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(*Ankara Ecz. Fak. Derg.*) **eISSN: 2564-6524**

Sahibi:
Prof. Dr. Gülbin ÖZÇELİKAY

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIOXIDANT, ANTIMICROBIAL AND ANTI-PROLIFERATIVE ACTIVITY OF *SUILLUS LUTEUS* (L.) ROUSSEL EXTRACTS

SUILLUS LUTEUS (L).ROUSSEL EKSTRESİ'NİN ANTİOKSİDAN, ANTİMİKROBİYAL VE ANTİ-PROLİFERATİF AKTİVİTESİ

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ABSTRACT

Objective: *Many drug discovery have used nature as an inspiration for the design of naturel products like compound classes. From ancient times edible mushrooms have been used both as food and medicine. People living in Turkey widely consume Suillus luteus (L.) Roussel wild edible mushrooms In this study, we were investigated antioxidant, antimicrobial and cytotoxic activities of various extracts of S.luteus.*

Material and Method: *Antioxidant activity of S.luteus was detected method by DPHH free radical scavenging and ferrous ion chelating ability. In addition, the concent of the components with antioxidant properties, such as total phenols,β-caratone and lycopene were determined by spectrophotometric methods. The antimicrobial potential was demonstrated with a agar well diffusion method on 14 microorganisms. Fınally, the cytotoxic effect of methanolic extract of S. luteus on MCF-7 cancer cell lines were evaluated by using MTT method.*

Result and Discussion: *The results indicated that S.luteus methanolic and ethanolic extracts have more abundant phenols (153, 49.33 mg GAE/g extract, respectively).In addıtıon β-caratone and lycopene content detected. (from 0.120 to 0.606µg/mL).S.luteus extracts had more potent free radical scavenging activity than standard antioxidants BHT. (Methanol extract (IC_{50}: 63.72µg/mL) > Ethanol extract (IC_{50}: 80.72 µg/mL) > BHT (IC_{50}: 96.47µg/mL). In addition, methanol extracts possessed higher ferrous ion chelating ability than ethanol extracts(2.72, 3.45 µg/mL, respectively) .Generally, the tested mushroom extracts had relatively low antimicrobial activity against the tested microorganisms (9 and 10 mm zone diameter). Also, S.luteus methanolic extract was found to kill all cancer cells at a concentration of 1mg/mL. These results showed that S.luteus, especially methanol extracts, have potential medical.*

Keywords: *antimicrobial activity, antioxidant activity, anti-proliferative activity, Suillus luteus*

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ÖZ

Amaç: *Birçok ilaç keşfinde doğa, doğal ürünler benzeri bileşik sınıflarının tasarımına ilham kaynağı olarak kullanmıştır. Eski zamanlardan beri yenilebilir mantarlar hem gıda hem de ilaç olarak kullanılmıştır. Türkiye'de yaşayan insanlar Suillus luteus (L.) Roussel yabani yenilebilir mantarlarını yaygın olarak tüketmektedir. Bu çalışmada, çeşitli S.luteus ekstraktlarının antioksidan, antimikrobiyal ve anti-proliferatif aktiviteleri araştırıldı.*

Gereç ve Yöntem: *S.luteus'un antioksidan aktivitesi, DPHH serbest radikal süpürme yöntemi ve demir iyonu şelatlama kabiliyeti saptandı. Ek olarak, toplam fenoller, β-karoten ve likopen gibi antioksidan özelliklere sahip bileşenlerin konsantrasyonu spektrofotometrik yöntemlerle belirlenmiştir. Antimikrobiyal potansiyel, 14 mikroorganizma üzerinde agar difüzyon yöntemi ile gösterilmiştir. Son olarak, S. luteus metanol ekstresinin MCF-7 kanser hücre hatları üzerindeki sitotoksik etkisi MTT yöntemi kullanılarak değerlendirildi.*

Sonuç ve Tartışma: *Sonuçlar S.luteus metanolik ve etanolik özütlerin daha bol fenollere sahip olduğunu gösterdi. (sırasıyla 153, 49.33 mg GAE/g ekstre,) Ayrıca β-karoten ve likopen içeriği saptandı (0.120 ile 0.606 µg/mL arası) S. luteus'un metanol ve etanol özütlerinin, DPPH radikaline karşı antioksidan aktiviteleri aynı konsantrasyondaki standart antioksidanlar olan BHT'den daha yüksek aktivite göstermiştir (Metanol özütü (IC_{50}: 63.72µg/mL) > Etanol özütü (IC_{50}: 80.72 µg/mL) > BHT (IC_{50}: 96.47µg/mL). Ek olarak, metanol ektresinin etanol ekstresine göre daha yüksek demir iyonu şelatlama kabiliyetine sahiptir (sırasıyla 2.72, 3.45 µg/mL). Genel olarak, test edilen mantar ekstreleri test edilen mikroorganizmalara karşı nispeten düşük antimikrobiyal aktiviteye sahiptir (9 ve 10mm zonçapı) Ayrıca, S. luteus'un metanol ekstresinin 1mg/mL konsantrasyonda kanser hücrelerinin tamamını öldürdüğü tespit edildi. Bu çalışmanın sonuçları incelendiğinde S.luteus'un özellikle metanol ekstresinin potansiyel medikal özelliklere sahip olduğu gösterilmiştir.*

Anahtar Kelimeler: *antimikrobial aktivite, antioksidan aktivite, antiproliferatif aktivite, Suillus luteus*

INTRODUCTION

Oxidation is an essential process for the production of energy to many organisms. Under physiological conditions, however, the concentrations of reactive oxygen species (ROS) are usually over physiological limits leading to oxidative stress [1,2]. Overproduction of free radicals can cause oxidative damage to biomolecules, (lipids, proteins, DNA), eventually leading to many chronic diseases such as cancer, cardio-vascular diseases and inflammation in humans. Oxidative stress in cells can result from either an increase in the levels of reactive oxygen species, or a reduction of the natural cell antioxidant capacities [3]. Antioxidants can be defined as molecules that can delay or prevent oxidation of the substrate when they encounter a low amount of oxidizable substrate [4]. In some cases, the amount of antioxidant in cell may be insufficient for intracellular protection. In such cases, external antioxidant supplementation will contribute to the renovation of this balance again [5]. Currently, synthetic antioxidants had unwanted side effects mostly [6]. Therefore, it is essential to develop and utilize effective natural antioxidants to replace the synthetic antioxidants [7].

Natural products (such as secondary metabolites) and their analogs are the source of inspiration in the production of new drugs. Active ingredients of many drugs such as antibiotics (penicillin, tetracycline and erythromycin), anti-parasites (such as avermectin), antimalarials (such as uinine, arminthymine), lipid control agents (lovastatin and analogues), immunosuppressants for organ transplantation (cyclosporine, rapamycin), anti-cancer drugs (Toxol, doxorubicin) are derived from natural resources [8].

Mushrooms are used by people since ancient times as they have significant nutritional values and medical property, especially in Asian countries [9]. Edible mushrooms are rich in high minerals (potassium, phosphorus, iron), essential amino acids, vitamins (B12 and D) and source of some fiber [10-12]. Mushrooms use their own metabolic pathways throughout their life cycles. Mushroom also produces a variety of secondary metabolites, such as numerous alkaloids, terpenes, steroids and phenolic compounds that can be used for therapeutic purposes [13]. The mushroom's compounds possess antimicrobial activity [14], antigenotoxic [15], antioxidant [16], antiproliferative [17], anticancer [18], antihyperlipidemic [19], anti-hypertensive, anti-nociceptive and immunostimulanting [20], hypocholesterolemic, anti-atherogenic [21], anti-allergic [22], neuroprotective and antidepressan effect [23,24].Bioactive compounds isolated from mushroom include small molecule compounds, polysaccharides, proteins, polysaccharide-protein compounds. Amongst bioactive compounds, polysaccharides have been studied in the broadest field [25-27]. These polysaccharides are actively involved in the life cycle of organisms and have biological activities such as anti-cancer, anti-fungal, antioxidant [28,29]. Among the polysaccharides found in mushroom, β-glucan is used as a chemotherapeutic drug in cancer treatments and various diseases [30,31].In recent years, therapeutic agents which affected apoptosis, angiogenesis, metastasis, cell cycle and signal transduction control has been used in oncology [32]. The use of polysaccharide and polysaccharide-protein complexes isolated from edible mushrooms has proven to be a source of therapeutic agents due to their immunomodulatory and anti-tumor effects [33].

Over 2600 macrofungi species have so been reported from Turkey and approximately 300 of them are edible [34-36]. Today, a significant amount of cork exports are made in Turkey and 171 million US dollars was recovered from exports from 2007 until 2017 [37, 38]. *S. luteu*s, a member of *Boletales* in *Agaricomycetes* is an ectomycorrhizal fungus that solely associates with *Pinaceae* plants in the Northern Hemisphere, such as *Pinus densiflora, Pinus thunbergii*, *Pinus sylvestris*, *Pinus strobus*, and *Picea glehnii*. The mushrooms are widely consumed in central Europe [39, 40]. In our country, it is necessary to determine the nutritional and medicinal properties of fungi because of this variety and economic value of their potential.

The main objectives of the current study were to evaluate the phenolic, β-carotene and lycopene content and antioxidant, antimicrobial and anti-proliferative activity of *S. luteu*s in Turkey.

MATERIAL AND METHOD

Mushroom material

S. luteus samples were collected from Ankara and Tokat province in 2013. The samples used in this study were identified by Dr. Ilgaz Akata from Ankara University. The identified specimens were deposited at the herbarium of Ankara University.

Chemicals

Chloroform, Folin-Ciocalteu's phenol reagent, ethanol, methanol were purchased from Merck (Darmstadt, Germany). 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), Tween 40, dimethylsulphoxide (DMSO), β-carotene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine), gallic acid, 2,6-di-tert-butyl-4-methylphenol (BHT) and linoleic acid were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, USA). All other chemicals were analytical grade and obtained from either Sigma or Merck.

Preparation of the extracts

The dried mushroom samples were extracted by maceration in 1:4 (w/v) biomass /solvent ratio with methanol and ethanol for 2 weeks at room temperature in a dark environment. The obtained methanolic and ethalolic extracts were filtered through filter paper. After filtration the solvent was evaporated in a rotary evaporator (Heidolph, Germany) at 50°C under reduced pressure and the solid extracts were stored at +4°C until further use.

Determination of Total Phenolics Content

Total phenolic of each mushroom extract was quantified according to the method of Folin-Ciocalteu [41] using gallic acid as standard. Briefly, 0.1 mL of extracts (1 mg/mL) were mixed with 0.2 mL of diluted Folin-Ciocalteu reagent (1:1 with water). After incubation at room temperature for 3 min,1 mL 2% sodium carbonate was added to the reaction mixture. The absorbance was read at 760 nm by spectrophotometer after 1 h of incubation at room temperature in the dark. The total phenolic concent values are expressed as gallic equivalent (GAE) in milligrams per gram of dried extract (mg GAE/g). All measurements were performed in triplicate.

Determination of *β*-Carotene and lycopene content

β-Carotene and lycopene content of the extracts were determined according to the method described by (42) with slight modification. Briefly, dried samples (100 mg) were mixed with acetone/hexane (4:6, v/v). After incubation for 1 min. The absorbance of the supernatants was read at 453, 505, 645 and 663 nm by spectrophotometer. Contents of *β*-carotene and lycopene were calculated according to the following equation:

Lycopene (mg/100 ml) = $-0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$; β-carotene (mg/100 ml) = $0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}$.

Antioxidant activity

1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging capacity of the extracts were analyzed according to the method described by (43) with slight modifications. Briefly,0.5 mL extracts with different concentrations were mixed with a methanolic solution of DPPH radical (0.1mM) freshly prepared. After incubation for 30 min at room temperature in the dark, absorbance was read at was added to extracts solutions at 517 nm by spectrophotometer (Shimadzu UV-1800,Japan) against a blank (extract only). Same procedure with a solution without the extract was applied as a control group. Butylated hydroxytoluene (BHT) was used as a reference standard. The percentage of DPPH radical scavenging effect was calculated according to the following equation:

DPHH scavenging activity (%inhibation) = [(Acontrol-Asample)/Aconrtol]×100, where A control is the absorbance of the control and Asample is the absorbance of the reaction mixture or standard. A curve of extract concentration versus % inhibation was created to determine the concentration of the extract needed to cause a %50 decrease of the beginning DPHH concentration. This value calculated by linear regression analysis is known as a IC_{50}.The lower IC_{50} value indicates better antioxidant activity.

Ferrous ion chelating ability

Ferrous ion chelating ability of the extracts were determined according to the method described by (44) with slight modifications. 0.5 mL of the extracts at different concentrations were mixed with 1.35 mL of methanol and ethanol. 50 µl of 2 mM $FeCl_2$ were added to extract solution and stand for 5 min. Thereafter, 100 µl of 5 mM ferrozine solution were added to this mixture and incubated for 10 min. After incubation, absorbance was read at 562 nm by spectrophotometer (Shimadzu UV-1800, Japan) against a blank (extract and $FeCl_2$ only). In the control group, extract was substituted with methanol and ethanol. EDTA (Ethylene diamine tetraacetate) was used as a positive control. Percentage of the ability of the sample to helate ferrous ion was calculated according to the following equation:

Ferrous ion chelating ability (%)=$[(A_{control}-A_{sample})/A_{control}]x100$, where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the reaction mixture. The IC_{50} value, which is the concentration of the extracts that chelate 50% of the ferrous ion, was calculated by linear regression curve.

Antimicrobial activity

The antimicrobial activities of mushroom extracts were determined by agar well method and evaluated against bacterial strains on *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* NRRL B-

3711, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Enterococcus hirae* ATCC 9790, *Klebsiella pneumaniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* RSKK 96029, *Salmonella typhimurium* ATCC 14028 and fungal strains *Candida tropicalis* Y-12968, *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258. For comparison ampicillin and chloramphenicol were used a standard antibiotics.

In the agar well method, bacterial strains were allowed to incubate at 37°C for 24 hours in Nutrient Agar medium and yeast strains were incubated for 48 hours at 30°C in Malt Extract Agar medium. The post-incubation microorganisms were adjusted to 0.5 McFarland blur. Muller – Hinton Agar (for bacterial strains) and Malt Extract Agar (for yeast strains) were spread on a petri with a 1% suspension of microorganism suspension. With the punch, 6 mm in diameter wells are opened at specific points of the medium. The opened wells were placed in a volume of 50 µL from mushroom extracts at a concentration of 150 mg/mL and left to incubate. The diameter of the inhibition zones formed after incubation is measured in mm. Chloramphenicol, ampicillin were used for antimicrobial activity.

Cultiring of cell lines

Human breast adenocarcinoma cell line MCF-7 was purchased from American Type Tissue Culture Collection (USA) and cultured in RPMI 1640 (Sigma Chemicals) media containing 10% FBS and 1% of sodium pyruvate, amphotericin B, penicillin and streptomycin. Cells were maintained at 37 °C and 5% CO_2 under humidified condition.

Antiproliferative activity

Cells were grown in culture flask at a range of 10,000-100,000 cells per ml. Mushroom extracts were applied at increasing concentrations (25, 50,100, 250,500 and 1000 mg/mL) for 24, 48 and 72 hours. Viable cells in the control and application groups were determined by MTT [3- (4,5-dimethyl thiazol-2-yl) -2,5-diphenyl tetrazolium bromide] staining method [45]. The solution was measured by spectrophotometer (Thermo/LabSystems 352 Multiskan MS Microplate Reader) at 590 nm. All experiments were performed with 3 replications.

$[(C_{72h+extract} - C_{24h+extract}) / (C_{72h- control} - C_{24h- control})] \times 100 = \%$ dividing cell viabilty

$C_{72h+ extract}$: Live cell measurement 72 hours after manipulation

$C_{24h+ extract}$: Live cell measurement 24 hours after manipulation

$C_{72h- control}$: 72 hours after live cell measurement without extract manipulation

$C_{24h- control}$: 24 hours after live cell measurement without extract manipulation

Statistical analysis

SPSS 11 were used for statistical analyses. Experimental results were expressed as mean ± S.D of three parallel measurements. *P*-values < 0.05 were regarded as significant.

RESULT AND DISCUSSION

The extracts of *S.luteus* was studied regarding antioxidant capacity potential. The standard curve equation is, y (absorbance) =0.008 5x (μg gallic acid)-0.0209, R^2=0.999 9. The data of the sample regarding the content of total phenolics, β-carotene and lycopene are presented in Table 1. As a shown in table, the mushrooms of *S.luteus* methanolic and ethanolic extracts presented phenolic contents with 153 ± 3.54 and 49.33 ± 0.14 mg GAE/g extract, respectively. The results suggest that most of the phenolic compounds in methanolic extract. In addition, ethanolic extract had more β-carotene and lycopene content (0.606 ± 0.05, 0.357 ± 0.02 μg/mL, respectively) than methanolic extract (0.220 ± 0.01, 0.120 ± 0.05 μg/mL, respectively).

Table 1. Total phenolic, β-Carotene and lycopene content in the extracts of *S.luteus* and ±SD*(n=3).

Sample	Total phenolic content (mg GAE/g extract)	β -Carotene (μg/mL)	Lycopene (μg/mL)
Methonolic extracts	153 ± 3.54	0.220 ± 0.01	0.120 ± 0.05
Ethanolic extracts	49.33 ±0.14	0.606 ± 0.05	0.357 ± 0.02

*Standart devidation

The antioxidant activity of mushrooms increased with the increased in the concentration of samples, higher the antioxidant properties lower the IC_{50} values. A lower IC_{50} values means better radical scavenging activity [46].The scavenging DPPH radicals of the studied methanolic and ethanolic extracts are indicated in Table 2. As a shown in table, the free radical scavenging activity of the mushroom extracts was evaluated by DPPH assay comparing the IC_{50} value of synthetic chemical BHT, which was 96,47 ± 0.57 μg/mL. Antioxidant activity was detected method by DPHH free radical scavenging. *S.luteus* methanolic and ethanolic extract had more potent free radical scavenging activity than BHT (Methanol extract IC_{50}: 63.72 ± 0.89 μg/mL > Ethanol extract IC_{50}: 80.72 ± 0.58 μg/mL > BHT: IC_{50}: 96.47 ± 0.57 μg/mL). Besides, ferrous ion chelating activities of the extracts expressed as IC_{50} values are shown in Table 3. As a shown in table, *S.luteus* methanolic extract had higher iron chelating activity than ethanolic extract (2.72 ± 0.06, 3.45 ± 0.05 mg/mL, respectively). EDTA showed very powerful activity.

