar belirteçlerini azaltarak serbest radikal oluşumunu indirgemektedir. Sporcularda 0,5-2 g/gün L-karnitin takviyesi güvenli olarak kabul edilmekte ve herhangi bir yan etki bildirilmemektedir [1,3,5,47-50].

Panax ginseng Meyer

"Kore ginsengi" olarak bilinen *Panax ginseng* C.A.Mey., Araliaceae familyası ve *Panax* cinsine ait olan bir bitkidir. İlk olarak Rus botanikçi C.A. Meyer tarafından kullanılan, Yunanca "pan (tüm)" ve "axos (ilaç)" kelimelerinden oluşan *Panax* ismini taşıyan bitki, günümüzde kökünün insana benzerliğinden dolayı Çince "insan" anlamına gelen "reshen" kelimesinden köken alan "ginseng" adıyla bilinmektedir [1,16,31].

Ginsengin, antik çağlarda gizemli bir ilaç olarak nitelendirildiği bilinmekle birlikte günümüzde bu nitelemenin karşılığı bilimsel kanıtlarla desteklenmiştir. Ginsengin, özellikle kök ve rizomlarının yapısındaki ginsenozitlerden dolayı antioksidan içeriğinin yüksek olduğu, yorgunluk, halsizlik ve stresi azalttığı bilinmektedir. Dünya'da en yaygın kullanılan ergojenik desteklerden biri olan Ginsengin, sporcularda kan laktat konsantrasyonlarını düşürdüğü, solunum fonksiyonlarını ve fiziksel performansı iyileştirdiği, lipid peroksidasyonunu inhibe ettiği, kortizol salınımını uyararak uykusuzluğu azalttığı yapılan çalışmalarla ortaya konmuştur. Bu etkileri nedeniyle *P. ginseng* C.A.Mey. (Asya veya Kore Ginsengi), *P. quinquefolius* L. (Amerikan Ginsengi) ve *Panax notoginseng* (Burkill) F.H.Chen (Çin Ginsengi) fonksiyonel olarak yaygın kullanılan türleridir [1,16,31,51-54].

Ginsengin çay olarak tüketilmesi durumunda günde 3-4 bardak; kapsül veya tablet formunda tüketilmesi halinde ise 2 defa/gün 1-2 adet şeklinde tüketiminin sporcular üzerinde potansiyel olumlu etki gösterebileceği bildirilmiştir. Ayrıca sporcunun fiziksel ve psikolojik performansını destekleyebilmek adına 2x100 mg/gün tüketim önerilmektedir [1].

Ginseng kullanımı, yasaklı maddeler listesinde olmayıp sporcular tarafından kullanılma durumu serum ve idrarda ginsenozitler ve metabolitlerinin belirlenmesi ile tespit edilebilmektedir [1,23,31,51-54].

Eurycoma longifolia Jack

Eurycoma longifolia Jack, Malezya'da "Pasak Bumi" veya "Tongkat Ali" adlarıyla bilinen Simarubaceae familyasına ait bir bitkidir. Başlıca yetiştiği yerler Malezya, Endonezya, Tayland, Myanmar, Laos ve Kamboçya'dır. Malezya'da sağlık üzerine olan olumlu etkilerinden dolayı halk arasında "Malezya Ginsengi" olarak da adlandırılmaktadır [34,55].

E. longifolia'nın antibakteriyel, antifungal, antienflamatuar, antimalaryal, antiparazitik, antioksidan ve antiülser etkileri olduğu bilinmektedir. Bununla birlikte sporcuların son yıllarda tercih ettiği ergojenik destekler arasında yer almaktadır. *E. longifolia'nın* sporcularda yüksek dozda ve uzun süreli kullanımının fiziksel performansı ve dayanıklılığı arttırdığı bildirilmiştir. Ayrıca egzersiz sonrası toparlanmayı kolaylaştırdığı ve sporcularda gelişebilecek aşırı antrenman sendromunu önlediği bilinmektedir. Özellikle testosteron seviyelerinde belirgin artışa yol açarak stres durumu ve uyku bozukluğu gibi psikolojik durumları iyileştirdiği saptanmıştır [56-58].

Zingiber officinale Roscoe

Zingiber officinale Roscoe, diğer bir adıyla zencefil, Zingiberaceae familyasına ait bir bitkidir. Başta Güney Asya olmak üzere pek çok bölgede doğal yayılış göstermektedir [31,59].

Z. officinale bitkisinin rizomlarından elde edilen zencefil, çeşitli hastalıkları tedavi etmek için yüzyıllardır kullanılmaktadır. Günümüzde ise bitkinin analjezik ve antienflamatuar etkileri olduğu bilinmektedir. Fakat sporculardaki potansiyel etki mekanizması tam olarak bilinmemektedir. Zencefilin selektif olmayan siklooksijenaz (COX) enzimlerini aktive ederek, prostaglandin ve lökotrien gibi proenflamatuar sitokinlerin sentezini inhibe ederek egzersiz sonrasında ağrıyı hafiflettiği bilinmektedir. Fakat COX enzimlerinin inhibisyonuna bağlı olarak egzersiz sırasında kas protein sentezindeki artışı köreltebileceği bildirilmiştir [60].

Bitki her ne kadar FDA tarafından "güvenli gıda" olarak kabul edilse de uzun süreli ve fazla miktarda tüketiminin gastrik epitel hücrelerinin irritasyonu, hiponatremi riski, bağ ve kemik dokusunun

defekasyonu, nefrotoksisite ve egzersize verilen inflamatuar yanıtın azalması gibi çeşitli advers etkilere yol açabileceği bilinmektedir. Sporcuların zencefil tüketimi ve egzersiz performansı arasındaki ilişkiyi inceleyen çalışmaların sonucunda herhangi bir advers etki bildirilmemiştir. Fakat tüm bu çalışmalarda sporcuların bitkiyi yüksek dozda tüketmemesi gerektiğine dair ortak uyarılar bulunmaktadır [59-64].

Curcuma longa L.

Curcuma longa L. Zingiberaceae familyasına ait "Zerdeçal, Zerdeçöp, Safran kökü, Sarıboya, Hint safranı ve Turmerik" gibi adlarla bilinen bir bitkidir. Dünyada başlıca yetiştiği ülkeler Çin ve Hindistan'dır. İçeriğinde önemli pek çok polifenolik bileşik bulundurmaktadır [31,63,64].