Table 2. DPPH radical scavenging activities of the *S. luteus* extracts. Scavenging activity is expressed as IC_{50} (µg/mL) ± SD (n=3).

Sample	**IC_{50} (µg/mL)**
Methanolic extract	63.72± 0.89
Ethanolic extract	80.72 ± 0.58
BHT	96.47± 0,57

*Standart devidation

Table 3. Ferrous ion chelating activities of the *S. luteus* extracts. Chelating activity is expressed as IC_{50} (mg/mL) ± SD (n=3).

Sample	IC_{50} (mg/mL)
Methanolic extract	2.72 ± 0.06
Ethanolic extract	3.45 ± 0.05
EDTA	0.018±0.001

*Standart devidation

Antimicrobial activities of the mushrooms extract against the test microorganisms is shown in Table 4. *S. luteus* methanolic extract formed against to *E. faecalis* ATCC 29212, *B.subtilis* ATCC 6633, *K.pneumaniae* ATCC 13883 9 mm inhibition zone diameter. Ethanolic extract formed against to *E. faecalis* ATCC 29212, *S. aureus* ATCC 25923, *S.typhimurium* ATCC14028 9 mm and *P. aeruginosa* ATCC 27853 10 mm inhibition zone diameter.

The antimicrobial activity was compared with the standard antibiotics, ampicillin and chloramphenicol. The results showed that standard antibiotics had stronger activity than tested samples as shown in Table 4. In a negative control, DMSO had no inhibitory effect on the tested organisms.

Table 4. Antimicrobial activity results (zone diameter / mm) and ± SD.

Test microorganisms	Methanolic extract	Ethanolic extract	Ampicillin	Chloramphenicol
E. faecalis ATCC 29212	9±1	9±0	27±0	20±0
K. pneumaniae ATCC 13883	9 ±0	-	-	31±1
B. subtilis ATCC 6633	9±1	-	23±1	21±0
S. aureus ATCC 25923	-	9±0	44±1	24±0
P. aeruginosa ATCC 27853	-	10±1	60±0	34±0
S.typhimurium ATCC 14028	-	9±0	19±1	38±1

*Standart devidation

Antiproliferative activity was studied in methanolic extract of *S.luteus*, since methanol extract is more effective than antioxidant activity and antioxidant containing ingredients than ethanol extract. The experimental data of antiproliferative activity of *S. luteus* methanolic extract on MCF-7 cell line by MTT method are shown in Figure1. It was found that the cells exposed to 25, 50, 100, 250, 500 µg/mL and 1 mg/mL concentrations of methanolic extract resulted in %,87.83 ,%78.82, %86.48, %34.68, %15.76 and % 0 cell viability reduction compared to the negative control group, respectively and these reductions were statistically significant in comparison to negative control group (P <0.05). The IC_{50} value of methanol extract was calculated to be approximately 173µ/mL. Observed to cause damage to the breast cancer cells.

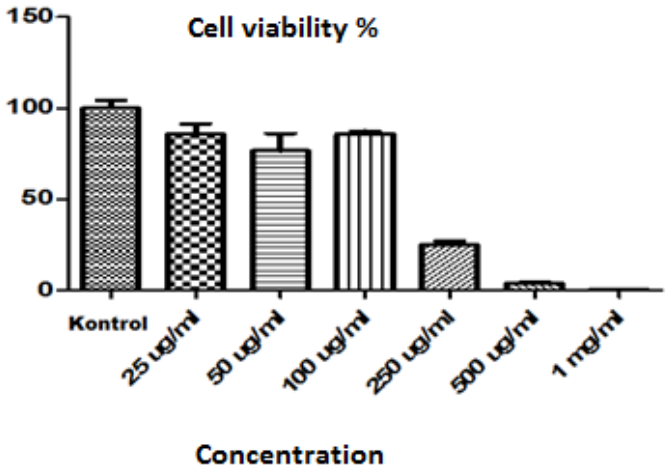

Figure 1. Percentage of viability of MCF-7 lysed breast cancer cells after exposure to various concentrations of methanol extract of *S. luteus at* 24 h

Many research studies have shown that *S.luteus* extract has antioxidant activity and total phenolic contents. Previous reports have demonstrated that the DPPH radical scavenging effect *S.luteus* of ethanol extract was found $IC_{50:}$ 0.66 mg/mL and total phenolic content was found 27.7 ± 4.0 mg GAE/g [47]. Another study indicated that DPPH radical scavenging effect methanolic extract of *S.luteus* IC_{50}: 1.92 ± 0.08 mg/mL. *S.luteus* showed the high concentration of phenolic acids (0.72 mg/100 g), due to the contribution of protocatechuic (0.47 mg/100 g) and cinnamic acid (0.41 mg/100 g) [48]. According to Keleş et al. reported that antioxidant activity was measured by the FRAP method, methanol extract of *S.luteus* was found EC_{50}: 4.76 mg/mL [49]. It was also concluded that totel total phenolic content of the methanol extract was found 1.72 ± 0.02 mg GAE/g. [50] and Jowarska et al. reported that DPPH activity in methanol extract was found IC_{50}: 3.48 ± 0.20 mmol TE and using the FRAP method it was 9.15 mmol Fe_2^+[51]. In the study of Heleno et al. the antioxidant DPPH activity of *Suillus collinitus* and *Suillus mediterraneenis* methanol extracts were examined IC_{50}: 14.05 ± 1.24 11 mg/mL and 2.90 ± 0.11

mg/mL, respectively. Methanol and ethanol extracts of *S.luteus* fungus used in our study have higher activity than *S.collinitus* and *S.mediterraneenis* [52]. Our results indicate that *S.luteus* have more abundant phenolic components, higher antioxidant activity and ferrous ion chelating ability. According to the results of our study, it is clearly indicated that the ethanolic and methanolic extracts of *S.luteus* have significant phenolic content and antioxidant activity. Furthermore, a good correlation was also observed between the total phenolic content and antioxidant activity. These differences can be attributed to differences in soil conditions in different geographical locations and the sub-species ability to synthesize phenolic compounds.

β-carotene is a light yellow or orange pigment that is the precursor of vitamin A. Antioxidant β-carotene prevents oxidation of unsaturated fats and the formation of free radicals [34]. Lycopene, an important derivative of carotenoids, is the most powerful antioxidant in vitro and has more radical scavenging activity [54,55]. The extracts of *S.luteus* of β-carotene and lycopene content are presented in Table 1.Jowarska et al. investigated *S. luteus* total polyphenol and flavonoids and B group vitamin contents. *Suillus* species are richer in polyphenols compared to other fungi in the literature [51].

Antimicrobial activities of the *S.luteus* extracts against the test microorganisms are shown in Table 4. Antimicrobial activities of the extracts were determined on five Gram-positive, six Gram-negative bacteria and three yeasts. In reported studies methanolic and ethanolic extracts from *S.luteus* showed similar antimicrobial activity against microorganisms [56,57]. In this study, the antibacterial properties of *S.luteus* were not as effective as the commercial drugs.

In this study, the antiproliferative activity of the methanol extract of *S. luteus* on MCF-7 cell lines in 24 hours was studied by MTT method. Methanol extract was found to kill all cancer cells at a concentration of 1mg/mL. The IC_{50} value of methanol extract was calculated to be approximately IC_{50}: 173µg/mL. Previous studies had investigated the effect of *S.luteus* methanolic exttract on colon cancer cell line by MTT method. The most sensitive amount was found to be IC_{50}= 17.75 ± 1.6 µg/mL on HCT-15 cell line which is the colon cancer cell line [58]. Vaz et al., also concluded antiproliferative activity of *S. collinitus* on ASG gastric cancer cell line. They found that the cell line had IC_{50}: 79.2 ± 15.5 µg/mL [59]. The results of the study show that *S. luteus* is a potential anticancer agent. No data are available against the antiproliferative activity of *S. luteus* MCF-7 on the cancer cell line. It is predicted that these new findings added to the literature will be effective in further studies.

As a result, *S. luteus* have high antioxidant activity at low concentrations of methanolic and ethanolic extracts, but have low antimicrobial activity. Especially methanol extract has antiproliferative activity on MCF-7 breast cancer cell line. Nowadays, the emergence of some side effects of the drugs used in the treatment of diseases causes increasing interest in the treatment with natural resources. Multidrug resistance is still a major problem in cancer chemotherapy [60]. Researchers should focus on solving this problem. Our studies have tried to determine some basic concepts about the applicability of

this kind of research in practice. *S. luteus*, especially methanol extract is recommended to be included in further studies.

REFERENCES

1. Liu, Q., Jiang, J. (2012). Antioxidative activities of medicinal plants from TCM. *Mini-Reviews in Medicinal Chemistry*, *12*(11), 1154-1172.

2. Liu, Z., Jiao, Y.C., Lu, H., Shu, X., Chen, Q. (2020). Chemical characterization, antioxidant properties and anticancer activity of exopolysaccharides from *Floccularia luteovirens. Carbohydrate Polymers*, *229*, 115432.

3. Racchi, M., Daglia, M., Lanni, C., Papetti, A., Govoni, S., Gazzani, G. (2002). Antiradical activity of water soluble components in common diet vegetables. *J. Agric. Food. Chem*, 50, 1272-1277.

4. Becker, E.M., Nissen, L.R. and Skibsted, L.H. (2004). Antioxidant evaluation protocols: Food quality or health effects. *European Food Research and Technology*, *19*, 561-571.

5. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*, 44-84.

6. Qi, H., Zhang, Q., Zhao, T., Chen, R., Zhang, H., Niu, X., Li, Z.(2005).Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from Ulva pertusa (Chlorophyta) in vitro. *International Journal of Biological Macromolecules*, *37*(4), 195-199.

7. Singh, N., Rajini, P.S. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry*, *85*(4), 611-616.

8. Li, J.W., Vederas, J.C. (2009). Drug discovery and natural products: End of an era or an endless frontier. *Science*, *32*, 161-165.

9. Hoshi, H., Yagi, Y., Iijima, H., Matsunaga, K., Ishihara, Y., & Yasuhara, T. (2005). Isolation and characterization of a novel immunomodulatory r-glucan-protein complex from the mycelium of *Tricholoma matsutake* in Basidiomycetes. *Journal of Agricultural and Food Chemistry*, *53*, 8948–8956.

10. Feeney, M.J., Dwyer, J., Hasler-Lewis, C.M., Milner, J.A., Noakes, M., Rowe, S., … Wu, D. (2014). Mushrooms and Health Summit Proceedings. *Journal of Food and Nutrition Research*, *144*(7), 1128-1136.

11. Manninen, H., Rotola-Pukkila, M., Aisala, H., Hopia, A., Laaksonen, T. (2018). Free Amino Acids and 5′-Nucleotides in Finnish Forest Mushrooms. *Food Chem*, *247*, 23-28.

12. Roupas, P., Keogh, J., Noakes, M., Margetts, C., Taylor, P. (2012). The role of edible mushrooms in health: Evaluation of the evidence. *J. Funct. Foods*, *4*, 687-709.

13. Jayakumar, T., Thomas, P.A., Sheu, J.R. and Geraldine, P. (2011). In-vitro and in-vivo antioxidant effects of the oyster mushroom Pleurotus ostreatus. *Food Research International, 44*, 851-861.

14. Osaki, K., Suyama, S., Sakuno, E., Ushijima, S., Nagasawa, E., Maekawa, N., Ishihara, A. (2019). Antifungal activity of the volatile compound ısovelleral produced by ectomycorrhizal Russula fungi against plant-pathogenic fungi. *J. Gen. Plant Pathol, 85*, 428-435.

15. Zivkovic, L., Bajic, V., Bruic, M., Borozan, S., Popic, K., Topalovic, D., Santibanez, J., Potparevic, B. (2019). Antigenotoxic and antioxidant potential of medicinal mushrooms (Immune Assist) against DNA damage induced by free radicals-an in vitro study. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 845*, 403078.

16. Song, X., Ren, Z., Wang, X., Jia, L., Zhang, C. (2020). Antioxidant, anti-inflammatory and renoprotective effects of acidic-hydrolytic polysaccharides by spent mushroom compost (*Lentinula edodes*) on LPS-induced kidney injury. *International Journal of Biological Macromolecules, 151*, 1267-1276.

17. Aytar, E., Özmen, A. (2020). Cytotoxic and apoptotic activities of *Rhizopogon roseolus* (Corda) Th. Fr. extracts. *International Journal of Secondary Metabolite, 7*(1), 54-62.

18. Kim, S.H., Jakhar, R., Kang, S.C. (2015). Apoptotic properties of polysaccharide ısolated from fruiting bodies of medicinal mushroom *Fomes fomentarius* in human lung carcinoma cell line. *Saudi J. Biol. Sci., 22*, 484-490.

19. Opletal, L., Jahodar, L., Chobot, V., Zdansky, P., Lukes, J., Bratova, M., Solichova, D., Blunden, G., Dacke, C.G. and Patel, A.V. (1997) Evidence for the antihyperlipidemic activity of edible fungus *Pleurotus ostreatu. Brit J Biomed Sci*., *54*,240-243.

20. Vaz, J.A., Barros, L., Martins, A., Santos-Buelga, C., Vasconcelos, M., Ferreira, I.C. (2011). Chemical composition of wild edible mushrooms and antioxidant properties of their water soluble polysaccharidic and ethanolic fractions. *Food Chemistry, 126*, 610-616.

21. Han, E.H., Hwang, Y.P., Kim, H.G., Choi, J.H., Im, J.H., Yang, J.H., Lee, H.U., Chun, S.S., Chung, Y.C., Jeong, H.G. (2011). Inhibitory effect of *Pleurotus eryngii* extracts on the activities of allergic mediators in antigen-stimulated mast cells. *Food Chem. Toxicol, 27*, 1199-1128.

22. Nguyen, T.M., Le, H.G., Le, B.V., Kim, Y.H.K., Hwang, I. (2020). Anti-allergic effect of inotodiol, a lanostane triterpenoid from Chaga mushroom, via selective inhibition of mast cell function. *International Immunopharmacology, 81*, 106244.

23. Choi, Y.J., Park, I.S., Kim, M.H., Choi, J.H.İ Im, J.H., Yang, J.H., Lee, H.K., Chun, S.S., Chung, Y.C., Jeong, H.G. (2018). The medicinal mushroom *Auricularia auricula-judae* (Bull.) extract has antioxidant activity and promotes procollagen biosynthesis in HaCaT cells. *Nat Prod Res, 4*,1-4.

24. Donatini, B. (2011). *Hericium erinaceus*: properties mostly related to the secretion of neuronal growth factor. *Phytothérapie, 9*, 48-52.

25. Kozarski, M., Klaus, A., Niksic, M., Jakovljevic, D., Helsper, J.P.F.G. and Van Griensven, L.J.L.D. (2011). Antioxidative and immunomodulating activities of polysaccharide extracts of

the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*, *Food Chemistry*, *129*, 1667-1675.

26. Pan, Y., Dong, S., Hao, Y., Zhou, Y., Ren, X., Wang, J., Wang, W., Cheu, T. (2010). Ultrasonicassisted extraction process of crude polysaccharides from Yunzhi mushroom and its effect on hydroxyproline and glycosaminoglycan levels. *Carbohydate Polymers*, *81*, 93-96.

27. Zhang, B.Z., Yan, P.S., Chen, H. and He, J. (2012). Optimization of production conditions for mushroom polysaccharides with high yield and antitumor activity. *Carbohydrate Polymers*, *87*, 2569-2575.

28. Lu, R., Conrad, P., Yacine, H. (2012). Antitumor activity of mushroom polysaccharides. *Food Function*, *3*, 1118–1130.

29. Trajkovic, L.M.H., Mijatovic, S.A., Maksimov –İclvanic, D.D., Stojanovic, I.D., Momcilovic, M.B. (2009). Anticancer properties of *Ganoderma lucidum* methanol extracts in vitro and in vivo. *Nutrition and Cancer*, *61*, 696–707.

30. Chan, G.C., Chan, W.K. and Sze, D.M. (2009). The effects of β-glucan on human immune and cancer cells. *Journal of Hematology Oncology*, *2*, 25.

31. Kidd, P.M. (2000). The use of mushroom glucans and proteoglycans in cancer treatment. *Alternative Medicine Review*, *5*, 4–27.

32. Ouyang, F., Wang, G., Guo, W., Zhang, Y., Xiang, Y. and Zhao, M. (2013). AKT signalling and mitochondrial pathways are involved in mushroom polysaccharide-induced apoptosis and G1 or S phase arrest in human hepatoma cells. *Food Chemistry*, *138*, 2130-2139.

33. Ooi, V.E., Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharidprotein complexes. *Current Medicinal Chemistry*, *7*, 715–729.

34. Akata, I., Altuntaş, D., Kabaktepe, Ş. (2019). Fungi determined ın Ankara University Tandoğan campus area (Ankara-Turkey). *Trakya Unıversity Journal of Natural Sciences*, *20* (1): 47-55.

35. Akata, I., Doğan, H.H. (2015). Orbiliaceae for Turkish Ascomycota: Three new records. *Bangladesh Journal of Botany*, *44*(1), 91-95.

36. Sesli, E., Denchev, C.M. (2008). Checklists of the myxomycetes, larger ascomycetes, and larger basidiomycetes in Turkey. *Mycotaxon*, *106*, 65– 67.

37. Bulam, S., Ustun, N.Ş., Pekşen, A. (2018). Mushroom Foreign trade of Turkey in the last decade. Conference: International Congress on Engineering and Life Science (ICELIS) 26-29 April 2018 at Kastamonu Turkey. Proceeding Book 779-784.

38. Bahadori, M.B., Sarikurkcu, C., Yalcin, O.U., Cengiz, M. and Gungor, H. (2019). Metal concentration, phenolics profiling, and antioxidant activity of two wild edible Melanoleuca mushrooms (*M. cognata* and *M. stridula*). *Microchemical Journey*, *150*, 140172.

39. Murata, H., Yamada, A., Yokota, S., Maruyama, T., Shimokawa, T., Neda, H. (2015). Innate traits of Pinaceae-specific ectomycorrhizal symbiont *Suillus luteus* that differentially associates with arbuscular mycorrhizal broad-leaved trees *in vitro*. *Mycoscience*, *56*(6), 606-611.

40. Kalogeropoulos, N., Mylona, A., Chiou, A., Ioannou, M.S., Andrikopoulos, N.K. (2007). Retention and distribution of natural antioxidants (α-tocopherol, polyphenols and terpenic acids) after shallow frying of vegetables in virgin olive oil *LWT, 40*(6),1008-1017.

41. Siddhuraju, P., Becker, K. (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *Journal of Agricultural and Food Chemistry, 51*(8), 2144–2155.

42. Nagata, M. and Yamashita, I. (1992). Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. *Nippon Shokuhin Kogyo Gakkaish, 39,* 925–928.

43. Braca, A., De Tommasi, N., Di Bari, L., Pizza, C., Politi, M., Morelli, I. (2001). Antioxidant principles from bauhinia tarapotensis. *Journal of Natural Products, 64*(7), 892-895.

44. Dinnis, T., Madeira, V., Almeida, L. (1994) Action of phenolic derivative (acetoaminophene salycilate and 5-amino solycilate as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers). *Archives Biochemistry and Biophyics, 315*, 161–169.

45. Dabur, R.R., Sharma, G. L. (2002). Studies on antimycotic properties of *Datura metel J. Ethnopharmacol, 80,* 193-197.

46. Abdelaaty, S., Abeer, I., Mansour, A.S. (2015). Antioxidant capacity and polyphenolic content of seven Saudi Arabian medicinal herbs traditionally used in Saudi Arabia. *Indıan J Tradit Knowledge, 14*(3), 28-35.

47. Macakovaa, K., Opletala, L., Polasekb, M., Samkovac, V.,Jahodara, L. (2009). Free-radical scavenging activity of some European Boletales. *Natural Product Communications, 2*, 261-264.

48. Reis, F.S., Heleno, S.A., Barros, L., Sousa, M.J., Martins, A.,Santos-Buelga,C., Ferreria, I.C.F.R. (2011). Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from Northeast Portugal. *Journal of Food Science, 76,* 6.

49. Keleş, A., Koca, İ.,Gençcelep, H. (2011). Antioxidant properties of wild edible. *Journal Food Process Technology, 2,* 130.

50. Zeng, X., Suwandi, J., Fuller, J., Doronila, A., Ng, K. (2011). Antioxidant capacity and mineral contents of ediblewild Australian mushrooms. *Food Science and Technology International, 18*(4), 367–379.

51. Jaworska, G., Pogon, K., Bernas, E., Gabor, A. (2014).Vitamins, phenolics and antioxidant activity of culinary prepared *Suillus luteus (L.)* Roussel mushroom. *Food Science and Technology, 99,* 701-706.

52. Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., Ferreira, I.C.F.R. (2010). Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. *Food Chemistry, 119,*1443-1450.

53. Miura, Y., Kondo, K., Saito, T., Shimada, H., Fraser, P. and Misawa, N. (1998). Production of the carotenoids lycopene, carotene, and astaxanhin in the food yeast candida utilis. *Applied and Environmental Microbiology, 64,* 1226–1229.

54. Lindshield, B.L., Canene-Adams, K., Erdman Jr, J.W. (2007). Lycopenoids. *Journal of Nutrition*, *122*(11), 2161-2166.

55. Yaping, Z., Suping, Q., Wenli, Y., Zheng, X., Hong, S., Side, Y.,Da-pu,W. (2002). Antioxidant activity of lycopene extracted from tomato paste towards tri chloro methyl peroxyl radical CCl3O2. *Food Chemistry*, *77*, 209-212.

56. Barranco, P.G., Ocanas, L.G., Cabrera, L.V., Carmona, M.C.S., Ocanas, F.G., Gomez, X.S.R., Rangel,R.L. (2010). Evaluation of antioxidant, immunomodulating, cytotoxic and antimicrobial properties of different strains of Basidiomycetes from Northeastern Mexico. *Journal of Medicinal Plants Research*, *4*(17), 1762-1769.

57. Duman, R., Doğan, H.H., Ateş, A. (2003).*Morchella conica* (Pers.) Boudier and *Suillus luteus* (L.) S. F. Gray Makrofunguslarının Antimikrobiyal Aktiviteleri. *Selçuk Üniversitesi Fen Edebiyat Dergisi*, *22*, 19-24.

58. Santos, T., Tavares, C., Sousa, D., Vaz, J.A., Calhelha, R.C., Martins, A., Ferreira, I.C.F.R.,Vasconcelos, H.M. (2013). *Suillus luteus* methanolic extract inhibits cell growth and proliferation of a colon cancer cell line. *Food Research Intertational*, *53*, 476-481.

59. Vaz, J.A., Ferreira, I.C., Tavares, C., Almedia, G.M., Martins, A., Vesconcelos, M. (2012). *Suillus collinitus* methanolic extract ıncreases P53 expression and causes cell cycle arrest and apoptosis in a breast cancer cell line. *Food Chem*, *135*, 596–602.

60. Lage, H. (2008).An overview of cancer multidrug resistance: a stil unsolved problem. *Cellular and Molecular Life Sciences*, *65*, 3145-3167.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

SÜLFONAMİDOBENZOTİYAZOL TÜREVİ hGSTP1-1 İNHİBİTÖRLERİNİN TASARIMI

DESIGN OF SULFONAMIDOBENZOTHIAZOLE DERIVATIVES AS hGSTP1-1 INHIBITORS

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ÖZ

Amaç: *Glutatyon Transferazlar (GST), ekzojen ve endojen kaynaklı, elektrofilik ve hidrofobik bileşiklerin GSH ile konjugasyonunu katalizleyen Faz-II detoksifikasyon enzim ailesidir. GST izozimlerinden en önemlisi olan hGSTP1-1'in çok farklı insan kaynaklı tümörde fazla miktarda salgılandığı ve kanser tedavisinde çoklu ilaç direnç (MDR) gelişimine sebep olduğu bilinmektedir. Tüm bu nedenlerden dolayı son zamanlarda hGSTP1-1 enzimi kanser tedavisi için bir hedef haline gelmiştir. Bu çalışmanın amacı yeni hGSTP1-1 inhibitörleri tasarlamaktır.*

Gereç ve Yöntem: *5F-203 bileşiğinden hareketle, önilaç şeklinde etki gösterebileceği düşünülen bir seri sülfonamidobenzotiyazol türevi bileşik tasarlanmıştır. Bu bileşiklerin hGSTP1-1 enzimi ile etkileşimlerini incelemek üzere Schrodinger Maestro programı kullanılarak moleküler doking çalışmaları yapılmıştır.*

Sonuç ve Tartışma: *Bu çalışmada 5F-203 bileşiğinden hareketle, önilaç şeklinde etki gösterebileceği düşünülen bileşiklerin, GST enzimi aracılığıyla metabolizasyonu sırasında gerçekleşen hidrolizle 5F-203 ve türevi bileşikler açığa çıkararak antitümör özellik gösterebilecekleri düşünülmüştür. Tasarlanan bileşiklerin hGSTP1-1 enzimi ile etkileşimlerini incelemek üzere yapılan doking çalışmalarına göre bileşiklerin tamamının hGSTP1-1 enzimi ile kuvvetli etkileşimlerinin olduğu gözlenmiştir. Bileşiklerin tamamının hGSTP1-1 enzim inhibisyonu için önemli olan Arg13 ve Tyr7 ile hidrojen bağı ve Tyr108 ile pi-pi etkileşimleri gösterdiği belirlenmiştir. Bir sonraki basamak olarak bu bileşiklerin sentezi gerçekleştirilecek ve hGSTP1-1 enzimi üzerindeki etkileri deneysel olarak test edilecektir. Böylece yapı-etki ilişkileri tanımlanarak daha etkili yeni bileşiklerin tasarlanması ve böylece direnç mekanizması inhibe edilerek antikanser ilaçların etkinliğinin artırılması söz konusu olabilecektir.*

Anahtar Kelimeler: *antikanser, doking, hGSTP1-1, ilaç direnci, sülfonamidobenzotiyazol*

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ABSTRACT

Objective: *The glutathione transferases (GSTs) are a family of widely distributed Phase II detoxification enzymes that catalyse the conjugation of exogenius and endogenius electrophilic and hydrophobic compounds. GSTP1-1 is frequently overexpressed in rat and human tumours. It is suggested that overexpression of hGSTP1-1 by human tumor cells may play a role in multi drug resistance (MDR) to cancer chemotherapy. Hence, hGSTP1-1 can be a promising target for cancer treatment. The aim of this study is to design new hGSTP1-1 inhibitors.*

Material and Method*: a series of sulfonamidobenzothiazole derivatives which are thought to act as prodrug have been designed based on the 5F-203 compound. To examine the interactions of these compounds with the hGSTP1-1 enzyme, molecular docking studies were carried out, by using the Schrodinger Maestro Software.*

Result and Discussion: *In this study, considering the compound 5F-203, it is thought that the compounds thought to be effective in the form of prodrugs may exhibit antitumor properties by releasing 5F-203 and its derivative compounds by hydrolysis during metabolism by GST enzyme. It has been observed that all of the compounds have strong interactions with the enzyme hGSTP1-1, according to the doking studies conducted to examine the interactions of the designed compounds with the enzyme hGSTP1-1. It was determined that all of the compounds showed hydrogen bonding with Arg13 and Tyr7, which are important for hGSTP1-1 enzyme inhibition, and pi-pi interactions with Tyr108. As a next step, the synthesis of these compounds will be carried out and their effects on the enzyme hGSTP1-1 will be tested experimentally. Thus, by defining structure-activity relationships, it can be possible to design more effective new compounds and increase the effectiveness of anticancer drugs by inhibiting the resistance mechanism.*

Keywords: *anticancer, docking, drug resistance, hGSTP1-1, sulfonamidobenzothiazole*

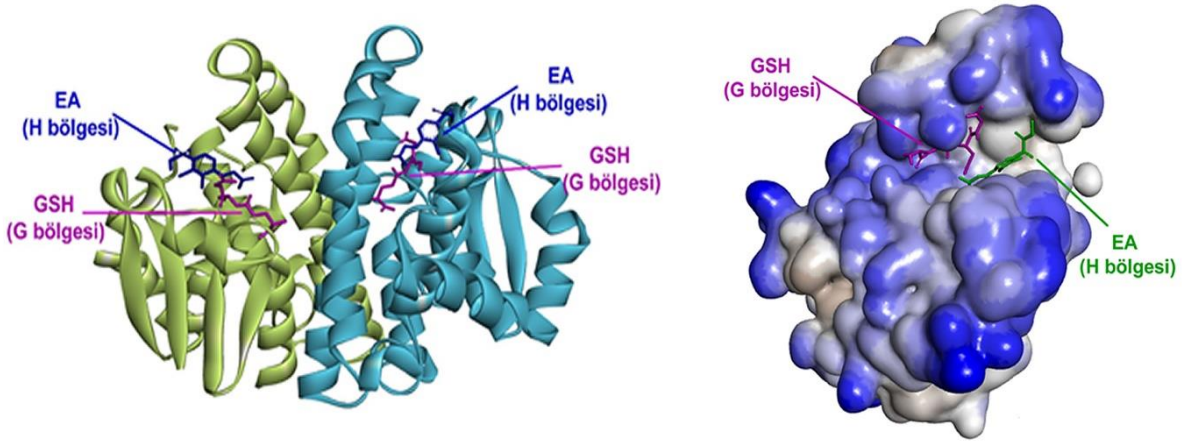
GİRİŞ

Çoklu ilaç direnci (MDR) günümüzde kanser tedavisinde en büyük engeli oluşturmaktadır. Bu direnç gelişimi, ilaca karşı permeabilitenin değişmesi ve hücrede GSH ile GST enzim seviyelerinin artmasıyla ilişkilendirilmiştir [1]. Bu bilgilere ilave olarak son yıllarda yapılan çalışmalar, π ve μ sınıfı GST enzimlerinin, hücresel yaşam ve ölüm sinyal iletimine katılan mitojenle aktive edilmiş protein kinaz (MAP kinaz) yolağındaki düzenleyici rolünün de kemoterapötik ilaçlara direnç gelişmesinde etkili olduğunu göstermiştir [2]. Bu nedenlerle kemoterapide, geleneksel kanser ilaçlarının (alkilleyici ajanlar) etkinliğinin düzenlenmesinde, GST enzim inhibitörlerinin kullanımının faydalı olabileceği düşüncesi doğmuştur.

Glutatyon transferazlar (GST), ekzojen ve endojen kaynaklı, elektrofilik bileşiklerin glutatyon (GSH) ile konjugasyonunu sağlayarak, genellikle daha kolay atılabilen ve daha az toksik metabolitlere dönüşümünü katalizleyen Faz II detoksifikasyon enzim ailesinin bir üyesidir [3]. GST izozimlerinden en önemlisi olan hGSTP1-1'in çok farklı insan kaynaklı tümörde fazla miktarda salgılandığı ve bu sistemle metabolize olan pek çok kanser ilacının metabolizmasını hızlandırarak; ilaçla hedeflenen etkiye ulaşılamamasına, bir başka deyişle çoklu ilaç direnç (MDR) gelişimine sebep olduğu bilinmektedir. Tüm bu nedenlerden dolayı son zamanlarda hGST P1-1 enzimi kanser tedavisi için bir hedef haline gelmiştir [4].

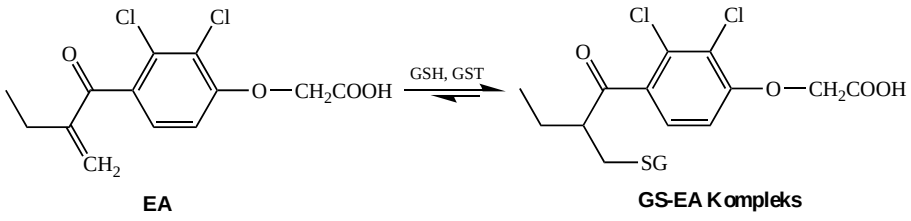
Doğada glutatyon S-transferaz aktivitesi gösteren enzimler, mitokondriyal, mikrozomal ve sitozolik olmak üzere üç alt gruptan oluşmaktadır [5,6]. Bu gruplardan, memelilerdeki sitozolik GST

enzimleri, kimyasal özellikleri, immünolojik reaktiviteleri ve aminoasit diziliş benzerliklerine göre alfa (α), zeta (ζ), teta (θ), pi (π), mü (μ), sigma (σ) ve omega (ω) olarak 7 alt gruba ayrılırlar [7]. Enzim, korunumlu dizileri içeren GSH bağlama bölgesi (G bölgesi) ve farklı substratları bağlayabilen hidrofobik bölge (H bölgesi) olmak üzere iki bölge içermektedir [8-10]. Herbir alt birimde de iki domain bulunmaktadır. Yaklaşık 80 rezidü uzunluktaki *N*-terminal domaininde α/β yapısal birimleri hakimdir. Tüm GST sınıflarında bu domain dizileri GSH'ı spesifik olarak bağlamak için korunmuşlardır. *C*-terminal domain ise tamamen α-helikal yapılardan oluşmaktadır. Hidrofobik bağlanma bölgesi (H bölgesi) iki domain arasında olup, çoğu hidrofobik bileşik bu bölge ile etkileşmektedir (Şekil 1) [4,7,11].



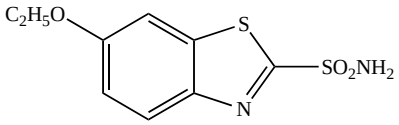
Şekil 1. Dimerik hGST P1-1 enziminin kristal yapısı (solda) ve hidrofobik yüzey alanı görünümü (sağda)

1968 yılında diüretik olarak kullanıma sunulan Etakrinik asit (EA), GST inhibitörü olarak incelenen ilk bileşiktir [12-14]. Bu bileşik, GSH'nin tiyol grubuna "Micheal eklemesi" konjugasyonu yoluyla bağlanıp (Şekil 2), GSH'ı tüketmesinin yanı sıra, substrat bağlanma bölgesi (H bölgesi)'ne doğrudan bağlanarak, GST A, M ve P'nin inhibe edilmesinden sorumludur [6,15-16].



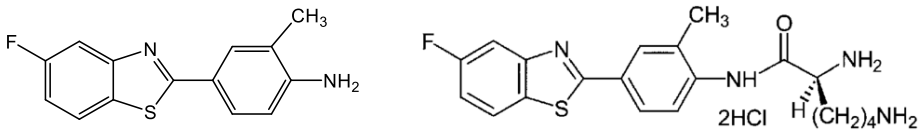
Şekil 2. GS-EA kompleks oluşumu

Zhao ve ark. [17], sülfonamit taşıyan bileşiklerin GST-katalizli hidrolizini incelemişler. Benzotiyazol-2-sülfonamit türevi bileşiklerin GST enzimi için iyi bir substrat olduğunu belirlemişlerdir (Şekil 3).