Sporcularda COX sinyal yolağını modüle ederek prostaglandin sentezini inhibe ettiği ve egzersiz sonrasında kas hasarını ve ağrıyı hafifleterek analjezik etki oluşturduğu bilinmektedir. Ayrıca *C. longa* 'dan elde edilen kurkumin maddesinin TNF-α (tümör nekroz faktörü-alfa), IL-6 (interlökin-6) ve IL-8 (interlökin-8) başta olmak üzere proinflamatuar sitokinlerin üretimini azaltarak nonsteroidal antienflamatuar ilaçlara (NSAİİ) alternatif olarak kullanılabileceği bildirilmiştir. Bununla birlikte kurkuminin lipit peroksidasyonunu ve hücresel hasarı önleyerek sporcu performansını desteklediği bilinmektedir. Egzersizde potansiyel olumlu etkilerin görülebilmesi için minimum doz konusunda literatürde bir fikir birliği bulunmamaktadır. Fakat 0,01-6 g/gün arasında kurkumin kullanımının yangı, oksitadif stres, kas hasarı ve ağrının azaltılması için ideal doz aralığı olduğu belirtilmektedir [65-68].

Tribulus terrestris L.

Tribulus terrestris L., ülkemizde "Demir dikeni" adıyla bilinen, Zygophylaceae familyasına ait tek yıllık bir bitkidir. Akdeniz bölgesine özgü olmakla beraber, Türkiye, Çin, Japonya, Kore ve bazı Afrika ülkelerinde yaygın olarak yetişmektedir [69,70].

T. terrestris, yapısında saponinler, flavonoitler, glikozitler, fitosteroller ve alkaloitler gibi önemli bileşenleri barındırmaktadır. Özellikle yapısındaki saponinlerin testosteron hormonunun salgılanmasını arttırarak iskelet kaslarındaki enflamasyonu ve oksitadif hasarı azaltarak antienflamatuvar ve antioksidan etki oluşturduğu bilinmektedir. Bunun sonucunda kas performansını desteklediği ve gücü arttırdığı yapılan çalışmalarla desteklenmiştir. Fakat *T. terrestris* kas performansı üzerindeki olumlu etkilerinin yanı sıra testosteron seviyelerinde artışa neden olup, doping kontrol testinde pozitif bir sonuca yol açabileceğinden dikkatli kullanılmalıdır. Bu nedenle uygun dozda ve sürede kullanımı sporcu sağlığı ve performansı açısından önem taşımaktadır [69-73].

Beta vulgaris L.

Beta vulgaris L., (Pancar) Amaranthaceae familyasına ait, besin açısından zengin önemli sebzelerden biri olup sarıdan kırmızıya kadar farklı renk varyetelerine sahip otsu bir bitkidir. Pancarın anavatanının Orta Doğu olduğu ve ABD, Avrupa ve Asya'ya buradan yayıldığı bilinmektedir [74]. Fitokimyasal içeriği polifenoller, betalainler, kateşin, epikateşin, C vitamini ve karotenoitler bakımından zengin olan pancar, yüksek antioksidan ve antienflamatuar etkiye sahip bir ergojenik destektir. Özellikle son yıllarda pancar suyu tüketiminin sporcularda vazodilatasyonu teşvik etme, hücresel solunumu düzenleme ve kas performansını iyileştirme özellikleri sayesinde sıklıkla kullanılmaya başlayan bir ergojenik destek olduğu bilinmektedir. Pancar suyunun içerisinde yer alan nitratın vücuttaki NO miktarını arttırarak dayanıklılık performansını iyileştirip metabolik adaptasyonu kolaylaştırdığı gözlemlenmiştir [75-77]. Kısa süreli pancar tüketiminin sporcularda ergojenik etki gösterdiğini fakat yüksek dozda ve uzun süreli kullanımda farklı yan etkiler ortaya çıkarabileceğini bildirmektedir. Bu nedenle pancarın vücuttaki potansiyel etkilerini netleştirmek ve dozunu belirlemek için daha fazla çalışmaya ihtiyaç duyulmaktadır [75-77].

Eski çağlardan beri bitkiler, pek çok rahatsızlığın önlenmesi ve tedavisi amacıyla kullanılagelmiştir. Fakat günümüzde yapılmış birçok çalışma ile bazı bitkilerin tedavilerinin etkili olmayabileceği hatta aksine güvenli olmayıp olumsuz etkilere yol açabileceği kanıtlanmıştır.

Literatürdeki çalışmalar değerlendirildiğinde günümüzde ergojenik amaçla kullanılan pek çok destek olduğu sonucuna varılmıştır. Bu destekler arasında en sık kullanılanların "Kafein, Kreatin, *Ephedra sinica* Stapf., Glutamin, Arjinin, Taurin, Sitrülin/Sitrülin Malat, Beta (*β*) Alanin, L-Karnitin, *Panax ginseng* Meyer, *Eurycoma longifolia* Jack, *Zingiber officinale* Roscoe, *Curcuma longa* L.,

Tribulus terrestris L. ve *Beta vulgaris* L." olduğu saptanmıştır. Bu çalışmalar sporcuların bazı ergojenik destekler arasında daha zararsız olduğunu düşündükleri doğal kaynaklı ürünlere yönelmesini destekler niteliktedir. Fakat son yıllarda sporcuların sıklıkla kullandığı bu ürünler bilimsel bir perspektifle değerlendirildiğinde, doğru ürünün uygun dozda, uygun formülasyonda ve doğru zamanda bilinçli bir şekilde kullanılmasının, sporcu sağlığı ve performansı açısından oldukça önem taşıdığı görülmektedir. Bu ürünler içeriklerindeki biyoaktif bileşenlerden dolayı pek çok farmakolojik aktiviteye sahiptir. Bu nedenle hangi amaçla kullanılacak olurlarsa olsun, sağlık profesyonellerine (hekim/eczacı) ve farmakope gibi önemli kaynaklara başvurularak kullanılmalıdır. Farmakope ve monograflar ile güncel literatür, ergojenik amaçla doğal kaynaklı destekleri kullanan sporcular için önemli bir rehber niteliği taşımakta olup burada yer almayan bu desteklerin ise yararlı ve toksik doz düzeylerinin araştırılması için kapı aralayacaktır.