Şekil 3. Benzotiyazol-2-sülfonamit türevi bileşikler

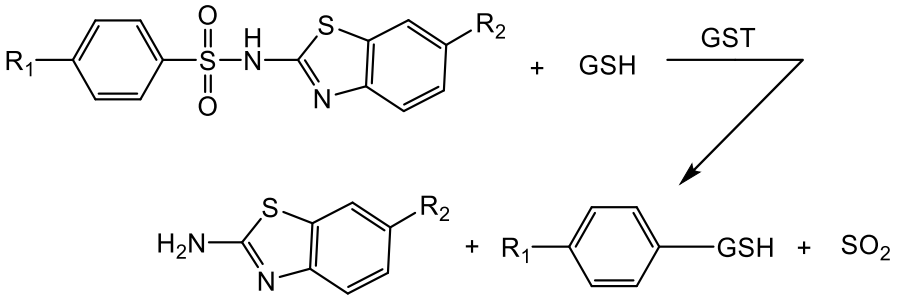
2-(4-Amino-3-metilfenil)-5-florobenzotiyazol (5F 203) bileşiği ve onun lizilamit yapısındaki ön ilacı, phortress kanser hastalığına karşı potent ve selektif etkileri nedeniyle klinik çalışmaları halen devam eden bileşiklerdir (Şekil 4) [18]. Ayrıca sülfonamit yapısı taşıyan birçok inhibitör bileşik de rapor edilmiştir [19].



5F 203 **Phortress**

Şekil 4. 5F 203 bileşiği ve onun lizilamit yapısındaki ön ilacı (Phortress)

Bu proje kapsamında 5F 203 bileşiğinden hareketle önilaç şeklinde etki gösterebileceği düşünülen bir seri sülfonamidobenzotiyazol türevi bileşik tasarlanmış ve sentezleri gerçekleştirilmiştir. Bu bileşiklerin, metabolizma sonucu gerçekleşen hidrolizle 5F 203 ve türevi bileşikler açığa çıkararak antitümör özellik gösterirken molekülden ayrılan diğer kısmın da hGSTP1-1 inhibitörü özellik gösterebileceği düşünülmektedir (Şekil 5). Böylece direnç mekanizması inhibe edilerek ilacın etkinliğinin artırılması söz konusu olacaktır.



Şekil 5. Sülfonamit türevi bileşiklerin GST-katalizli olası hidroliz reaksiyonu (**R_1:** H, Cl, F, Br, CF_3; **R_2:** H, F)

GEREÇ VE YÖNTEM

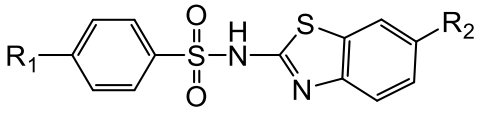
Moleküler doking teknikleri, bilgisayar destekli rasyonel ilaç tasarımında ilaç ya da ilaç adayları (ligantlar) ile enzim, nükleik asit, reseptör proteinlerinin birbirine nasıl uyum gösterdiklerini araştırmak için kullanılan önemi tartışılmaz bir yöntemdir [20-22]. Doking çalışmalarında, üç boyutlu yapısı belli olan enzime bağlanma enerjileri belirlenebilmekte ve enzimin bağlanma bölgesinde ligandın pozisyonu canlandırılabilmektedir. Bu yöntem, ilaç adayı moleküllerin protein hedeflerine karşı affinitesini, dolayısıyla biyolojik aktivitesini önceden tahmin edebilmek için uygulanmaktadır. Doking, ligandın uygun konformasyonu ile enzim arasında anahtar-kilit ilişkisine benzer bir uyum olması durumu olarak da açıklanabilmektedir [23].

Bu çalışmada, 5F-203 bileşiğinden hareketle, önilaç şeklinde etki gösterebileceği düşünülen bir seri sülfonamidobenzotiyazol türevi bileşik tasarlanmıştır. Bu bileşiklerin enzim ile etkileşimlerini incelemek üzere, Schrödinger Moleküler Modelleme Programı Glide modülü kullanılarak doking çalışmaları yapılmıştır. Doking çalışmaları için 2GSS pdb kodlu hGSTP1-1 enzimi kristal yapısı kullanılmıştır. Homodimer yapıda olan bu enzimin aktif yöresi grid oluşturma çalışmalarında kullanılmıştır. Protein yapısını iyileştimek için Schrödinger Suite içerisinde yer alan Protein Preparation Wizard arayüzü kullanılmıştır. Bağ uzunlukları düzenlenerek kristal yapısı içerisinde bulunmayan hidrojen atomları protein yapısına eklenmiştir. Zincirler arasında kalan aktif bölge seçilerek burada yer alan aminoasitler OPLS2005 kuvvet alanı kullanılarak yeniden düzenlenmiştir. Ligandların doking öncesi hazırlık işlemleri için protokol oluşturulması X-ray ligandı (etakrinik asit) ile yapılmıştır. Atom tiplerinin belirlenmesi, iyonizasyon/nötralizasyon süreçleri LigPrep programı ile pH 7.0 ± 2.0'de yapılmıştır. Grid oluşturma ve docking işlemleri Single Precision Mode (GlideScore SP) kullanılarak Glide [24-25] (Schrödinger Release 2018-1: Glide) ile gerçekleştirilmiştir. Oluşturulan grid dosyası üzerinde sentezleri gerçekleştirilen tüm bileşiklere doking işlemi uygulanmış ve olası etkileşimler incelenmiştir.

SONUÇ VE TARTIŞMA

Tasarımı gerçekleştirilen bileşiklerin hGSTP1-1 enzimi üzerindeki olası etkileşimleri incelenmek üzere yapılan doking çalışmalarına göre, bileşiklerin enzim ile etkileşimlerini gösteren 2D diagramlar Şekil 6'da, doking skorları ve etkileşimde yer alan aminoasitler Tablo 1'de verilmiştir.

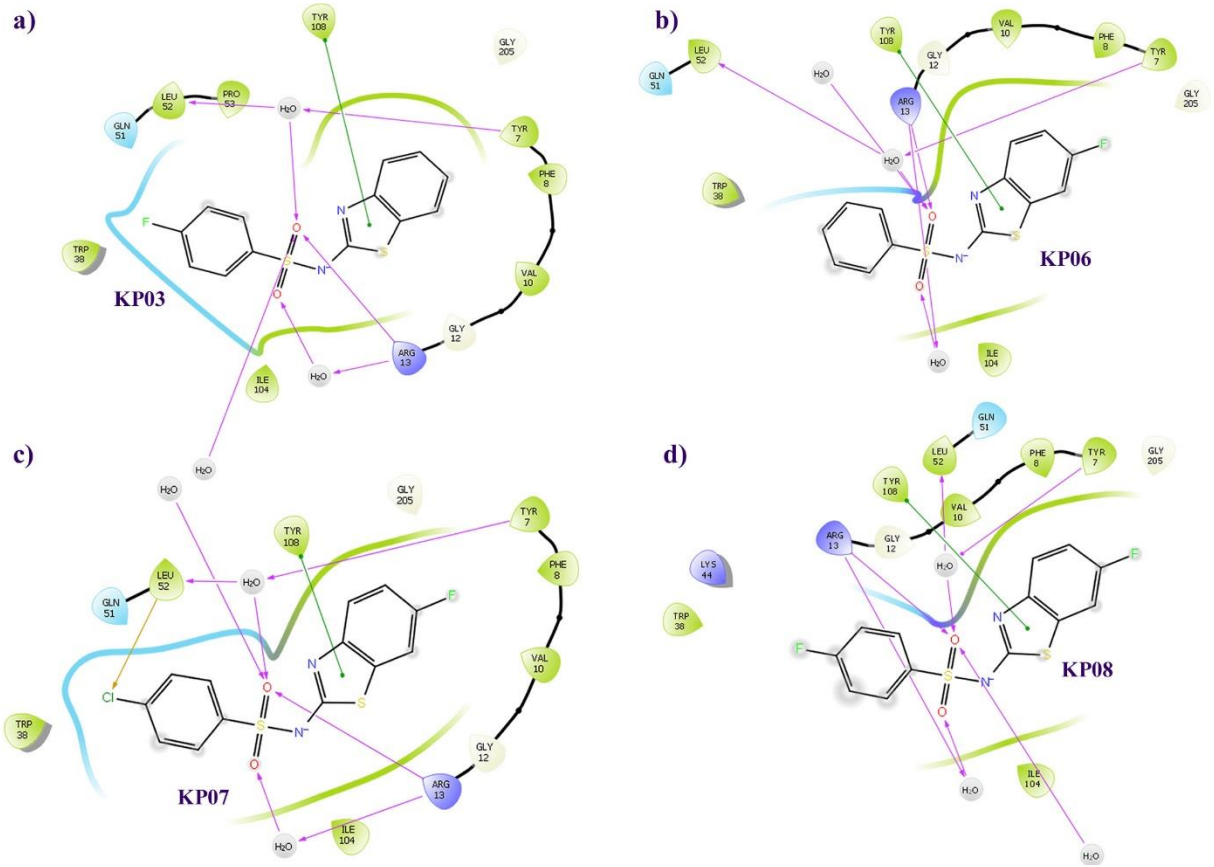
Tablo1. Doking sonuçları.



Bileşik	**R_1**	**R_2**	**Doking Skoru**	**Glide Skoru**	**Etkileşimler**
KP01	H	H	-5,426	-5,755	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f] (2)**, Trp38[a], Gln51[d], Leu52[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (3)
KP02	Cl	H	-5,351	-5,642	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f] (2)**, Trp38[a], Lys44[b], Gln51[d], Leu52[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (2)
KP03	F	H	-6,020	-6,330	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f] (2)**, Trp38[a], Gln51[d], Leu52[a], Pro53[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (3)
KP04	Br	H	-4,808	-5,102	**Tyr7[a,f]**, Phe8[a], Val10[a], **Arg13[b,f] (2)**, Trp38[a,g], Lys44[b], Gly50[c], Gln51[d], Leu52[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (3)
KP05	CF_3	H	-4,416	-4,799	Tyr7[a], Phe8[a,e], Val10[a], **Arg13[b,f] (2)**, Trp38[a], Ile104[a], Ser105[d], Tyr108[a,e], Thr109[d], Gly205[c], **H_2O** (2)
KP06	H	F	-6,282	-6,320	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f] (2)**, Trp38[a], Gln51[d], Leu52[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (3)
KP07	Cl	F	-6,602	-6,636	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f] (2)**, Trp38[a], Gln51[d], Leu52[a,g], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (3)
KP08	F	F	-6,221	-6,257	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f] (2)**, Trp38[a], Lys44[b], Gln51[d], Leu52[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (3)
KP09	Br	F	-4,696	-4,730	Tyr7[a], Phe8[a,e], Val10[a], **Arg13[b,f] (2)**, Trp38[a], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (2)
KP10	CF_3	F	-4,991	-5,038	Tyr7[a], Phe8[a], Val10[a], Gly12[c], Arg13[b], Val35[a], Trp38[a], Gln39[d], Lys44[b], Ile104[a], Tyr108[a,e], Gly205[c], **H_2O** (2)
Etakrinik Asit			-6,418	-6,418	**Tyr7[a,f]**, Phe8[a], Val10[a], Gly12[c], **Arg13[b,f,h] (2)**, Val35[a], Trp38[a], Ile104[a], Tyr108[a], Gly205[c], **H_2O^g** (5)

Koyu: H-bağı, a: hidrofobik (yeşil), b: pozitif yük (mor), c: glisin (açık yeşil), d: polar (turkuaz), e: pi-pi, f: su (gri), g: Halojen bağı h: tuz köprüsü

Özetle, bu çalışmada 5F 203 bileşiğinden hareketle, önilaç şeklinde etki gösterebileceği düşünülen bir seri sülfonamidobenzotiyazol türevi bileşik tasarlanmıştır. Bu bileşiklerin, GST enzimi aracılığıyla metabolizasyonu sırasında gerçekleşen hidrolizle 5F 203 ve türevi bileşikler açığa çıkararak antitümör özellik gösterebilecekleri düşünülmüştür. Tasarlanan bileşiklerin hGSTP1-1 enzimi ile etkileşimlerini incelemek üzere yapılan doking çalışmalarına göre bileşiklerin tamamının hGSTP1-1 enzimi ile kuvvetli etkileşimlerinin olduğu gözlenmiştir. Bileşiklerin tamamının (KP10 hariç), hGSTP1-1 enzim inhibisyonu için önemli olan Arg13 ve/veya Tyr7 ile hidrojen bağı ve Tyr108 ile pi-pi etkileşimleri gösterdiği belirlenmiştir. Bir sonraki basamak olarak bu bileşiklerin sentezi gerçekleştirilecek ve hGSTP1-1 enzimi üzerindeki etkileri deneysel olarak test edilecektir. Böylece yapı-etki ilişkileri tanımlanarak daha etkili yeni bileşiklerin tasarlanması ve böylece direnç mekanizması inhibe edilerek antikanser ilaçların etkinliğinin artırılması söz konusu olabilecektir.



Şekil 6. **a)** KP03 kodlu bileşiğe ait doking pozu. **b)** KP06 kodlu bileşiğe ait doking pozu. **c)** KP03 kodlu bileşiğe ait doking pozu. **d)** KP07 kodlu bileşiğe ait doking pozu. **e)** KP08 kodlu bileşiğe ait doking pozu.

TEŞEKKÜR

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KAYNAKLAR

1. Townsend, D.M., Tew, K.D. (2003). The role of glutathione S-transferase in anti-cancer drug resistance. *Oncogene, 22(47),* 7369-7375.

2. Adler, V., Yin, Z., Fuchs, S.Y., Benerza, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J., Ronai, Z. (1999). Regulation of JNK signaling by GSTp. *EMBO J, 18(5),* 1321-1334.

3. Armstrong, R.N. (1987). Enzyme-catalyzed Detoxication Reactions: Mechanisms and Stereochemistry. *Crit. Rev. Biochem. Mol. Biol., 22(1),* 39-88.

4. Bolelli, K., Musdal, Y., Aki-Yalcin, E., Mannervik, B., Yalcin, I. (2017). Synthesis and activity mechanism of some novel 2-substituted benzothiazoles as hGSTP1-1 enzyme inhibitors. *SAR QSAR Environ. Res., 28(11),* 927-940.

5. Jakobsson, P.J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., Persson, B. (1999). Common structural features of MAPEG-a widespread super family of associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci., 8,* 689-692.

6. Mathew, N., Kalyanasundaram, M., Balaraman, K. (2006). Glutathione S-transferase (GST) inhibitors. *Exp. Opin. Ther. Pat., 16(4),* 431-444.

7. Armstrong, R.N. (1997). Structure, Catalytic Mechanism, and Evolution of the Glutathione Transferases. *Chem. Res. Toxiol., 10(1),* 2-18.

8. Raghunathan, S., Chandross, R.J., Kretsinger, R.H., Allison, T.J., Penington C.J., Rule, G.S. (1994). Crystal structure of human class mu glutathione transferase GSTM2-2. Effects of lattice packing on conformational heterogeneity. *J. Mol. Biol., 238(5),* 815-832.

9. Reinemer, P., Dirr, H.W., Ladenstein, R., Schaffer, J., Gallay, O., Huber, R. (1991). The three-dimensional structure of class pi glutathione S-transferase in complex with glutathione sulfonate at 2.3 A resolution. *EMBO J., 10(8),* 1997-2005.

10. Sinning, I., Kleywegt, G.J., Cowan, S.W., Reinemer, P., Dirr, H.W., Huber, R., Gilliland, G.L., Armstrong, R.N., Ji, X., Board, P.G. (1993) Structure determination and refinement of human alpha class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. *J. Mol. Biol., 232(1),* 192-212.

11. Oakley, A. (2011). Glutathione transferases: a structural perspective. *Drug Metab. Rev., 43(2),* 138-151.

12. Tew, K.D., Bomber, A.M., Hoffman, S.J. (1988). Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res., 48(13),* 3622-3625.

13. Tozkoparan, B., Aytaç, S.P. (2007). Kanser kemoterapisinde terapötik hedef olarak glutatyon S-transferazlar. *Hacettepe Üniv. Ecz. Fak. Derg., 27(2),* 139-164.

14. Wang, Z., Jin, L., Wegrzyn, G., Wegrzyn, A. (2009). A novel method for screening the glutathione transferase inhibitors. *BMC Biochemistry, 10(6),* 1-11.

15. Chen, C., Wu, C., LU, X., Yan, Z., Gao, J., Zhao, H., Li, S. (2013). Coniferyl Ferulate, a Strong Inhibitor of Glutathione S-Transferase Isolated from Radix Angelicae sinensis, Reverses Multidrug Resistance and Downregulates P-Glycoprotein. *Hindawi Publ Corp,* 639083, 1-10.

16. Oakley, A.J., Lo Bello, M., Mazzetti, A.P., Federici, G., Parker, M.W. (1997). The glutathione conjugate of etharynic acid can bind to human pi class glutathione transferase P1-1 in two different modes. *FEBS Letters, 419,* 32-36.

17. Zhao, Z., Koeplinger, K.A., Peterson, T., Conradi, R.A., Burton, P.S., Suarato, A., Heinrikson, R.L., Tomasselli, A.G. (2009). Mechanism, Structure-Activity Studies, and Potential Applications of Glutathione S-Transferase-catalyzed Cleavage of Sulfonamides. *Drug Metab Dispos, 27(9),* 992-998.

18. Trapani, V., Patel, V., Leong, C.-O., Ciolino, H.P., Yeh, G.C., Hose, C., Trepel, J.B., Stevens, M.F.G., Sausville, E.A., Loaiza-Perez, A.I. (2003). DNA damage and cell cycle arrest induced by 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) is attenuated in aryl hydrocarbon receptor deficient MCF-7 cells. *British Journal of Cancer, 88,* 599–605.

19. Ertan-Bolelli, T., Musdal, Y., Bolelli, K., Yılmaz, S., Aksoy, Y., Yıldız, I., Akı-Yalcın, E., Yalcın, I. (2014). Synthesis, Biological Evaluation of 2-Substituted-5-(4-nitrophenylsulfonamido) benzoxazoles as Human GST P1-1 Inhibitors and Description of the Binding Site Features, *ChemMedChem, 9(5-SI),* 984-992.

20. Chikhi, A., Bensegueni, A. (2008). Comparative study of the efficiency of three protein-ligand docking programs. *J. Proteomics Bioinf., 1(3),* 161-165.

21. Schneider, G., Böhm H. J. (2002). Virtual screening and fast automated docking methods. *Drug Discovery Today, 7(1),* 64-70.

22. Toledo-Sherman, L. M., Chen, D. (2002). High-throughput virtual screening for drug discovery in parallel. *Curr. Opin. Drug Discovery Dev., 5(3),* 414–421.

23. Lengauer, T., Rarey, M. (1996). Computational methods for biomolecular docking, *Curr. Opin. Struct. Biol., 6(3),* 402–406.

24. Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., Perry, J. K., Shaw, D. E., Francis, P., Shenkin, P. S. (2004). Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy, *J. Med. Chem., 47(7),* 1739-1749.

25. Halgren, T. A., Murphy, R. B., Friesner, R. A., Beard, H. S., Frye, L. L., Pollard, W. T., Banks, J. L. (2004). Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening, *J. Med. Chem., 47(7),* 1750-1759.

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Doi: 10.33483/jfpau.742224

ORIGINAL ARTICLE / ÖZGÜN MAKALE

WOUND HEALING EFFECT OF NARINGIN GEL IN ALLOXAN INDUCED DIABETIC MICE

ALLOKSAN İLE DİYABET OLUŞTURULMUŞ FARELERDE NARİNGİN JELİN YARA İYİLEŞTİRİCİ ETKİLERİ

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ABSTRACT

Objective: *The aim of this study was to investigate the effects of naringin, a known flavonoid, on in vitro cytotoxicity, irritation and in vivo potential efficacy when topically applied to the diabetic wound.*

Material and Method: *In vitro direct contact assay and hen's egg chorio-allantoin membrane tests were used to evaluate irritation, and cytotoxicity potential of Naringin. In vitro antimicrobial activity was also tested. Topical treatments were administered once a day on the wound. Wound lesions were photographed and statistically analyzed. After the 10th day, histopathological parameters of tissues were assessed.*

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Result and Discussion: *The results showed that naringin gel has no cytotoxicity and irritation potential. Also, it displayed a weak antimicrobial effect. Aside from these, in vivo studies revealed that naringin gel showed statistically higher wound contraction and re-epithelialization, the thickness of granulation tissue, angiogenesis scores. The results indicated that naringin gel effectively enhanced diabetic wound healing, and so this formulation could be considered a promising therapeutic alternative for treating diabetic wounds.*

Keywords: *Cell culture, Diabetes mellitus, HET-CAM, Naringin, Wound healing*

ÖZ

Amaç: *Bu çalışmanın amacı, bir flavonoid olan Naringin'in in vitro sitotoksisite, irritasyon ile diyabetik yara üzerindeki in vivo potansiyel etkilerini araştırmaktı.*

Gereç ve Yöntem: *Naringin'in irritasyon ve sitotoksisite potansiyelini değerlendirmek için in vitro doğrudan temas deneyi ve tavuk yumurtası korio-allantoin membran testleri kullanıldı. Aynı zamanda in vitro antimikrobiyal etkinlikte test edildi. Topikal tedaviler günde bir kez yaraya uygulandı. Yara lezyonları fotoğraflandı ve istatistiksel olarak analiz edildi. 10. günden sonra dokuların histopatolojik parametreleri değerlendirildi.*

Sonuç ve Tartışma: *Sonuçlar, naringin jelin sitotoksisite ve tahriş potansiyeli olmadığını göstermiştir. Ayrıca, zayıf bir antimikrobiyal etki gösterdi. Bunların yanı sıra, in vivo çalışmalar naringin jelin istatistiksel olarak daha yüksek yara kapanması ve re-epitelyalizasyon, granülasyon dokusunun kalınlığı ve anjiyogenez skorları gösterdiğini ortaya koymuştur. Sonuçlar, naringin jelin diyabetik yara iyileşmesini etkili bir şekilde arttırdığını gösterdi ve bu nedenle bu formülasyon, diyabetik yaraların tedavisi için umut verici bir terapötik alternatif olarak düşünülebilir.*

Anahtar Kelimeler: *Diabetes mellitus, HET-CAM, Hücre kültürü, Naringin, Yara iyileşmesi*

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia over a prolonged period [1]. The majority of DM-related complications occur and are separated into macrovascular (cardiovascular disease) and microvascular (retinopathy, nephropathy, and neuropathy) complications [2].

Delayed wound healing and nonhealing ulcers (especially foot ulcers) are mainly caused by DM; more than 50% of these wounds become infected and require hospitalization [3]. Previous reports suggested that most of the nontraumatic lower extremity amputations are related to diabetes complications. Moreover, nearly half of the total cost of diabetes care is spent on the management of diabetes-related wounds. It is claimed that an excess of the United States (US) $25 billion is spent annually on the treatment of chronic wounds [4].

Physiological wound healing is a complex, finely balanced process that is generally thought to comprise four interconnected and overlapping stages: haemostasis (clot formation), inflammation, proliferation, and remodeling [5]. The wound healing process involves a variety of specialized cells such as platelets, macrophages, fibroblasts, epithelial and endothelial cells. The platelets have a key role in primary hemostasis. It is followed by the neutrophils, leading to the inflammatory phase and later taken over by the macrophages [6].

In diabetic patients, the healing process is stalled in the inflammatory phase that is manifested by the upregulation of proinflammatory cytokines, proteases, and reactive oxygen species (ROS), as well as cellular dysfunctions. Wound healing impairment in diabetic patients is associated with decreased levels of various growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b), or impaired collagen deposition and delayed inflammatory response [5].

Diabetics have impaired leukocyte function, and the metabolic abnormalities of diabetes lead to inadequate migration of neutrophils and macrophages to the wound, along with reduced chemotaxis. Due to these cellular changes, diabetic wounds are more prone to infection [7,8]. Diabetic foot ulcer patients have severe neuropathy, elevated leucocyte counts, and lower endothelium-dependent and independent vasodilation in the macro-circulation. These events decrease the cellular and growth factor responses, diminish the peripheral blood flow and local angiogenesis [6].

Standard treatments for diabetic wounds include debridement of necrotic tissue, revascularization surgery, infection control, mechanical offloading, control of blood glucose, foot care education, and limb elevation. However, these treatments are often insufficient to ensure satisfactory wound healing and are associated with high rates of limb amputation [9]. Therefore, alternative therapies are urgently needed to shorten the process of healing diabetic wounds using animal models.

The wound healing effect of several medicinal plants and/or plant-derived substances such as phenolic compounds have been investigated. More than 70% of wound healing pharmaceutical products are derived from plants, 20% are mineral based and the remaining are animal-derived products. Flavonoids are phenolic substances isolated from a wide range of vascular plants, with over 8000 individual compounds known and these compounds possess several biological activities such as antimicrobial, anti-inflammatory, antioxidant, and antidiabetic [10,11].

Naringin (4',5,7-trihydroxy flavanone 7-rhamnoglucoside) (Fig.1) is a major and active flavanone glycoside, isolated from the grape and citrus fruit species, which has been shown to have several pharmacological properties such as neuroprotective, hepatoprotective, antimicrobial, cardioprotective, anti-inflammatory, antioxidant, and renoprotective effects [12].

Hence, the main purpose of the current study was to enlighten the therapeutic ability of Naringin loaded gel with an excision wound model by evaluating several macroscopic and histological parameters in alloxan induced diabetic mice. On the other hand, *in vitro* alternative approaches for the assessment of safety and biocompatibility of novel formulations are of great importance. Therefore, cytotoxicity and irritation potential of Naringin loaded gel was evaluated using direct contact assay and hen's egg chorio-allantoin membrane (HET-CAM) test method.

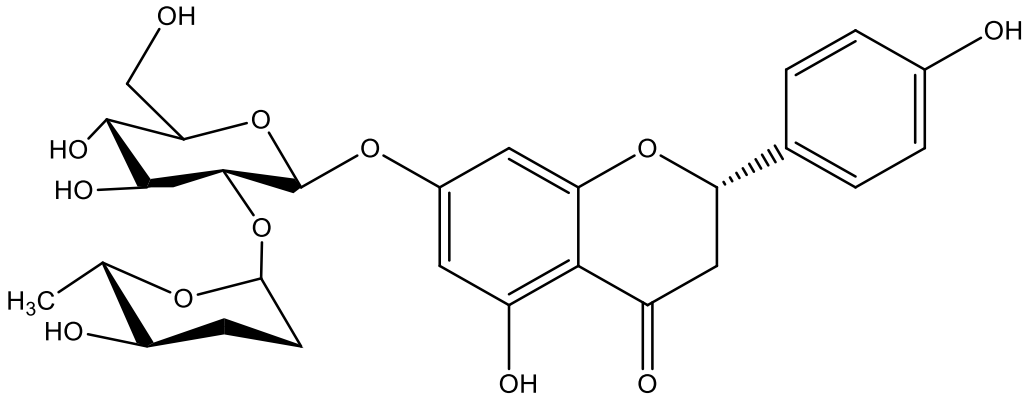

Figure 1. The chemical structure of Naringin

MATERIAL AND METHOD

Materials

Staphylococcus aureus ATCC 6538, *Candida albicans* ATCC 64548, *Listeria monocytogenes* ATCC 35152, and L929 healthy mouse fibroblast cell strains were purchased from American Type Culture Collection (ATCC, USA). Alloxan, Naringin, hydroxyethyl cellulose, and isopropanol were purchased from Sigma (Germany) and DMEM was purchased from Gibco (USA). All other reagents used in this study were of analytical or HPLC grade.

Preparation of topical extract gel

The base gel was composed of 2% hydroxyethyl cellulose. Firstly, 2 g hydroxyethyl cellulose was dispersed in distilled water by slowly stirring using a mechanical stirrer, at room temperature. Distilled water was added to the mixture slowly and the final weight of the mixture was completed to 100 g. Following, the blend was stirred constantly until the formation of a clear gel is observed. Then, Naringin gel (5%, w/w) was formulated by mixing base gel in a 95:5 ratio with Naringin by stirring gently.

In vitro Biological Assays

Antimicrobial activity

The *in vitro* antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) via the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) [13]. The commonly found microorganisms on colonized wounds were studied; *S. aureus* ATCC 6538, *Escherichia coli* NRLL B-3008, *L. monocytogenes* ATCC 35152 and

C. albicans ATCC 64548 strains were grown in Mueller Hinton Broth (MHB, Merck, Germany); *C. albicans* species were grown in RPMI broth for 24 h at 37°C in aerobic conditions. The bacterial suspension was adjusted to match 0.5 McFarland turbidity standards (corresponding to 10^8 colony forming unit (CFU)/mL) with sterile saline [14].

Cytotoxicity measurement by direct contact assay

A direct contact test that allows measuring quantitative evaluation of cytotoxicity was performed according to the International Organization of Standardization (ISO) recommended protocol [15]. L929 cell was cultured in DMEM supplemented with 10% FBS and 1% penicillin (10.000 units/mL) and streptomycin (10.000 µg/mL) at 37°C under a humidified atmosphere of 5% CO_2 [16]. The L929 cell is plated in a 24-well culture dish and incubated until it forms a semi-confluent layer for 24 hours. Then, for direct contact, positive control (4% SDS), negative control (PBS), Naringin gel and were applied to the filter papers with a pore size of 0.45 µm and were placed into the wells on the cell surface. The filter papers were prepared in sizes corresponding to the 1:10 of the surface of the well are and sterilized. After 24 h, cell medium and filter paper were discarded. MTT solution (0.5 mg/mL) was added to wells and cells were incubated (37°C) for a further 2 h. At the end of the incubation period, the cell culture medium was removed and 100 µL of isopropanol was added into each well for dissolving the formazan. The optical density (OD) was determined at 570 nm using an ELISA microplate reader (BioTek, USA). The reduction in viability compared to negative control is calculated using the equation below.

$$\text{Cell viability (\%)} = (OD_{570\ (\text{sample})} \times 100) / OD_{570\ (\text{negative control})}$$

Hen's Egg Chorio-Allantoin Membrane Test

For the assessment of the irritation capability of the formulation, the HET-CAM assay was conducted according to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [17]. The test system consists of fresh, clean, and fertile White Leghorn chicken eggs (50-60 g). Eggs are candled to detect the viability and development of embryo's prior to use and nonviable or defective eggs are eliminated. Eggs were placed in an incubator with a rotating tray and were incubated at 38±0.2°C and 58±2% relative humidity.

On day 9, eggs were removed from the incubator and the air cells of the eggs were marked and eggs were cut from the marked sections via a rotating saw blade and pared off. After moistening with 0.9% NaCl, eggs were placed into the incubator for up to 30 min. Afterward, 0.9% NaCl solution was decanted from the eggs. The egg white membrane was removed while avoiding any damage to the fine blood vessels. 0.3 mL of test substances, 0.9% NaCl (negative control; NC), and 0.1 N NaOH (positive

control; PC) were directly applied to the chorioallantoic membrane (CAM) surface and allowed in contact for 300 seconds. Monitored and recorded the time for the appearance of each of the noted endpoints in seconds.

The ICCVAM recommended irritation score (IS) analysis method was used for the evaluation. This method is based on the development of the three HET-CAM endpoints at fixed time intervals of 0.5, 2, and 5 min. By summing up the numerical time-dependent scores for lysis (blood vessel disintegration), hemorrhage (bleeding from the vessels), and coagulation (intra- and extra-vascular protein denaturation), a single numerical value is obtained with a maximum value of 21 (Table 1).

Table 1. Scoring scheme for irritation testing with the HET-CAM test method

Effect	**Score**		
	0.5 Min	**2 Min**	**5 Min**
Lysis	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

If the non-irritant response with NC and a severe irritant response with PC are monitored respectively, then the irritation test is considered acceptable. According to historical control studies, the IS value of negative control (0.9% NaCl) was 0.0, and the IS value of positive controls (1% SDS and 0.1 NaOH) ranged between 10 and 19, respectively. When using the IS analysis method, the severe irritancy classification for a test substance is assigned for values greater than nine.

In vivo Experiments

Test animals

The balb-c mice (26–30 g) were kept in standard cages and maintained in a climate room under controlled conditions of temperature (23±2°C), relative humidity (50±10%), with an inverted 12 h light: dark cycle (lights off at 8 a.m.) and they had access to standard nutritionally balanced diet and tap water *ad libitum.*

In this study, all animal procedures were in strict accordance with the guidelines of the European Council Directive (EU2010/63). All experiment procedures and protocols used in the study were reviewed and approved by the Ethics Committee of Istanbul Medipol University (No:38828770-604.01.01-E.66328) in accordance with the Standards for the Care and Use of Laboratory Animal.

Induction of diabetes mellitus

DM was induced by intraperitoneal (i.p.) injection of freshly prepared alloxan (150 mg/kg) after overnight (12 hours) fasting of mice. Alloxan was applied in an isotonic saline solution three times over a 48-hour period. Diabetic mice were confirmed by the fasting blood glucose concentration above 200 mg/mL on the 7th day after alloxan administration [18].

Experimental wounding and treatment

The animals were divided into the following 4 groups at random (n=7); Untreated (as control), Blank gel (as a vehicle), Naringin gel, Madecassol® (Bayer, Switzerland) (as standard). The mice were anesthetized with a single i.p. injection of Ketamine/Xylazine (100/10 mg/kg), after dorsal hair was removed with an electric razor, and the exposed skin area was cleaned with a povidone-iodine solution. Then, circular full-thickness wounds were made bilaterally on the back of each animal by a 5 mm punch biopsy. All treatments were conducted once a day and lasted for 10 days [19].

Macroscopic wound healing assessment

The day when wounds were made was designated as day 0, the process of wound healing was observed from then until day 10 after wounding. On days 0, 6, and 10 after the operation, the wounds were photographed with a digital camera (Canon, Japan). The wound area was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA), and wound contraction rate was calculated by the following equation:

$$\%\text{Wound contraction} = (\text{Wound Area}_{actual} / \text{Wound Area}_{initial}) \text{ X } 100$$

Histology

Mice were sacrificed on the 10th day and the whole wound with a margin of around 5 mm of ambient unwounded skin was excised for histological evaluation. All samples were fixed in 10% neutral formalin. After 24 h, the biopsies were bisected, embedded in paraffin, and sectioned in 5 μm thick layers. The 5 μm thick sections were mounted on glass slides, dewaxed, rehydrated with distilled water, and stained with hematoxylin-eosin (HE) to be evaluated via light microscopy.

Wound healing rates of each group was assessed by the ranking method defined by Galeano et al. [20]. Epidermal and dermal regeneration score system was; 1: Epidermal formation (poor) ≥ 20%; 2: Epidermal formation (incomplete) ≥ 40%; 3: Epithelial proliferation (moderate) ≥60%; 4: Epidermal re-modelling (complete) ≥ 80%. Score system of the granulation tissue thickness was; 4: very thick layer; 3: thick layer; 2: moderate layer; 1: thin layer.

The angiogenesis was evaluated by counting and identifying only mature vessels the existence of erythrocytes in the lumen. The presence/absence of edema, thrombosis, hemorrhage, congestion, and intra/intervascular fibrin formation was evaluated to identify poorly formed and well-formed capillary vessels. Score system of angiogenesis was; 1: High level of hemorrhage, edema, occasional congestion, and thrombosis; 2: Capillary vessels (newly formed, 3-4/site), occasional congestion, moderate edema and hemorrhage, intravascular fibrin deposition and absence of thrombosis; 3: Capillary vessels (newly formed, 5–6/site); 4: Capillary vessels (newly formed and normal appearing, >7/site).