Sporcular tarafından sıklıkla kullanılan doğal kaynaklı ergojenik desteklerin farmakopelerde kayıtlı olma durumu değerlendirildiğinde; bu desteklerin birçoğunun Türk Farmakopesi ve Avrupa Farmakopesinde kayıtlı olduğu fakat ergojenik yönüne vurgu yapacak herhangi bir bilgi bulunmadığı sonucuna varılmıştır. Bu nedenle endikasyonları, kontraendikasyonları, gıda-ilaç etkileşimleri, kullanım şekli ve dozaj formu, minimum/maksimum doz önerileri, bu desteklerin kullanımında ortaya çıkabilecek yan etkiler konusunda bilgi bulunmamaktadır. Literatürde yapılmış çalışmalar sonucunda elde edilen bu bilgilerin farmakope ve monograf kayıtlarında bulunması halinde, bunun sporcu sağlığı için bir yol haritası niteliği taşıyacağını düşünmekteyiz. Ayrıca literatürde doğal kaynaklı ergojenik destekler ile ilgili açıklığa kavuşturulması gereken konular olduğu göz ardı edilmemelidir. Bu nedenle doğal kaynaklı ergojenik desteklerin sporcu performansı üzerindeki etkisini belirleyebilmek adına daha fazla çalışmaya ihtiyaç duyulmaktadır.

YAZAR KATKILARI

Kavram: E.B., G.E.C., B.Ö.; Tasarım: E.B., G.E.C., B.Ö.; Denetim: E.B., G.E.C., B.Ö.; Kaynaklar: E.B.; Malzemeler: E.B.; Veri Toplama ve/veya İşleme: E.B., G.E.C., B.Ö.; Analiz ve/veya Yorumlama: E.B., G.E.C. B.Ö.; Literatür Taraması: E.B.; Makalenin Yazılması: E.B., G.E.C., B.Ö.; Kritik İnceleme: E.B., G.E.C., B.Ö.; 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., 47(3): 1170-1176, 2023
Doi: 10.33483/jfpau.1270767

SHORT COMMUNICATION / KISA BİLDİRİ

INTERACTION OF PTERIDOPHYTIC BIOACTIVE COMPOUNDS WITH FUNGAL DIHYDROFOLATE REDUCTASE ENZYME AS INHIBITOR

PTERİDOPİTİK BİYOAKTİF BİLEŞİKLERİN İNHİBİTÖR OLARAK MANTAR DİHİDROFOLAT REDÜKTAZ ENZİMİ İLE ETKİLEŞİMİ

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ABSTRACT

Objective: *Fungal infections which are relatively common mainly invades the body of an immunosuppressed patients and people undergoing therapy. These pathogens act through different pathways like the Dihydrofolate reductase (DHFR) has a role in the folate synthetic pathway which is responsible for DNA synthesis. Since the early ages herbal remedies were used and have been tested for treating these fungal infections. Previous studies have revealed the use of bioactive molecules of pteridophytes to demonstrate antifungal activity.*

Material and Method: *In the present study different pteridophytes were selected from available library which showed the presence of bioactive phytoconstituents. In-silico studies on DHFR target (PDB ID: 6DRS and PDB ID: 3QLW) was carried out using PyRx program (India) to determine the affinity of bioactive molecules against the fungal strain.*

Result and Discussion: *Molecular docking was performed with 11 bioactive molecules showing activity against the selected target proteins. So, we can conclude that the selected bioactive molecules are active against fungal strain and can be further investigated for both in-vivo and in-vitro studies.*

Keywords: *Dihydrofolate reductase (DHFR), fungal infection, molecular docking, pteridophytes*

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Submitted / Gönderilme : 25.03.2023
Accepted / Kabul : 31.07.2023
Published / Yayınlanma : 20.09.2023

ÖZ

Amaç: *Nispeten yaygın olan mantar enfeksiyonları esas olarak immün sistemi baskılanmış hastaların ve tedavi gören kişilerin vücudunu istila eder. Bu patojenler, DNA sentezinden sorumlu olan folat sentezinde rol oynayan dihidrofolat redüktaz (DHFR) gibi farklı yolaklar üzerinden etki gösterir. İlk çağlardan beri bitkisel ilaçlara bu mantar enfeksiyonlarını tedavi etmek için kullanılmış ve test edilmiştir. Önceki çalışmalar, antifungal aktiviteyi göstermek için biyoaktif pteridofit moleküllerinin kullanıldığını ortaya koymaktadır.*

Gereç ve Yöntem: *Bu çalışmada, biyoaktif fito bileşenlerinin varlığını gösteren mevcut kütüphaneden farklı pteridofitler seçilmiştir. Biyoaktif moleküllerin mantar suşuna karşı afinitesini belirlemek için Pyrx (Hindistan) programı kullanılarak, DHFR hedefi (PDB ID 6DRS ve PDB ID 3QLW) üzerine in-siliko çalışmalar gerçekleştirilmiştir.*

Sonuç ve Tartışma: *Seçilen hedef proteinlere karşı aktivite gösteren 11 biyoaktif molekül ile molekler yerleştirme çalışması gerçekleştirilmiştir. Buna göre, seçilen biyoaktif moleküllerin mantar suşuna karşı aktif olduğu ve hem in-vivo hem de in vitro çalışmalar için daha fazla araştırılabileceği sonucuna varılmıştır.*

Anahtar Kelimeler: *Dihidrofolat redüktaz (DHFR), mantar enfeksiyonu, moleküler yerleştirme, pteridofitler*

INTRODUCTION

In humans, fungal infections vary from topical mild rashes and itching to systemic diseases such as fungal pneumonia, meningitis, and bloodstream infections which can be fatal in nature [1]. Dermatophytes such as *Tinea capitis* or *Tinea corporis* are mainly responsible for topical infections [2] whereas Candida species like *Candida albicans* or *Candida glabrata* and Aspergillus species, viz, *Aspergillus flavus* are major examples of causing invasive fungal infections [3]. Currently, there are three classes of antifungal drugs that are used, viz, azoles, polyenes, and allyl amines [4]. These drugs act on various targets such as P-450 demethylase, squalene epoxidase, ornithine decarboxylase, alpha and beta tubulin, Dihydrofolate reductase (DHFR), etc. [3]. DHFR is an important target in cancer, microbial infections, malaria, tuberculosis, fungal infections etc. [5]. DHFR is involved in the folate synthetic pathway responsible for DNA synthesis which is initiated by the cellular uptake of folic acid through a specialized mechanism followed by the reduction of dihydrofolate to tetrahydrofolate (THF) in the presence of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) [3]. THF is a major coenzyme that serves as a carrier of one carbon unit for different enzymes during their interconversion between several oxidative states which is required for the biosynthesis of purines, methionine, and other important metabolites [6]. Various studies have been carried out using *in-silico* methods for the inhibition of DHFR (an important enzyme involved in the folate pathway) [3].

Development of resistance and relapse of disease are the major drawback of antifungal drugs. Therefore, there is a requirement to discover new antifungal therapies derived from plant sources having lesser side effects than conventional synthetic medicines [7]. In the current study, many Himalayan pteridophytes have been reported to have various bioactive molecules with appreciable antifungal activity [8,9]. The purpose of this study is to evaluate the selected compounds for antifungal potential against DHFR of Aspergillus flavus and *Candida albicans* using CADD (Computer Aided Drug Design).

MATERIAL AND METHOD

Various Himalayan pteridophytes were identified through literature search from different databases such as PubChem, PubMed, Google Scholar, ScienceDirect, etc. Among these, 20 were selected whose chemical constituents were disclosed based on GCMS analysis [10-12]. These selected pteridophytes provided a total of 180 bioactive compounds which were further explored for their fungal activity prediction. Additionally, *in-silico* studies were performed on the identified phytoconstituents.

Protein and Ligand Preparation

The foremost step in molecular docking is protein preparation which was done using the BIOVIA Discovery studio visualizer [13]. In this study, the target protein, Dihydrofolate Reductase, i.e., DHFR (PDB ID- 6DRS and PBD ID- 3QLW) were selected from Prot ein Data Bank (https://www.rcsb.org/). Protein preparation involves removing water molecules, heteroatoms, and ligands from the active site and further it was saved in .pdb format. Furthermore, all the 180 phytocompounds were retrieved from PubChem Database (https://pubchem.ncbi.nlm.nih.gov/). These ligands were converted into 3D structures using ChemDraw 16.0 [14] and their smiles were generated for docking studies.

Molecular Docking

Molecular docking is an effective tool for identifying the most appropriate binding site of the protein where the ligand fits energetically as well as geometrically [15]. It investigates important molecular events including ligand binding modes and intermolecular interactions of the protein-ligand complex [16]. This study uses PyRx software to conduct docking studies. The selected 19 bioactive compounds were docked with DHFR to generate docking scores to predict the binding energies of the protein-ligand complex. The scoring function gives score based on the best docked ligand complex which is represented as a negative value in kcal/mol. The compounds with promising binding affinity are chosen for further analysis and visualization.

Visualization of Protein-Ligand Complex

Protein-ligand interactions are visualized and analysed via BIOVIA Discovery studio visualizer. Visualization of the docked protein-ligand model provides 2D and 3D structures of the complex with interacting bonds, bond category, bonding distance, and so on [17]. 2D structures display the various interacting amino acid residues bonded by hydrogen and hydrophobic bonds between the ligand and target protein [18] whereas 3D structures help to understand the molecular arrangement and how protein and ligand are bonded to each other.

RESULT AND DISCUSSION

Previous research unfolded the significance of DHFR activity in DNA synthesis and inhibition of DHFR is a well-established mechanism of action. To discover new potent antifungal agents, 180 phytoconstituents were identified on which docking studies were conducted against the target DHFR proteins. Molecular docking provides the binding affinities of the selected phytoconstituents. The results of the docking studies have been mentioned in Table 1.

Docking studies revealed that only 11 compounds (structures depicted in Figure1A) were recognized with very good binding affinity towards DHFR (PDB ID – 6DRS) of *Aspergillus flavus.* All these compounds depicted binding affinities even higher than the reference compound, i.e., 3-{[(3R)-7,9-diamino-3-methyl-2,3-dihydrofuro[2,3-*f*]quinazolin-4-yl]oxy}benzonitrile (-6.3kcal/mol), represented in Figure 1B and 1C. Furthermore, docking studies of the same compounds on DHFR (PDB ID-3QLW) of *Candida albicans* revealed that these 11 phytoconstituents showed good binding affinities as depicted in and among them, 6 phytoconstituents (PC-1, PC-2, PC-6, PC-8, PC-9, PC-11) exhibited exceptionally good binding affinities which are even higher than the reference compound, 5-[3-(2,5-dimethoxyphenyl) prop-1-yn-1-yl]-6-ethylpyrimidine-2,4-diamine with binding affinity -6.9kcal/mol, represented in Figure 1D and 1E. The docking results are stated in Table 1. Visualization of the compounds was carried out using BIOVIA Discovery studio visualizer for which results of 3D interactions are shown in Figure 2 and molecular interactions with amino acid residues are mentioned in Table 1 for both target proteins. The findings of this study can be applied to future research on several pathways for both *in-vitro* and *in-vivo* analysis against the fungus.

Table 1. Molecular docking results and amino acid interactions of phytoconstituents