Statistical analysis

Results were given as means±standard error of the mean (Mean±SEM). Statistical analyses were evaluated with GraphPad Prism 7.0 program. Statistical significance between groups was analyzed by one-way ANOVA. Values for $p<0.05$ were considered statistically significant.

RESULT AND DISCUSSION

Antimicrobial Activity

Various studies have demonstrated that the antimicrobial effects of various plant extracts have been attributed to their flavonoid contents [21]. Flavonoids are oxygen-containing aromatic compounds in which their wound healing properties are well known. These compounds can promote rapid wound healing due to their antimicrobial, antioxidant, and astringent properties [22].

In this study, the antimicrobial activity of the naringin was tested against various human pathogenic microorganisms such as *E. coli, S. aureus, L. monocyt,* and *C. albicans*. Table 1 shows the antimicrobial activity of naringin. Naringin showed weak antimicrobial activity against the tested microorganisms as compared to Amoxicillin and Ketoconazole. However, a MIC value of naringin of 31.2 µg/mL against *S. aureus* was determined (Table 2). *S. Aureus* has been reported as a common pathogen found on chronic wounds as diabetic wounds [3].

Table 2. Antimicrobial activity results of naringin (MICs in µg/mL)

	E. coli	***S. aureus***	***L. monocyt***	***C. albicans***
Naringin	500	31.2	250	500
Amoxicillin	0.5	≤0.125	0.5	
Ketoconazole				0.25

It was reported that Naringin inhibited *Actinomyces naeslundii, Actinomyces viscosus, Aggregatibacter actinomycecomitans, Enterococcus faecalis, E. coli, S. aureus, Lactobacillus casei,* and

C. albicans [23]. The mechanisms behind a possible antimicrobial effect of naringin are virtually unknown. However, it has been suggested that the antibacterial activity was related to the presence of the hydroxyl side groups [24].

Cytotoxicity results

For the *in vitro* cytotoxicity study, the direct contact test was chosen among the methods recommended by ISO [15] while the formulation's ability to cause irritation was tested using HET-CAM [25]. In this study, the cytotoxicity of naringin gel was evaluated on L929 fibroblast cell lines by direct contact assay after treatment with Naringin gel for 24 h. The percentage viability of the negative control group was considered 100%. As it is seen in the Figure 2, the naringin gel did not show any cytotoxic effect on L929 cells. According to the study by Auner *et al.* which investigate the antioxidant activity and cytotoxicity of naringin, there was no strong cytotoxic effect detected demonstrating that naringin is non-toxic [26]. Similarly, Fan *et al.* measured the cell viability of free naringin on KB cells and no cytotoxicity was observed [27].

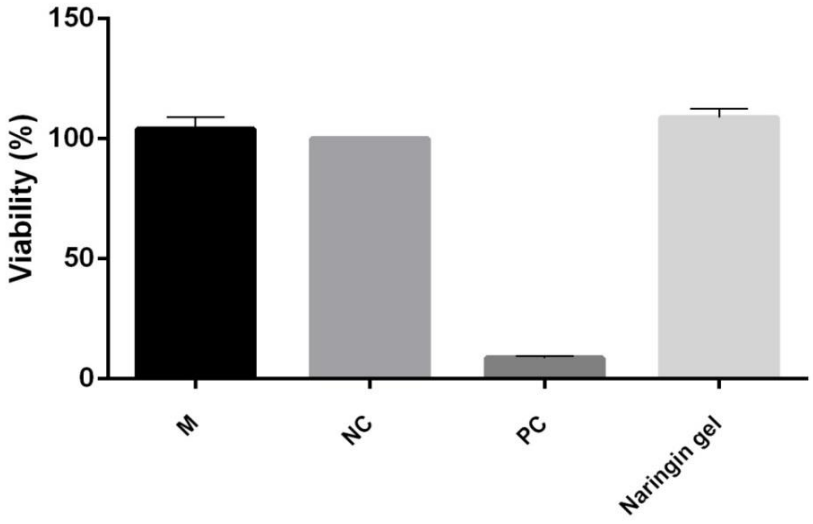

Figure 2. Effect of Naringin gel on L929 cell viability. Cells were treated with naringin gel for 24 h. M: Medium control, NC: Negative control (PBS), PC: Positive control (4% SDS). Statistical significances were indicated for each compound vs NC *(*p<0.05*).

HET-CAM

ICCVAM recommends the HET-CAM method as a highly preferred test for the prediction of eye injury hazard potential of chemicals, despite being not validated yet [28]. It is considered a more sensitive method due to chorioallantoic membrane's property of being thinner than mucous membranes

of the eye [29]. Even though the method is used for the testing of ocular irritation potential, it is also suggested for the skin irritation with a similar level of efficacy, comparably [25].

In the HET-CAM test method, 0.3 mL of NC, PC, naringin gel, and blank gel were directly applied to the CAM surface after removal of the inner membrane. The responses on the CAM surface were observed and scored over a period of 300 seconds with intervals of 0.5, 2, and 5 minutes. The results are given in Table 3.

Table 3. Irritation score of formulations according to HET-CAM protocol.

Tested Substance	IS Score	Result
NC	0	No irritation potential
PC	17	Strong irritation potential
Blank gel	0	No irritation potential
Naringin gel	0	No irritation potential

NC: negative control (0.9% NaCl); PC: positive control (0.1 N NaOH)

According to the IS analysis method, a value greater than 9 indicates the severe irritancy potential of a substance. As s stated by the results, the IS value of NC (0.9% NaCl) was scored as 0, and PC (0.1 N NaOH) was scored as 17. These results are considered within an acceptable range as stated in the ICCVAM protocol. Blank gel and the analyzed substance naringin gel did not induce lysis, hemorrhage, and coagulation therefore classified as non-irritant according to HET-CAM scoring (Figure 3).

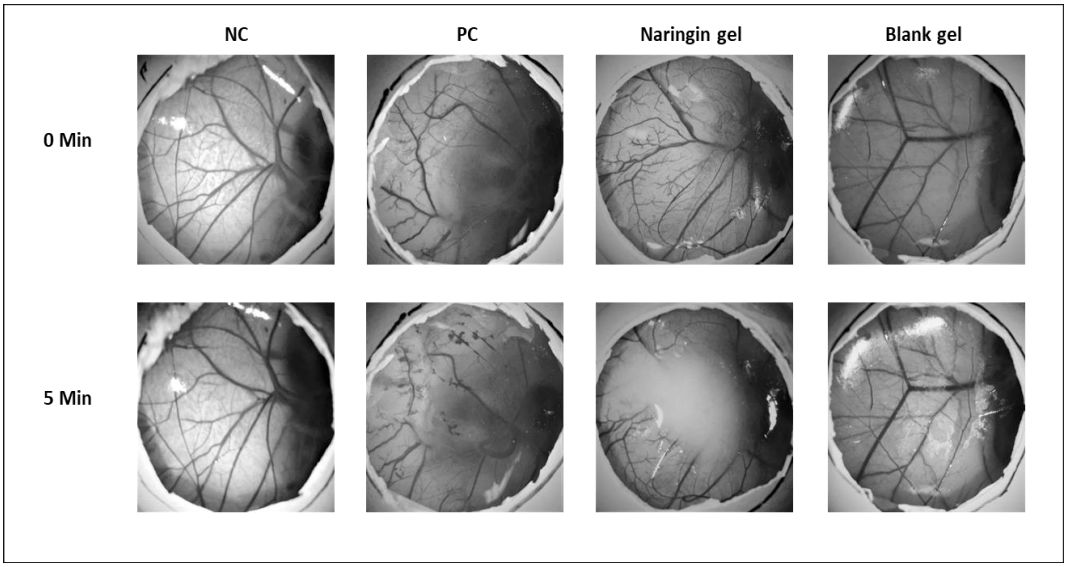

Figure 3. Macroscopic illustration of the HET-CAM test among NC (negative control), PC (positive control), Naringin gel, Blank gel groups before applying the samples, and after 5 min.

In vivo Experiments

Macroscopic wound healing and wound contraction

Excision wounds are used to study the rate of wound contraction and epithelialization [30]. Wound shrinkage in the early period of the healing process, granulation tissue formation, and re-epithelialization are crucial for wound closure [8]. Topically administered drugs are effective for faster wound contraction because of the larger availability at the wound site [31].

The macroscopic changes of the wound area in the different groups over a period of 10 days are presented in Figure 4. A crust forming was detected on the lesion area after a few days. A residual lesion was felt on the skin after the crust fell off. It was also observed that naringin and blank gel treatments show no dermal irritation or any allergic skin reaction.

The rate of wound closure was calculated as the percentage of wound reduction from the original wound on the 5^{th}, and 10^{th} days to estimate the wound repair ability of treatments in diabetic mice (Figure 5). The quantitative measurements of wound size are routinely used to assess initial wound size before and after debridement, as well as progress towards wound closure [32]. Naringin and Madecassol displayed a considerably improved wound healing on day 6 ($p<0.001$), and 10 ($p<0.001$) in comparison with control.

The healing percentage of scar tissue surface area ranged from 42.05% to 25.29% in the control group, 40.31% to 22.48% in blank gel group, 24.25% to 16.18% in madecassol group, and 31.85% to 17.57% in naringin group in the period from 6 to 10 days. Scars treated with Madecassol and naringin recovered quickly and the wound area rapidly decreased in size by the 10^{th} day compared to the control group.

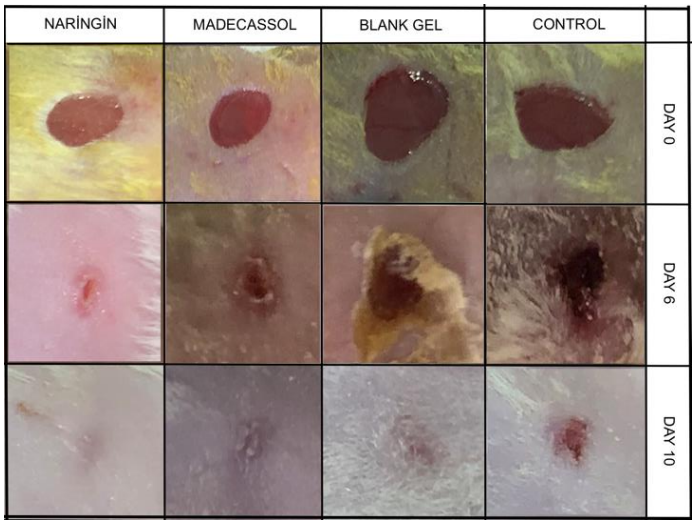

Figure 4. Effects of treatments on wound contraction. Photographic representation of contraction rate on different days of groups.

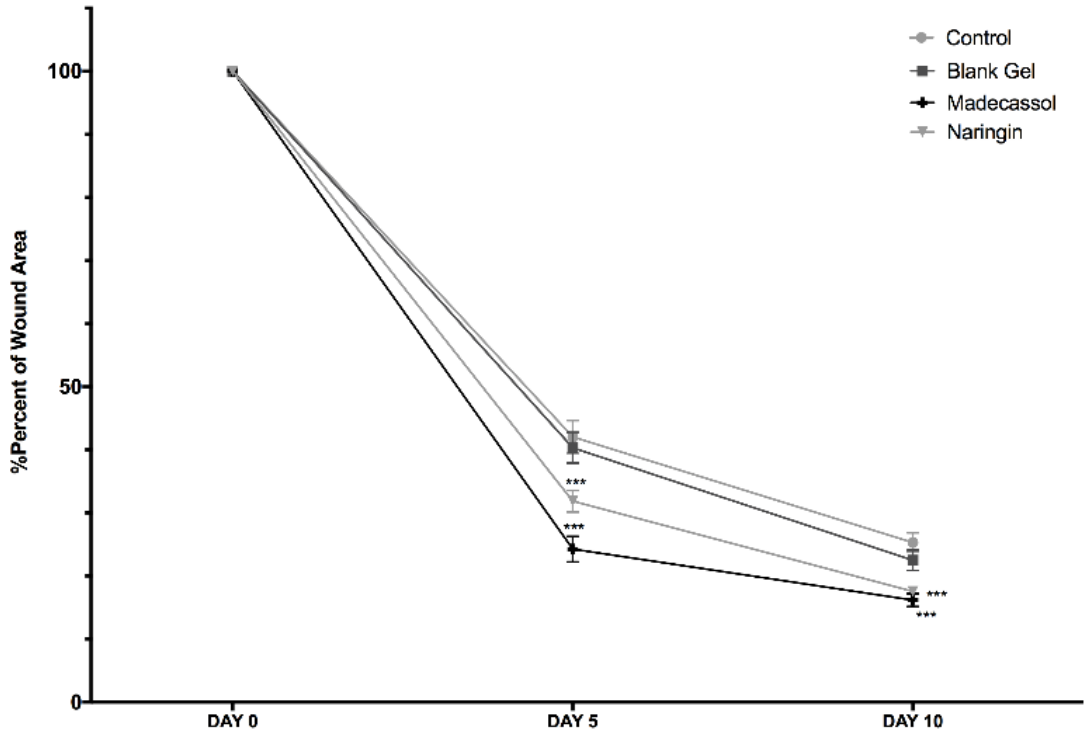

Figure 5. The healing rates of wound area among groups on days 0–10. Each data point represents the mean ± SEM.

Fibroblast performs a key position in the wound contraction in consequence of activation of collagen formation. It was revealed that topical naringin (4%) application improves wound healing in normoglycemic rats which could be due to the increased proliferation of fibroblasts [8].

Histology

The wound healing process consists of different phases such as granulation, collagenization, collagen maturation and scar maturation, which are concurrent but independent to each other [7]. Phases of the diabetic wound could be trapped in either of the phases for a longer time and also vanish the model synchrony of cascade that causes rapid healing [2]. The microscopic images captured during the histological analysis of the wound tissues on the 10th day are illustrated in Figure 6.

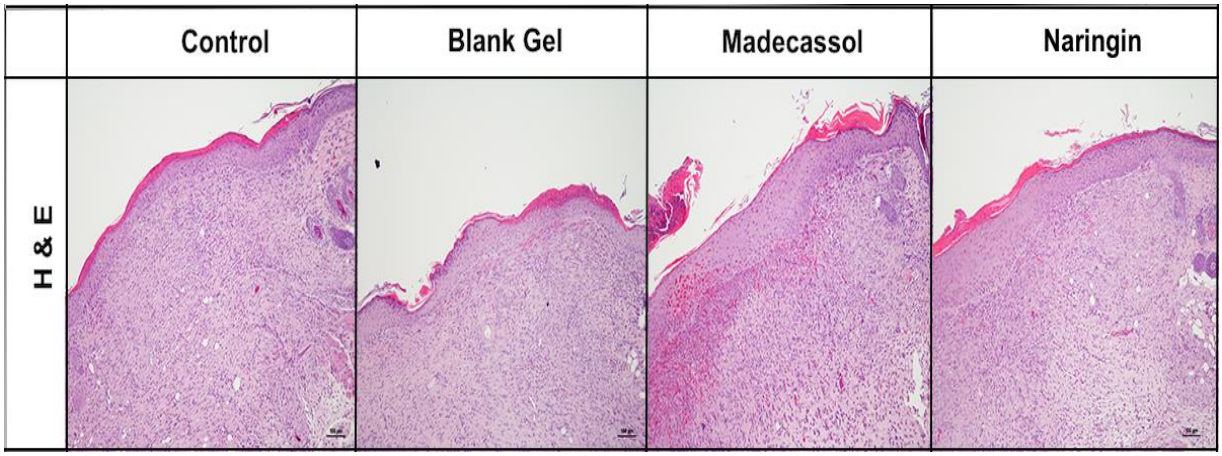

Figure 6. Histological examination of healed wound sections stained with H&E. The photomicrographs show healed wound sections isolated from mice treated with madecassol, blank, and naringin gel on day 10 after wounding.

Angiogenesis performed a key function in the matrix composition during the wound healing process. It included the development of endothelial cells involving neutrophils, macrophages, thrombocytes, keratinocytes, macrophages, and fibroblasts from main blood vessels, followed by migration, proliferation, and anastomosis to other vessels [12]. Histological outcomes revealed that there were significantly more blood vessel formations with the madecassol ($p<0.001$) and naringin ($p<0.001$) as compared to the control group (Figure 7).

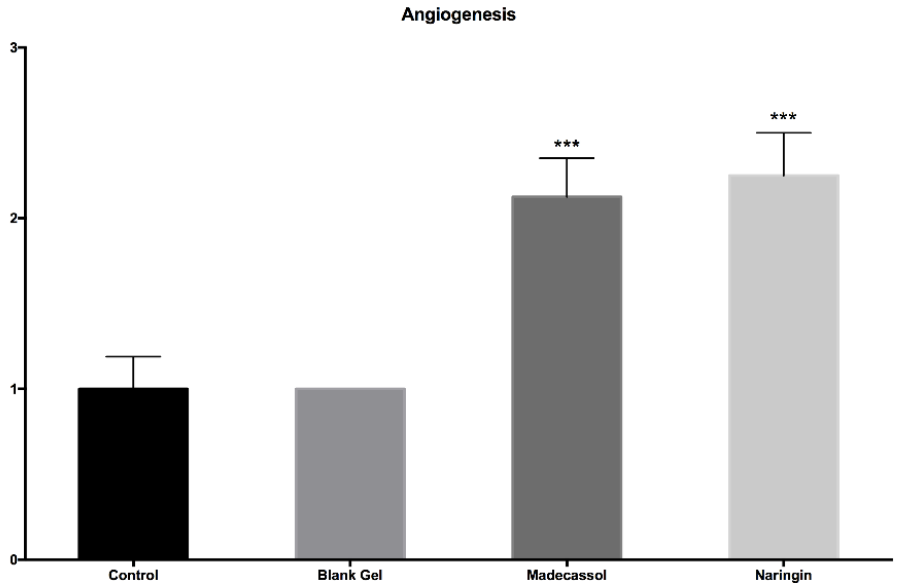

Figure 7. Microscopic assessment of angiogenesis among groups by histological scores. Significant to control; p<0.001(***)

It was reported that Naringin treatment showed promising wound healing ability by decreasing the expression of inflammatory cytokines such as TNF-a, IL-1b, IL-8, and IL-6 and apoptotic mediators along with the increased expression of growth factors such as VEGF and TGF-b, hence modulating collagen-1 gene expression to induce angiogenesis leading to wound healing [12]. It was also reported that naringin depressed the STZ-induced raised blood glucose concentration, enhanced antioxidant capacity, decreased expression of inflammatory cytokines such as TNF-a, IL-6, IL-1b and increased the expression of growth factors such as TGF-b1, VEGF-c, IFG-1, therefore, induced angiogenesis leading to less delay in healing of chronic diabetic ulcers [8].