Phyto-Consituents	Source	PDB-6DRS		PDB-3QLW	
		Docking Score (kcal/mol)	Amino Acid Interactions	Docking Score (kcal/mol)	Amino Acid Interactions
PC-1	*Blechnum orientale*	-7.4	TRP A:34, ILE A:10, ALA A:12, TYR A:162, VAL A:11, LEU A:32, ILE A:26, THR A:66, PHE A:44, LEU A:77, VAL A:70, ILE A:156	-7.3	ILE A:62, ILE A:112, ARG A:72, LEU A:69, PHE A:66, LYS A:37, PRO A:63, ILE A:33, PHE A:36, MET A:25,
PC-2	*Blechnum orientale*	-7.8	ASP A:40, VAL A:11, ILE A:26, GLY A:27, LEU A:32, THR A:31, SER A:69, THR A:66, GLY A:157, GLY A:158, TYR A:162, ILE A:156, ALA A:12, ILE A:10, PHE A:44	-7.8	PRO A:63, SER A:61, LEU A:69, MET A:25, ILE A:62, THR A:58, PHE A:36, ILE A:112, ILE A:33
PC-3	*Blechnum orientale*	-7.5	GLY A:27, GLY A:158, GLY A:157, ILE A:26, ALA A:12, TYR A:162, VAL A:11, ILE A:10, PHE A:44, ILE A:156, LEU A:32, ASP A:40, THR A:66, TRP A:34	-5.8	ILE B:135, ILE B:33, ILE B:112, TYR B:118, PHE B:36, ILE B:9, VAL B:10, GLE B:32, ALA B:11, MET B:25, TRP B:27, LEU B:29
PC-4	*Blechnum orientale*	-6.4	LEU A:77, THE A:44, ASP A:40, ILE A:10, LEU A:32, GLY A:158, GLY A:157, ILE A:156, THR A:66, TYR A:162, ALA A:12, VAL A:11, VAL A:70	-6.2	ILE B:33, PHE B:36, MET B:25, ILE B:112, THR B:58, LEU B:69, PRO B:63, ILE B:62, PHE B:66, ARG B:72, PRO B:70, LYS B:37
PC-5	*Calaguala*	-6.6	GLY A:157, GLY A:158, TYR A:162, ILE A:10, VAL A:11, PHE A:44, ALE A:12, LEU A:32, VAL A:70, LEU A:78, THR A:31, THR A:197, LEU A:77, GLY A:27, GLY A:30, ASP A:196, ILE A:156, THR A:66, ILE A:26	-6.3	GLU A:32, VAL A:10, ILE A:112, PHE A: 36, ILE A:33, PHE A:66, LEU A:69, LYS A:37, ARG A:72, PRO A:70
PC-6	*Drynoria quercifolia*	-7.5	ASP A:40, ALA A:12, TRP A:34, ILE A:26, LEU A:32, SER A:69, THR A:31, GLY A:30, THR A:66, GLY A:158, GLY A:157, PHR A:197, GLY A:27, TYR A:162, ILE A:156, VAL A:11, ILE A:10, PHE A:44	-7.2	VAL B:10, ILE B:9, PHE B:36, ILE B:62, LEU B:69, ILE B:33, LEU B:29, MET B:25, GLU B:32, TRP B:27, ALA B:11, TYR B:118
PC-7	*Drynoria quercifolia*	-7.0	THR A:197, GLY A:157, GLY A:27, TYR A:162, ILE A:156, PHE A:44, ASP A:40, ILE A:10, VAL A:11, ALA A:12, ILE A:26, LEU A:32, THR A:66, THR A:31, GLY A:30, SER A:69, GLY A:158, ALA A:159	-6.6	PHE A:36, ILE A:112, ILE A:33, PHE A:66, ILE A:62, MET A:25, PRO A:63
PC-8	*Drynoria quercifolia*	-6.9	GLU A:160, GLY A:158, THR A:197, SER A:169, THR A:66, LEU A:32, GLY A:27, ILE A:26, ASP A:196, GLY A:30, LYS A:65, ALA A:159	-7.7	GLU A:32, TRP A:27, MET A:25, ILE A:33, ARG A:72, LEU A:69, LYS A:37, PHE A:66, ALA A:11, ILE A:112, PHE A:36, ILE A:9, TYR A:118, VAL A:10
PC-9	*Pteris vitata*	-6.9	VAL A:11, ASP A:40, GLY A:157, TYR A:162, SER A:69, THR A:66, ILE A:26, LEU A:32, ALA A:12, TRP A:34, ILE A:156, PHE A:44	-7.4	PRO A:63, MET A:25, ILE A:62, ILE A:9, TYR A:118, VAL A:10, PHE A:36, GLU A:32, ILE A:112, THR A:58, LEU A:69
PC-10	*Equisetum arvense*	-6.4	ILE A:26, ALA A:12, VAL A:11, TRP A:34, ILE A:10, TYR A:162, ASP A:40, ILE A:156, LEU A:32, PHE A:44, GLY A:157, GLY A:158, THR A:66	-6.3	LEU A:69, PHE A:36, ILE A:112, THR A:58, MET A:25, ILE A:62, VAL A:10, ILE A:9, ALA A:11, GLU A:32, ILE A:33
PC-11	*Equisetum arvense*	-6.8	MET A:41, PHE A:44, ASP A:40, ALA A:12, LEU A:32, VAL A:11, TRP A:34, TYR A:162, ILE A:26, GLY A:157, GLY A:158, THR A:66, ILE A:156	-7.2	LYS A:24, SER A:61, THR A:58, ILE A:112, PHE A:36, LEU A:69, ARG A:72, LYS A:37, PHE A:66, ILE A:33, ILE A:62, PRO A:63, MET A:25

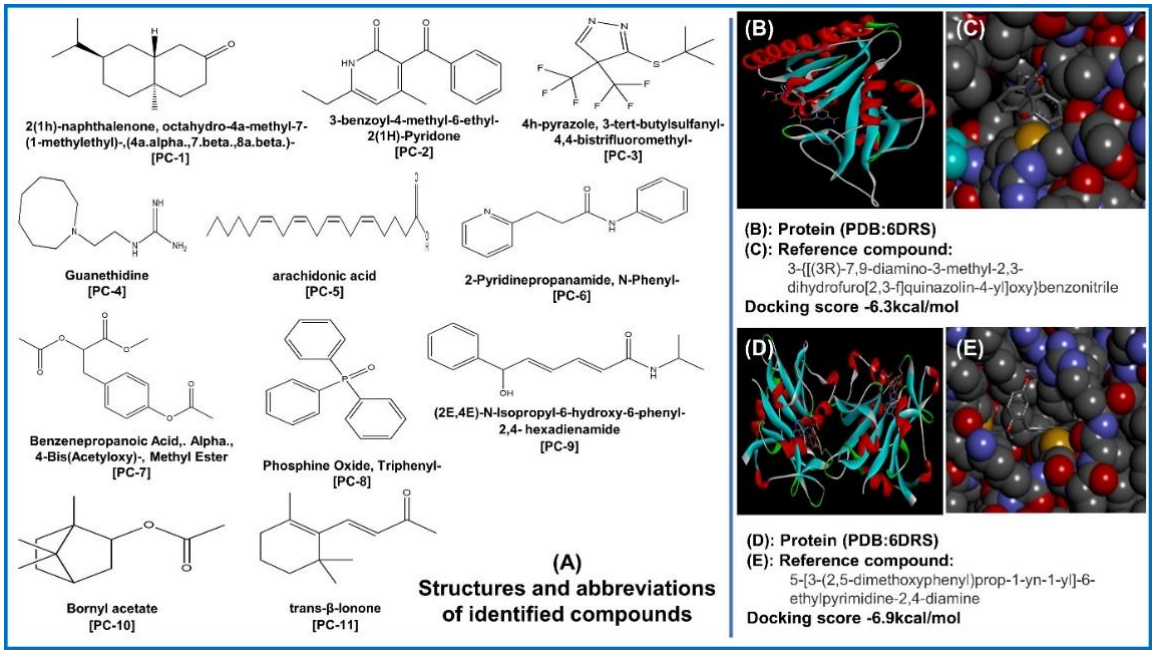

Figure 1. (A) Structure and abbreviations of identified pteridophytic molecules; (B) Protein structure of DHFR enzyme of *A. niger;*(C) Docking of reference molecule; (D) Protein structure of DHFR enzyme of *C. albicans*