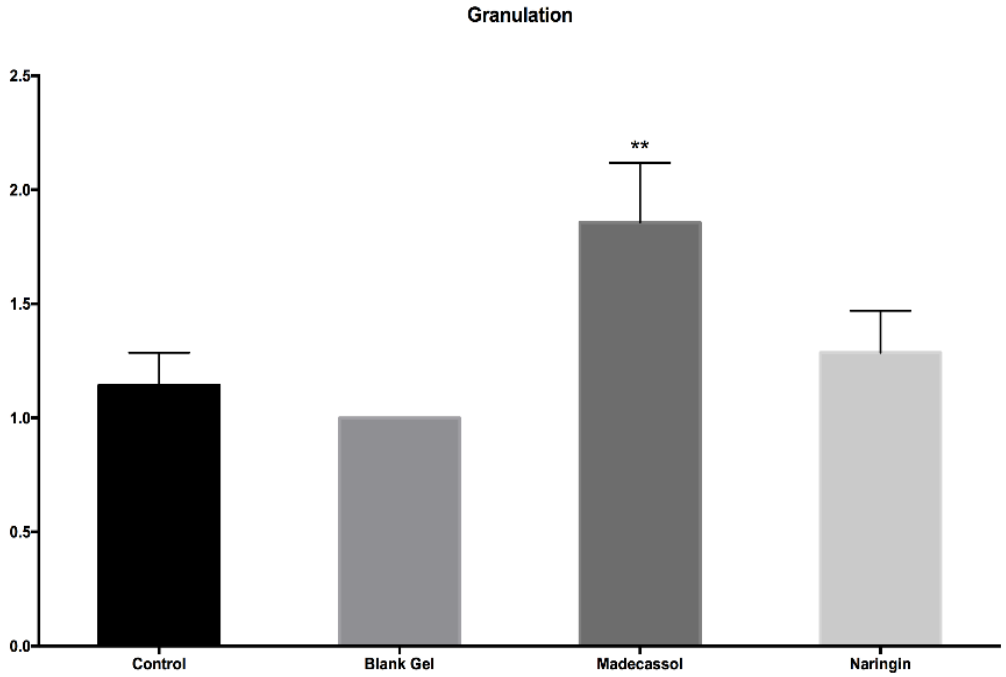

Figure 8. Microscopic assessment of granulation tissue thickness among groups by histological scores. Significant to control; $p<0.01$(**)

Diabetic wounds exhibit a persistent inflammatory phase associated with an impediment in the formation of mature granulation tissue and a reduction in wound tensile strength. This could be due to vascular damage resulting in ischemia [33]. According to the results obtained from the tissue samples; it was determined that madecassol ($p<0.01$) had a significant effect compared to the control group. In addition, it was observed that thicker granulation tissue was formed in the naringin group compared to the control after treatment (Figure 8).

The granulation tissue formation, dermis, and epidermis thickness are all closely related to the healing course. The granulation layer is initially formed from the base of the wound. Granulation tissue

additionally supports a matrix utilized by keratinocytes to migrate along which activates re-epithelialization from the wound edges [34].

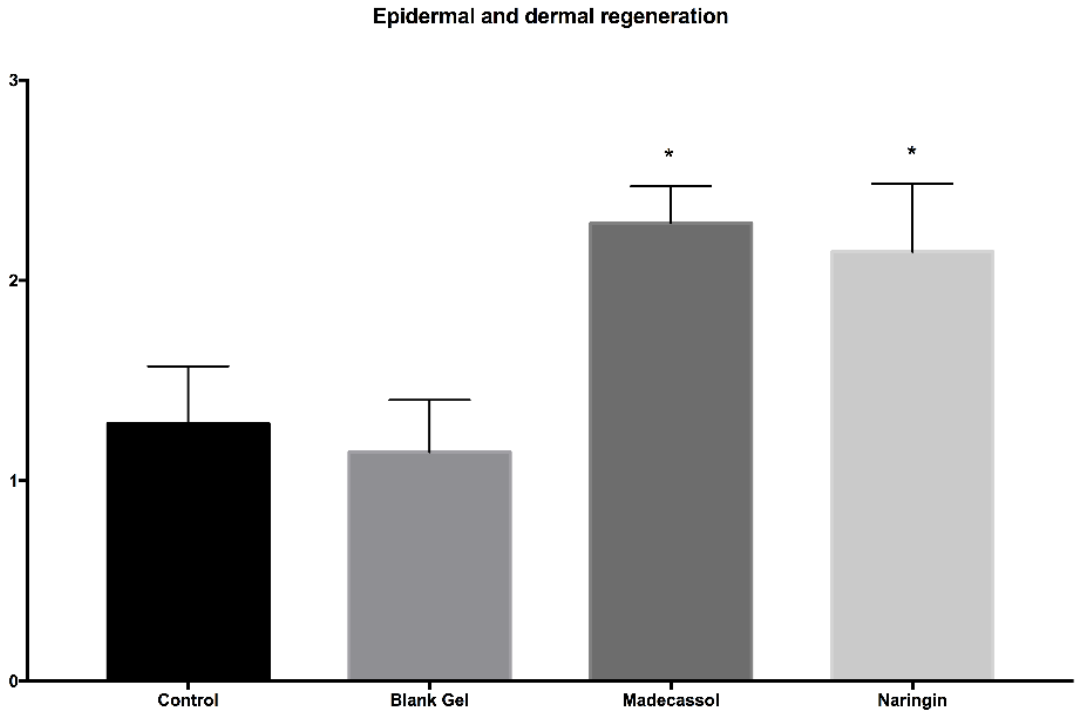

Figure 9. Microscopic assessment of epidermal-dermal regeneration among groups by histological scores. Significant to control; p<0.05(*)

Madecassol ($p<0.05$) and Naringin ($p<0.05$) groups showed significant dermal and epidermal regeneration scores in comparison to the control group (Fig. 9). Similarly, in a previous study which was conducted by Kandhare *et al.*, it was shown that daily Naringin (20, 40 and 80 mg/kg, p.o.) treatment was able to shorten foot ulcer healing time in diabetic rats [8].

In conclusion, the present study displayed that the Naringin gel did not cause lysis, hemorrhage, coagulation, and also did not possess cytotoxic activity, according to the irritation (HET-CAM) and cytotoxicity test results. Besides these, *in vitro* antimicrobial test revealed that Naringin exhibited weak antimicrobial activity. The data obtained from *in vivo* study demonstrated that naringin ameliorated wound closure and re-epithelization, enhanced granulation tissue formation, and improved epidermal regeneration in a diabetic wound model. In the current study, the topical administration of naringin gel was capable of effectively stimulating the wound healing process.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Okur, M.E., Karantas, I.D., Siafaka, P.I. (2017). Diabetes Mellitus: A Review on Pathophysiology, Current Status of Oral Pathophysiology, Current Status of Oral Medications and Future Perspectives. *Acta Pharmaceutica Sciencia, 55,* 61.

2. Shah, S.A., Sohail, M., Khan, S., Minhas, M.U., de Matas, M., Sikstone, V., Hussain Z., Abbasi M., Kousar M.et al. (2019). Biopolymer-based biomaterials for accelerated diabetic wound healing: A critical review. *International Journal of Biological Macromolecules, 139,* 975–993.

3. Siafaka, P.I., Zisi, A.P., Exindari, M.K., Karantas, I.D., Bikiaris, D.N. (2016). Porous dressings of modified chitosan with poly(2-hydroxyethyl acrylate) for topical wound delivery of levofloxacin. *Carbohydrate Polymers, 143,* 90–99.

4. Veith, A.P., Henderson, K., Spencer, A., Sligar, A.D., Baker, A.B. (2019). Therapeutic strategies for enhancing angiogenesis in wound healing. *Advanced Drug Delivery Reviews, 146,* 97–125.

5. Okur, M.E., Karantas, I.D., Şenyiğit, Z., Okur, N.Ü., Siafaka, P.I. (2020). Recent trends on wound management; new therapeutic choices based on polymeric carriers. *Asian Journal of Pharmaceutical Sciences*, January 23, 2020

6. Rao, S.S., Venkatesan, J., Prabhu, A., Rekha, P.D. (2020). Natural polymeric biomaterials in growth factor delivery for treating diabetic foot ulcers. *Journal of Drug Delivery Science and Technology, 55,* 101385.

7. Cho, H., Blatchley, M.R., Duh, E.J., Gerecht, S. (2019). Acellular and cellular approaches to improve diabetic wound healing. *Advanced Drug Delivery Reviews, 146,* 267–288.

8. Kandhare, A.D., Ghosh, P., Bodhankar, S.L. (2014). Naringin, a flavanone glycoside, promotes angiogenesis and inhibits endothelial apoptosis through modulation of inflammatory and growth factor expression in diabetic foot ulcer in rats. *Chemico-Biological Interactions, 219,* 101–112.

9. Gadelkarim, M., Abushouk, A.I., Ghanem, E., Hamaad, A.M., Saad, A.M. and Abdel-Daim, M.M. (2018). Adipose-derived stem cells: Effectiveness and advances in delivery in diabetic wound healing. *Biomedicine and Pharmacotherapy, 107,* 625–633.

10. Ahmed, O.M., Mohamed, T., Moustafa, H., Hamdy, H., Ahmed, R.R. and Aboud, E. (2018). Quercetin and low level laser therapy promote wound healing process in diabetic rats via structural reorganization and modulatory effects on inflammation and oxidative stress. *Biomedicine and Pharmacotherapy, 101,* 58–73.

11. Özay, Y., Güzel, S., Yumrutaş, Ö., Pehlivanoğlu, B., Erdoğdu, İ.H., Yildirim, Z., Türk, B.A., Darcan, S. (2019). Wound healing effect of kaempferol in diabetic and nondiabetic rats. *Journal of Surgical Research, 233,* 284–296.

12. Kandhare, A.D., Alam, J., Patil, M.V.K., Sinha, A. and Bodhankar, S.L. (2016). Wound healing potential of naringin ointment formulation via regulating the expression of inflammatory, apoptotic and growth mediators in experimental rats. *Pharmaceutical Biology, 54,* 419–432.

13. Karadağ, A.E., Demirci, B., Çaşkurlu, A., Demirci, F., Okur, M.E., Orak, D., Sipahi, H., Başer, K. H. C. (2019). In vitro antibacterial, antioxidant, anti-inflammatory and analgesic evaluation of Rosmarinus officinalis L. flower extract fractions. *South African Journal of Botany, 125,* 214–220.

14. Siafaka, P., Okur, M.E., Ayla, Ş., Er, S., Cağlar, E.Ş. (2019). Design and characterization of nanocarriers loaded with Levofloxacin for enhanced antimicrobial activity ; physicochemical properties , in vitro release and oral acute toxicity. *Brazilian Journal of Pharmaceutical Sciences, 55,* 1–13.

15. Standardization, C.-E.C. for (2009). ISO 10993-5:2009 - Biological evaluation of medical devices-Part 5: Tests for in vitro cytotoxicity. https://www.iso.org/standard/36406.html (21 April 2020).

16. Üstündağ Okur, N., Filippousi, M., Okur, M.E., Ayla, Ş., Çağlar, E.Ş., Yoltaş, A., Siafaka, P.I. (2018). A novel approach for skin infections: Controlled release topical mats of poly(lactic acid)/poly(ethylene succinate) blends containing Voriconazole. *Journal of Drug Delivery Science and Technology, 46,* 74–86.

17. HET-CAM (2010). ICCVAM-Recommended Test Method Protocol: Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method.

18. Okur, M.E., Polat, D.C., Ozbek, H., Yilmaz, S., Yoltas, A., Arslan, R. (2018). Evaluation of the antidiabetic property of capparis ovata desf. Var. Paleastina zoh. Extracts using in vivo and in vitro approaches. *Endocrine, Metabolic & Immune Disorders - Drug Targets, 18,* 489–501.

19. Okur, M.E., Ayla, Ş., Çiçek Polat, D., Günal, M.Y., Yoltaş, A. and Biçeroğlu, Ö. (2018). Novel insight into wound healing properties of methanol extract of Capparis ovata Desf. var. palaestina Zohary fruits. *Journal of Pharmacy and Pharmacology, 70,* 1401–1413.

20. Galeano, M., Altavilla, D., Bitto, A., Minutoli, L., Calò, M., Cascio, P. Lo, et al. (2006). Recombinant human erythropoietin improves angiogenesis and wound healing in experimental burn wounds*. *Critical Care Medicine, 34,* 1139–1146.

21. Özçelik, B., Kartal, M., Orhan, I. (2011). Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharmaceutical Biology, 49,* 396–402.

22. Budovsky, A., Yarmolinsky, L., Ben-Shabat, S. (2015). Effect of medicinal plants on wound healing. *Wound Repair and Regeneration, 23,* 171–183.

23. Gutiérrez-Venegas, G., Gómez-Mora, J.A., Meraz-Rodríguez, M.A., Flores-Sánchez, M.A., Ortiz-Miranda, L.F. (2019). Effect of flavonoids on antimicrobial activity of microorganisms present in dental plaque. *Heliyon, 5*(12), *e03013.*

24. Jang, M.H., Piao, X.L., Kim, J.M., Kwon, S.W., Park, J.H. (2008). Inhibition of cholinesterase and amyloid-&bgr; aggregation by resveratrol oligomers from Vitis amurensis. *Phytotherapy Research, 22,* 544–549.

25. Ishaka, A., Ismail, M., Imam, M.U., Mahmud, R., Sani, I.M., Zakaria, Z.A.B. (2017). Toxicity Evaluation, HET-CAM Irritation, and Anti-Irritant Potential of Rice Bran Wax Policosanol Nanoemulsion. *Journal of Nano Research, 49,* 44–55.

26. Auner, B.G., Wirth, M., Valenta, C. (2005). Antioxidative activity and cytotoxicity of four different flavonoids for dermal applications. *Journal of Drug Delivery Science and Technology, 15,* 227–232.

27. Fan, H., Zhang, P., Zhou, L., Mo, F., Jin, Z., Ma, J., Lin, R., Liu, Y., Zhang J. (2020). Naringin-loaded polymeric micelles as buccal tablets: formulation, characterization, in vitro release,

cytotoxicity and histopathology studies. *Pharmaceutical Development and Technology*, *25(5),547-555.*

28. Sipahi, H., Reis, R., Dinc, O., Kavaz, T., Dimoglo, A., Aydın, A. (2019). In vitro biocompatibility study approaches to evaluate the safety profile of electrolyzed water for skin and eye. *Human and Experimental Toxicology*, *38,* 1314–1326.

29. Dahl, J.E. (1999). Irritation of dental adhesive agents evaluated by the HET-CAM test. *Toxicology in Vitro*, *13,* 259–264.

30. Liu, W.Y., Tzeng, T.F., Liu, I.M. (2017). Healing potential of zerumbone ointment on experimental full-thickness excision cutaneous wounds in rat. *Journal of Tissue Viability*, *26,* 202–207.

31. Suriyamoorthy, S., Subramaniam, K., Jeevan Raj Durai, S., Wahaab, F., Pemila Edith Chitraselvi, R. (2014). Evaluation of wound healing activity of Acacia caesia in rats. *Wound Medicine*, *7,* 1–7.

32. Abu-Al-Basal, M.A. (2010). Healing potential of Rosmarinus officinalis L. on full-thickness excision cutaneous wounds in alloxan-induced-diabetic BALB/c mice. *Journal of Ethnopharmacology*, *131,* 443–450.

33. Patel, S., Srivastava, S., Singh, M.R., Singh, D. (2019). Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomedicine and Pharmacotherapy*, *112,* 108615.

34. Devalliere, J., Dooley, K., Yu, Y., Kelangi, S.S., Uygun, B.E., Yarmush, M.L. (2017). Co-delivery of a growth factor and a tissue-protective molecule using elastin biopolymers accelerates wound healing in diabetic mice. *Biomaterials*, *141,* 149–160.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

TÜRKİYE'DE YETİŞEN BAZI *CENTAURIUM* HILL TAKSONLARININ ANTİMİKROBİYAL AKTİVİTESİ

ANTIMICROBIAL ACTIVITY OF SOME CENTAURIUM HILL TAXA GROWING IN TURKEY

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ÖZ

Amaç: *Bu çalışmada, Türkiye'de yetişen beş Centaurium türünün (Centaurium erythraea subsp. rhodense, C. erythraea subsp. turcicum, C. maritimum, C. spicatum ve C. tenuiflorum subsp. acutiflorum) toprak üstü kısımlarından elde edilen etil asetat ve metanol ekstrelerinin seçilen Gram pozitif ve Gram negatif bakteriler ve Candida albicans mayasına karşı in vitro antimikrobiyal etkinliğinin test edilmesi amaçlanmıştır.*

Gereç ve Yöntem: *Ege (Muğla, Denizli, İzmir) ve Akdeniz (Antalya) bölgelerinden toplanan bitki örneklerinin toprak üstü kısımları kullanılarak elde edilen metanol ve etil asetat ekstrelerinin, Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Pseudomonas aeruginosa ATCC 27853 bakterileri ve Candida albicans ATCC 10231 mayasına karşı in vitro antimiktobiyal aktivitesi sıvı mikrodilüsyon yöntemi kullanılarak araştırılmıştır.*

Sonuç ve Tartışma: *Üzerinde çalışılan Centaurium türlerinin metanol ekstrelerinin S. aureus, E. faecalis, E. coli, K. pneumoniae, P. aeruginosa bakterileri ve C. albicans mayasına karşı orta düzeyde antimikrobiyal aktivite gösterdiği, etil asetat ile hazırlanan ekstrelerin ise etkinliğinin olmadığı tespit edilmiştir*

Anahtar Kelimeler: *Antimikrobiyal aktivite, Centaurium erythraea subsp. rhodense, C. erythraea subsp. turcicum, C. maritimum, C. spicatum, C. tenuiflorum subsp. acutiflorum.*

ABSTRACT

Objective: *In this study, in vitro antimicrobial activity of ethyl acetate and methanol extracts of the aerial parts of five Centaurium species (Centaurium erythraea subsp. rhodense, C. erythraea subsp. turcicum, C. maritimum, C. spicatum, and C. tenuiflorum subsp. acutiflorum), which are naturally grown in Turkey were*

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intended to be tested against selected Gram positive and Gram negative bacteria; and a yeast Candida albicans by using broth microdilution method.