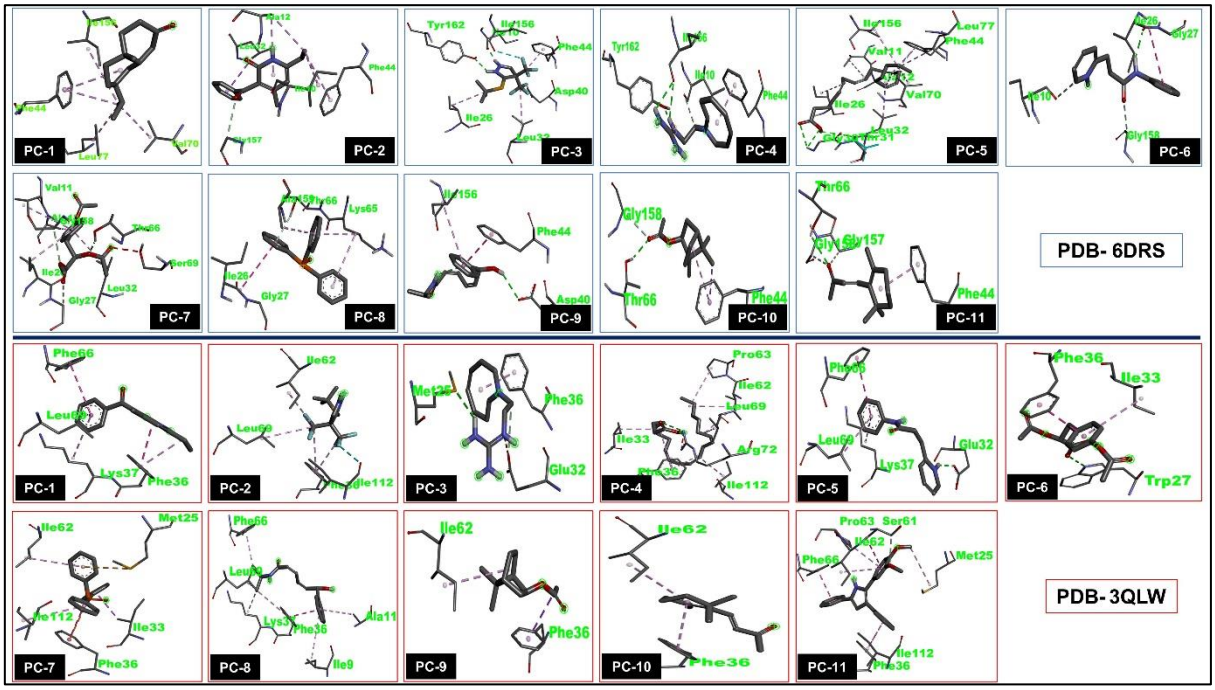

Figure 2. Pose view and binding scaffold of phytochemical compounds (PC-1 to PC-11) with DHFR enzyme (PDB: 6DRS and 3QLW)

DHFR inhibitors are a significant class of drugs, which is evident from their utilization as antibacterial, antimalarial, antifungal, and anticancer agents [19]. The DHFR protein plays a crucial role in the process of DNA synthesis during the development of bacterial and human cells [20]. To assess the potential antibacterial and anticancer properties, we performed docking studies of the selected ligands with the DHFR enzyme against *Aspergillus flavus* and *Candida albicans*. Surprisingly, we

observed docking profiles with significantly higher binding affinity compared to their respective standard molecules. The analysis of the docking study discovered that in the selected phytoconstituents, having large size and polar groups is essential for establishing favourable interactions with the proteins targeted for anti-fungal effects. The physical and chemical characteristics of these biologically active compounds, along with their intriguing binding interactions with specific proteins involved in treating fungal infections, can be utilized for combating fungal diseases and to progress the development of newer antifungal agents through *in-vitro* and *iv-vivo* studies targeting DHFR and other proteins.

ACKNOWLEDGEMENTS

Author acknowledges the KIET Group of Institutions, Ghaziabad, for the infrastructural support.

AUTHOR CONTRIBUTIONS

Concept: A.K., P.B.; Design: A.K., P.B.; Control: S.P., Sources: A.K., P.B.; Materials: M.S., M.R.; Data Collection and/or Processing: M.S., M.R.; Analysis and/or Interpretation: A.S., A.K.; Literature Review: M.S., M.R.; Manuscript Writing: M.S., M.R., A.S., A.K.; Critical Review: A.K., P.B., S.P.; 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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Yayım Koşulları

1. Ankara Üniversitesi Eczacılık Fakültesi Dergisi (Ankara Ecz. Fak. Derg. – J. Fac. Pharm. Ankara) yılda üç kez (Ocak-Mayıs-Eylül) yayımlanır.

2. Dergiye Eczacılığın her alanında daha önce hiç bir yerde yayınlanmamış, Türkçe veya yabancı dilde (İngilizce, Fransızca, İspanyolca ve Almanca) olarak hazırlanmış makaleler kabul edilir. Deneylerde, insan için "the Declaration of Helsinki" ve hayvan için "European Community Guidlines"'a bağlı kalınmalıdır.

3. Yayın Komisyonuna gelen makaleler en az 2 danışmana gönderilir.

4. Makaleler yayına kabul ediliş sırasına göre yayınlanır.

5. Danışmanlar tarafından önerilen düzeltmelerin yapılması için yazar/ yazarlara geri gönderilen makaleler, düzeltilip yayınlanmak üzere 3 ay içinde tekrar yayın kuruluna gönderilmezse, yeni başvuru olarak işlem görür. Makale yayımlandan önce yazarların yayımcıya makalenin "Copyright Transfer Form'unu doldurarak telif hakkını göndermesi gerekmektedir.

6. Yayımlarda intihal olup olmadığı kontrol edilmelidir.

7. Dergimize aşağıdaki makale türleri kabul edilir:

    a) **Araştırma makalesi**: Türkçe veya ingilizce hazırlanmış, şekiller ve tablolar dahil tamamı en çok 20 A4 kağıdı sayfası olan, orjinal araştırmaların bulgu ve sonuçlarını açıklayan makalelerdir.
    b) **Derleme:** Türkçe veya ingilizce hazırlanmış, şekil ve tablolar dahil tamamı en çok 25 A4 kağıdı sayfası olan, yeterli sayıda bilimsel makale taranarak, o güne kadarki gelişmeleri özetleyerek ortaya koyan ve sonuçlarını yorumlayarak değerlendiren makalelerdir.
    c) **Önbilgiler:** Devam etmekte olan bir çalışmanın bulgularını zaman kaybetmeden duyurmak için Türkçe veya ingilizce yazılan en çok 5 A4 kağıdı sayfası olan makalelerdir.

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 ve yazışma yapılacak yazarın açık adresi, telefon ve e-mail adresi belirtilmeli ve ortalı yazılmalıdır. İlk sayfada başlıktan önce yukarıdan 5 satır aralığı bırakılmalıdır. Başlık ile Öz/Abstract arası 1.5 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 24 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 fazla 5 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.
  - Metinde sonuçlar (conclusions) başlığı yer **almamalıdır.**

- **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 kullanılmamalıdır**.), makalenin tam başlığı, derginin adı, cilt no, varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa 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.**

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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.

Etik İlkeler ve Yayın Politikası

Editörün Sorumlulukları

Yayın politikası:

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 uluslararası bir yayım ortamıdır.

Editör, dergiye gönderilen makalelerden hangilerinin yayınlanması gerektiği kararından tek başına sorumludur. Editörün kararı, derginin editör kurulunun prensipleri doğrultusunda olabileceği gibi, onur kırıcı yayın yapmak, telif hakkı ihlali ve intihal gibi konularla ilgili olarak yürürlükte olan yasal gereklilikler ile sınırlandırılmıştır. Editör, karar verirken editör kurulunun üyelerine ya da hakemlere danışabilir. Makale yayımlanmadan önce yazarların yayımcıya makalenin "*Copyright Transfer Form*"unu doldurarak telif hakkını göndermesi gerekmektedir. Dergiye gönderilen makaleler editör ve editör yardımcıları tarafından biçimsel olarak incelenir. Dergi kurallarına göre hazırlanmamış makaleler kesinlikle değerlendirmeye alınmayarak reddedilmektedir.

Yayın değerlendirmesi:

Editör, yayın değerlendirme sürecinin adil, tarafsız ve zamanına uygun şekilde gerçekleştiğini garanti altına almaktadır. Araştırma makaleleri genel olarak dışardan ve bağımsız en az iki hakem ile değerlendirilmekte ve gerek olması durumunda editör üçüncü bir hakemden ek görüş istemektedir. Editör, gerekli, kapsayıcı ve çeşitli düzeylerde değerlendirmelere ihtiyaç duyulacağını göz önüne alarak, ilgili alanda uygun uzmanlığı olan hakemler seçmektedir. 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 gözden geçirmektedir.

Editör, bir makaledeki hataları yayınlanmadan önce tespit ederse düzeltmeli, daha sonra tespit edilmesi durumunda ise düzeltmeleri yayınlamalıdır. Tüm düzeltme veya geri çekme bildirimleri, dergide belirgin bir şekilde yayınlanmalı ve orijinal makaleye veya özete tam bibliyografik referansı içermelidir. Ayrıca içindekiler sayfasında listelenmeli ve belirgin bir şekilde etiketlenmelidir (ör. Yazım hatası, geri çekme veya özür dileme).

Adil değerlendirme:

Editör, makalaleri yazarların ırk, cinsiyet, cinsel eğilim, inanç, etnik köken, vatandaşlık ya da politik görüşlerine bakmaksızın bilimsel içeriklerine göre değerlendirmektedir. Derginin editöryal 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 yapmaktadır. Editör, editöryal kararlara itiraz durumunda şeffaf bir mekanizma işletmektedir.

Gizlilik ilkesi:

Editör, dergiye yapılan başvurudaki tüm materyallerin ve hakemlerle yapılan tüm iletişimin gizliliğini (ilgili yazar ve hakemlerle aksi onaylanmadığı sürece) korumakla yükümlüdür. Hakemler isimlerinin açıklamasını kabul etmediği sürece, editör, hakemlerin kimliklerini korumaktadır. Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan editörün kendi çalışmaları/araştırmaları için kullanılmamalıdır. Değerlendirme sürecinde edinilen ayrıcalıklı bilgi ya da fikirler gizli tutulmalı ve kişisel amaçlar için kullanılmamalıdır.

Hakemlerin Sorumlulukları

Editöryal kararlara katkı:

Etik ile ilgili sorumluluklara ek olarak, hakemlerin yazarlara ve sundukları çalışmalara uygun şekilde davranmaları ve iyi bir değerlendirme performansı göstermeleri istenmektedir. Makalede yer alan araştırmayı değerlendirmeye yetkin olmadığını düşünen ya da verilen sürede değerlendirmeyi yapamayacağını farkeden hakemin, editörü bilgilendirmesi ve değerlendirme sürecinden çıkartılması gerekmektedir.

Gizlilik:

Hakemler makale hakkındaki değerlendirmelerini ya da bilgilerini hiç kimseyle paylaşmamalı, editörden izin almadan yazar ile iletişime geçmemelidirler. Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan hakemin kendi çalışmaları/araştırmaları için

kullanılmamalıdır. Değerlendirme sürecinde edinilen bilgi ya da fikirler gizli tutulmalı ve kişisel amaçlar için kullanılmamalıdır.

Etik sorunları farketme:

Hakem, makalede yer alan olası etik sorunları farketmeli ve editörün dikkatine sunmalıdır. Bahsedilen olası sorunlar içinde, değerlendirmede olan makale ile daha önce basılmış bir makale arasındaki önemli ölçüdeki benzerlik ya da örtüşme de bulunmaktadır. Daha önce yayınlanmış olan herhangi bir gözlem ve/veya argüman, ilgili kaynak/referans ile birlikte verilmelidir.

Tarafsızlık ve rekabet standartları:

Hakemler tarafsız olarak değerlendirmelerini yapmalıdırlar. Hakemler, makale değerlendirmesi sırasında önyargılı olabilecekleri konusunda farkında olmalıdırlar. Yazarın kişi olarak eleştirilmesi uygun değildir. Hakemler görüşlerini destekleyici argümanlarla ifade etmelidirler. Hakemler makaleyi değerlendirmeyi kabul etmeden önce olası çıkar çatışması durumuna karşı (makale ile ilişkili yazar, şirket ya da kurum ile rekabetçi, işbirliği içinde olan ya da diğer ilişkiler/bağlantılar için) editöre bilgi vermelidirler. Hakem, yazarın hakemin (ya da hakemle birlikte çalışan kişilerin) çalışmalarının kaynak olarak alındığını ileri sürerse, gerçek bilimsel gerekçeler sunulmalı, bu durumun hakemin kaynak gösterilme sayısını ya da çalışmalarının görünürlüğünü arttırmaya yönelik bir girişim olmamasına özen gösterilmelidir.

Yazarın Sorumlulukları

Bildirim standartları:

Orijinal araştırmanın makalesini hazırlayan yazarlar, çalışmanın önemine ilişkin tarafsız bir tartışma ile gerçekleştirilen araştırmayı net bir şekilde sunmalıdırlar. Makalede veri açık bir şekilde sunulmalıdır. Çalışma, diğer araştırmacıların çalışmayı tekrar edebilmesine izin verecek şekilde yeterli detay ve kaynak içermelidir. Yanıltıcı ya da bilinçli olarak net olmayan ifadelerin verilmesi etik olmayan davranış olarak görülür ve kabul edilmemektedir.

Veri ulaşımı ve saklama:

Yazarlardan editöryal değerlendirme için makalelerini destekleyici araştırma verisi istenebilir. Yazarların belirtilen veriyi erişime açmaları, yayınlandığı tarihten belli bir süre sonra da gerekli görülmesi durumunda belirtilen veriyi sunabilmeleri gerekmektedir.

Orijinallik, intihal 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 çalışmalarının tümüyle orijinal olduğunu garanti etmelidirler. Eğer yazarlar başkalarına ait iş ve/veya metinleri kullanıyorlarsa, mutlaka uygun şekilde kaynak ya da alıntı gösterilmeli ve gerekliyse izin alınmalıdır. 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. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderilen tüm makaleler, yazarlar tarafından, intihal tarama programları ile taranmış olmalıdır.

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.

Yazar katkıları:

Yazar katkıları, çalışmanın konseptine, tasarımına, gerçekleştirilmesine ya da yorumlanmasına önemli katkı sağlayan kişiler ile sınırlandırılmalıdır. Belirtilen süreçlere önemli katkı sağlayan kişiler eşyazar (co-author) olarak belirtilmelidir. Çalışmaya başka açılardan (dil düzenlemesi ya da tıbbi yazım gibi) katkı sunan kişiler "Teşekkür" (Acknowledgement) bölümünde yer almalıdırlar. 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. Yazarlar, makale başvurusu yapmadan önce yazar listesini ve sıralamasını dikkatli bir şekilde değerlendirmeli ve orijinal başvuru sırasında kesin bir yazar listesi sunmalıdırlar. 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. Ankara Üniversitesi Eczacılık Fakültesi Dergisine makale ile birlikte "Yazar Katkı Formu"nun da doldurulup gönderilmesi gerekmektedir.

Çıkar çatışması beyanı:

Tüm yazarlar, çalışmalarını uygunsuz bir şekilde etkileyebilecek olarak gördükleri diğer kişi veya organizasyonlarla her türlü maddi ve kişisel ilişkilerini beyan etmelidirler. Araştırmanın yürütülmesinde ve/veya makalenin hazırlanmasında maddi destek sağlayan tüm kaynaklar ile çalışmanın planlanması, verinin toplanması, analizi ve yorumlanması, raporlanması ve makalenin yayın için başvurma kararının alınmasında rol oynayan kişi ve/veya kurumlar belirtilmelidir. Fon kaynaklarının belirtilen şekilde bir katkısı olmamışsa, bu durum da ifade edilmelidir. Açıklanması gereken potansiyel çıkar çatışmalarına örnek olarak istihdam, danışmanlıklar, hisse sahipliği, ücret, ücretli uzman tanıklığı, patent başvuruları/tescilleri ve hibeler veya diğer fonlar sayılabilir. Olası çıkar çatışmaları mümkün olan en erken aşamada açıklanmalıdır.

Temel hataların bildirimi:

Yazar, yayınlanmış olan bir çalışmasında önemli bir hata ya da eksiklik farkettiğinde, acil olarak dergi editörüne/yayınevine bildirmek ve editör tarafından gerekli görülmesi durumunda makaleyi geri çekmek veya düzeltmek için editörle işbirliği yapmak ile yükümlüdür. Editör/yayınevi yayınlanmış olan makalenin bir hata içerdiğini üçüncü bir taraftan öğrenirse, editör ile işbirliği yapmak ve gerektiğinde destekleyici kanıt sağlamak, yazarın yükümlülüğüdür.

Olası riskler ve insan veya hayvan konuları:

Eğer kullanımları sırasında olağandışı risk yaratan kimyasallar, işlemler ya da malzemeler çalışmada yer alıyorsa, yazar bu durumu metin içinde açıkça belirtmelidir. 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. 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. İnsanlar üzerinde gerçekleştirilen tüm araştırmalar Helsinki Bildirgesi ilkelerine göre yapılmalıdır (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). 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.

Guide for Authors

1. The Journal of Faculty of Pharmacy of Ankara University (J. Fac. Pharm. Ankara) 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 a foreign language (English, French, Spanish or German). The experiments used have to be adhered to the Declaration of Helsinki for humans and European Community Guidlines for animals.

3. All manuscripts will be submitted to a review process by the editors and by qualified at least 2 outside reviewers.

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, the by authors are considered to transfer all rights of the manuscript to the Publisher.

6. Manuscript will be controlled using plagiarism checker.

7. Manuscripts with the following charactheristics are accepted:

    a) **Research article**: Articles written in English or Turkish in scientific format presenting original research. Articles should be printed on A4 size papers not exceeding 20 pages (including tables and figures).
    b) **Review:** 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 25 pages (including tables and figures).
    c) **Rapid communication:** Rapid announcement of the results of a continuing research written in English or Turkish, no longer than 5, A4 size pages.

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 (5 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 and the full address/es 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 24 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.

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Subheadings to be used for **ABSTRACT**:

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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.*

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Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

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Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler:

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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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https://doi.org/10.1016/0006-2952(89)90403-6

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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Example:

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- **Book chapters:** Author's surname, initials of the name, the title of the section, the editor / editors' surname, the initials of the name, the phrase (Ed./Eds.), The title of the book, if any, the book house, the city where it was published, the page number, the year it was published (in parentheses) should be written.

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- **For patent:** The surname of the author, the initials of the name, the year should be written (in parentheses) after the author's names and a full stop. The title and number of the patent should be indicated.

Example:

Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

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JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

Cilt / Vol : 47
Sayı / No : 3
Yıl / Year : 2023
eISSN : 2564-6524
ISSN: 1015-3918 (1971-2010)

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