Material and Method: *Methanol and ethyl acetate extracts of aerial parts were obtained from each of the Centaurium plants collected from Ege (Muğla, Denizli, İzmir) and Akdeniz (Antalya) Regions in Turkey tested on S. aureus ATCC 29213, E. faecalis ATCC 29212, E. coli ATCC 25922, K. pneumoniae ATCC 13883, P. aeruginosa ATCC 27853 as bacteria and C. albicans ATCC10231 as yeast by using broth microdilution method.*

Result and Discussion: *The methanol extracts of all Centaurium species have indicated moderate antimicrobial activities against S. aureus, E. faecalis, E. coli, K. pneumoniae, P. aeruginosa and C. albicans, but ethyl acetate extract have no activity.*

Keywords: *Antimicrobial activity, Centaurium erythraea subsp. rhodense, C. erythraea subsp. turcicum, C. maritimum, C. spicatum C. tenuiflorum subsp. acutiflorum.*

GİRİŞ

Geleneksel bitkisel tedavi uygulamalarındaki ilerleme insanlık tarihinin gelişimi ile paralellik gösterir (1,2). Modern tıpla birlikte bitkilerden çok sayıda aktif bileşen izole edilmiş ve aktiviteleri aydınlatılmıştır. Tüm dünyada hızla artan, tıbbi bitki ve kullanımlarını içeren literatürün derlenmesiyle Dünya Sağlık Örgütü, Avrupa Farmakopesi ve Avrupa İlaç Ajansı monografları gibi bitkilerin terapötik etkilerinin ortaya konduğu eserler sistematik olarak güncellenmektedir. Tıbbi bitkiler, özellikle kırsal bölgelerde hastalıkların önlenmesinde ve tedavisinde yerel halk tarafından yaygın olarak kullanılmaktadır (3). Bitkiler ilaç sanayisinde son derece önemli potansiyel antimikrobiyal ajanlar olarak kabul edilmektedirler. Yüzlerce bitki türü antimikrobiyal etkinlikleri bakımından test edilmiş olmasına rağmen, dünya üzerinde halen bitkilerin büyük bir çoğunluğu üzerinde yeterince çalışma yapılmadığı görülmektedir (4). Günümüzde, antibiyotik direncinin hızla artması, araştırmacıları alternatif çözüm yolları aramaya yönlendirmektedir. Bunlardan biri de doğal ürünlerden elde edilen yeni aktif bileşiklerin tespitidir. Bu nedenle bitki ekstreleri ve uçucu yağları ile antimikrobiyal aktivite çalışmaları güncelliğini yitirmeyecek bir konudur.

Bitkilerin içerdiği sekonder metabolitler (fenolik bileşikler, alkaloidler, glikozitler, terpenoidler ve uçucu yağlar) ve biyolojik aktiviteleri üzerine çok sayıda bilimsel çalışma mevcuttur. Bunlardan alkoloidler, flavonoidler, fenoller, kinonlar, tanenler, terpenler ve steroidler antimikrobiyal aktivitesi bilinen gruplardır (5,6).

Gentianaceae familyasında yer alan *Centaurium* Hill cinsinin Türkiye'de 7 tür ve 6 alt tür olmak üzere toplamda 11 taksonu doğal olarak yetişmektedir (7,8). Ülkemizde halk arasında "kırmızı kantaron, kızılkantaron, küçük kantaron, sıtma otu, tukul otu" adlarıyla bilinen *Centaurium* türleri taşıdığı acı maddeler nedeniyle halk arasında hazım kolaylaştırıcı ve iştah açıcı olarak kullanılmaktadır (9). Geleneksel tıpta, eski zamanlardan beri yiyecek ve içeceklere acı tat vermek için yaygın kullanımının yanında tonik, sakinleştirici, ateş düşürücü ve sindirimi düzenleyici olarak kullanıldığı da bilinmektedir (10). Özellikle Balkan halk tıbbında ateşte, diyabette, hepatitte, gutta (11, 12) iltihapta, hazımsızlıkta, gastritte (13) ve ateş düşürücü olarak kullanılmıştır (14).

C. erythreaya türünün bitkisel çay olarak dâhili ve harici kullanımları bulunmaktadır. Dekoksiyon halinde ateş düşürücü (14), infüzyon halinde ise ateş düşürmede, diyabette, hepatit ve gut tedavisinde (11,12) iltihap giderici, sindirim problemlerinde, gastritte, diyabette ve uyarıcı olarak kullanılmaktadır (10,15). İltihaplanmalarda, yara tedavisinde (16,17) yılan sokmalarında ve egzama benzeri gibi durumlarda harici olarak kullanımları bulunmaktadır (18). Almanya'da *Centauri* ekstreleri gastrointestinal ve ürolojik rahatsızlıklarda kullanılan ilaçların bileşiminde yer almaktadır (19,20).

Son yıllarda ülkemizde yapılan çeşitli etnobotanik çalışmalarda Türkiye'de yetişen *Centaurium* türlerinin halk arasında sıtma, anoreksi, hemoroit, mide ülseri, guatr ve egzama, mide ağrısı, bel ağrısı ve bağırsak iltihabı gibi hastalıkların tedavisinde kullanıldığı tespit edilmiştir (21,22).

Orhan ve ark. (2017) bazı *Centaurium* türlerinin asetilkolin esteraz enzim inhibisyonu aktivitesini araştırmışlar ve *C. erythraea* subsp. *rhodense* türünün AChE'yi %50'den fazla inhibe ettiğini belirtmişlerdir (23).

C. erythraea bitkisi üzerinde çok sayıda *in vivo* ve *in vitro* çalışmalar bulunmaktadır. *İn vivo* olarak antienflamatuar, antipiretik, antidiyabetik, diüretik, hepatoprotektif aktive çalışmaları, *in vitro* olarak gastroprotektif, antidiyabetik, antioksidan, antibakteriyel, sitotoksik, antimutajenik, sindirim sistemi üzerine ve insektisit aktivite çalışmaları yapılmış ve etkili bulunmuştur (24).

Bu çalışmada Türkiye'de yetişen bazı *Centaurium* (*Centaurium erythraea* Rafn. subsp. *rhodense* (Boiss. & Reuter) Melderis, *C. erythraea* subsp. *turcicum* (Velen.) Melderis, *C. maritimum* (L.) Fritsch, *C. spicatum* (L.) Fritsch ve *C. tenuiflorum* (Hoffm. & Link) Fritsch subsp. *acutiflorum* (Schott) Zeltner) türlerinin toprak üstü kısmından elde edilen etilasetat ve metanol ekstrelerinin antimikrobiyal aktivitesinin bazı Gram pozitif ve Gram negatif bakteriler ile *Candida albicans* mayasına karşı *in vitro* olarak test edilmesi amaçlanmıştır.

GEREÇ VE YÖNTEM

Bu çalışmada kullanılan bitkisel materyaller (*Centaurium erythraea* subsp. *rhodense, C. erythraea* subsp. *turcicum, C. maritimum, C. Spicatum, C. tenuiflorum* subsp. *acutiflorum*) Türkiye'nin çeşitli yerlerinden toplanmıştır. Bitkilerin toprak üstü kısımlarından örnekler alınmış, herbaryum örnekleri hazırlanmıştır. Herbaryum örnekleri Ankara Üniversitesi Eczacılık Fakültesi Herbaryumu'na (AEF) kayıt edilip, dolaplara yerleştirilmiştir. Çalışmada kullanılan türler Tablo 1'de verilmiştir.

Tablo 1. Çalışma materyallerinin Toplandığı yerler, tarihler ve Herbaryum numaraları

Tür adı	Toplandığı Yer	Toplandığı Tarih	Herbaryum Numarası
C.erythraea subsp. *rhodense*	Muğla: Marmaris, Hisarönü, 10 m	30.05.2010	AEF 26014
C. erythraea subsp. *turcicum*	Denizli: Acıpayam, Kelekçi, Olukbaşı üstü, Bozdağ, Geyran Yaylası, 1380-1400 m	22.07.2010	AEF 26015
C. maritimum	İzmir: Çeşme'nin 15 km doğusu, 80 m.	04.05.2011	AEF 26016
C. spicatum	Muğla: Dalyan İztuzu arası, 7 m.	31.05.2010	AEF 26017
C. tenuiflorum subsp. *acutiflorum*	Antalya: Demre, Demre Kuşcenneti, deniz seviyesi	04.06.2010	AEF 26018

Bitkisel materyallerinin ekstraksiyonu: Bitki numuneleri gölgede kurutulduktan sonra toz haline getirilmiştir. İlk olarak 10 gr bitki toz edilip 150 ml etil asetat içerisinde 3 gün (150 x 3=450 ml etil asetat), daha sonra ise her seferinde 150 ml metanolde (150 x 3=450 ml metanol), 3 gün olmak üzere maserasyon yolu ile ekstre edilmiştir. Ekstreler süzüldükten sonra kuruyana kadar vakum altında buharlaştırılmıştır. Antimikrobiyal çalışma için her numune 40 mg/10 ml olarak kendi çözücüsünde çözülerek kullanılmıştır.

Antimikrobiyal aktivite çalışmaları

Mikroorganizma süspansiyonlarının hazırlanması: Deneyde kullanılan mikroorganizmalar, Ankara Üniversitesi Eczacılık Fakültesi Farmasötik Mikrobiyoloji Anabilim Dalı kültür koleksiyonunda yer alan Gram pozitif bakteriler *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212; Gram negatif bakteriler *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 ve maya olarak *Candida albicans* ATCC 10231'dir.

Antimikrobiyal aktivite çalışmaları, maya için bazı modifikasyonlar yapılarak sıvı mikrodilüsyon metodu ile CLSI kılavuzuna uygun olarak gerçekleştirilmiştir (25,26). Bakteriler Mueller-Hinton Broth (MHA, Merck) ve maya Sabouraud Dextrose Broth (SDA, Oxoid) besiyerine ekilmiş ve 35 ± 2°C'de 20 saat inkübasyona bırakılmıştır. Bir gecelik kültürden izole edilen

kolonilerden, % 0.85 NaCl çözeltisinde 0.5 McFarland standardında süspansiyon hazırlanmıştır. Bu oran daha sonra bakteriler için MHB besiyeri ile 2,5 x 10^5 cfu / ml nihai konsantrasyonu, maya için SDB besiyeri ile 2.5 x 10^3 cfu / ml nihai konsantrasyonu verecek şekilde seyreltilmiştir.

Sıvı Mikrodilüsyon Yöntemi

Centaurium türlerinin metanol ve etil asetat ekstrelerinin antimikrobiyal aktivitesi sıvı mikrodilüsyon yöntemi ile test edilmiştir. İlk olarak, bakteri için 100 µl MHB besiyeri ve maya için 100 µl SDB besiyeri, mikroplakların her kuyucuğuna ilave edilmiştir. Her bir örnek için, ilk kuyucuğa 100 µl ekstre eklenmiş ve iki katlı 8 seri dilüsyon yapılmıştır. Seri seyreltmeden sonra, kuyucuklara 100'er µl bakteri süspansiyonları ilave edilmiş ve mikroplaklar 35 ± 2 ° C'de 24 saat inkübasyona bırakılmıştır. Standart antibiyotik olarak siprofloksasin, antifungal olarak mikonazol kullanılmıştır. Üreme ve sterilite kontrol kuyucukları eklenmiştir. İnkübasyon süresinin sonunda, mikroorganizma üremesi olmayan son kuyucuktaki konsantrasyon Minimum İnhibisyon Konsantrasyonu (µg/ml) olarak değerlendirilmiştir. Tüm deneyler kontrol amacıyla iki paralel olarak yürütülmüştür.

SONUÇ VE TARTIŞMA

Antimikrobiyal aktivite

Bu çalışmada Gram pozitif *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212; Gram negatif *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 bakteri ve *Candida albicans* ATCC 10231 maya standart suşları kullanılmıştır.

Tüm metanol ekstrelerinin Gram pozitif bakterilerden *S. aureus* (250-500 µg/ml) ve *E. faecalis*'e (250-500 µg/ml), Gram negatiflerden *E. coli* (250-500 µg/ml), *K. pneumoniae* (500 µg/ml) ve *P.aeruginosa*'ya (125µg/ml) ve maya *C. albicans'a* (125-250 µg/ml) karşı etkileri Morales ve ark. (27) tarafından belirtilen ekstre aktivite konsantrasyon aralığı değerlerine göre orta düzeyde olup, standart antibiyotik siprofloksasine ve antifungal mikonazole kıyasla çok düşük bulunmuştur. Etil asetat ekstrelerinin aktivitesinin olmadığı gözlenmiştir. *C. tenuiflorum* subsp. *acutiflorum* test edilen *Centaurium* ekstreleri arasındaki tüm mikroorganizmalara karşı en etkili takson olduğu; *C. erythraea subsp. turcicum*'un ise en az etkili takson olduğu görülmüştür. Bütün ekstrelerde *Pseudomonas aeruginosa'*ya karşı etkinin diğer Gram negatiflere göre daha iyi olduğu tespit edilmiştir. Yine bütün ekstrelerde Gram negatifler arasında E. *coli'* ye karşı etki *K. pneumoniae*'ye karşı etkiden daha iyi olarak belirlenmiştir. Bu çalışma sonucunda elde ettiğimiz *in vitro* antimikrobiyal aktivite sonuçları Tablo 2' de verilmiştir.

Sonuç olarak, bu çalışma *Centaurium* türlerinin metanol ekstrelerinin değişken antimikrobiyal aktiviteye sahip olduğunu göstermektedir. Kırbağ ve ark. (2009) çeşitli bitki ekstreleri ile yaptıkları çalışmada disk difüzyon yöntemini kullanmış ve *C. erytraea* ekstresinin antimikrobiyal aktivitesini *P. aeruginosa*, *E. coli*, *S. aureus*, *C. albicans*, *Candida glabrata* ve *Candida tropicalis*' e karşı etkisiz; *Bacillus megaterium* (13 mm), *K. pneumoniae* (8 mm) ve *Proteus vulgaris* (7 mm)'e karşı çok düşük olarak belirtmişlerdir (28). Siler ve ark. (2014) sıvı mikrodilüsyon yöntemiyle yürüttükleri çalışmada *C. erythraea*, *C. tenuiflorum*, *C. littorale* ve *C. pulchellum* türlerinden elde ettikleri metanol ekstrelerinin antibakteriyel etkisini *Bacillus cereus* (0.05-0.20 mg/ml), *Micrococcus flavus* (0.10-0.25 mg/ml), *S. aureus* (0.10-0.20 mg/ml), *Listeria monocytogenes* (0.10-0.25 mg/ml), *E. coli* (0.05-0.20 mg/ml), *Enterobacter cloacae* (0.10-0.25 mg/ml), *P. aeruginosa* (0.10-0.25 mg/ml), *Salmonella typhimurium* (0.05-0.25 mg/ml) üzerinde; antifungal etkisini *Penicillium funiculosum* (0.10-0.20 mg/ml), *Penicillium ochrochloron* (0.20-0.40 mg/ml), *Trichoderma viride* (0.20-0.40 mg/ml), *Aspergillus fumigatus* (0.20-0.40 mg/ml), *Aspergillus niger* (0.20-0.40 mg/ml), *Aspergillus flavus* (0.20-0.40 mg/ml), *Aspergillus versicolor* (0.10 mg/ml) ve *C. albicans* (0.10-0.40 mg/ml) üzerinde çalışmış ve sonuçlarını standart antimikrobiyallerle karşılaştırdıklarında çok iyi olarak rapor etmişlerdir (29). Bouyahya ve ark. (2019) *C. erythraea* uçucu yağının antibakteriyel etki değerini sıvı mikrodilüsyon yöntemiyle araştırmış; Gram pozitif bakteriler üzerindeki etkinin [*S. aureus* CECT 994 (0.125-0.25 µg/ml), *L. monocytogenes* serovar 4b CECT 4032 (0.25 µg/ml), *B. subtilis* 6633 DSM (0.25-1 µg/ml)] Gram negatif bakteriler üzerindeki etkinden [*E. coli* K12 (1 µg/ml), *Proteus mirabilis* CECT (0.25-0.5 µg/ml), *P. aeruginosa* IH (1-2 µg/ml)] daha yüksek olduğunu belirtmişlerdir (30).

Çalışmamızdan elde ettiğimiz verilere göre *Centaurium* subsp. ekstreleri orta düzeyde aktiviteye sahip olup, literatür verileri ile uyumlu bulunmuştur. İleri çalışmalar ile gıdalarda koruyucu veya tatlandırıcı olarak kullanımında mikroorganizmaların üremesini önleyen bitki türleri arasında değerlendirilebileceği ve antimikrobiyal aktiviteye neden olan bileşenleri tespit edilip, yeni ilaç tasarım modellerinde kullanılabileceği düşünülmektedir.

Tablo 2. *Centaurium* türlerinin metanollü ekstrelerinin antimikrobiyal aktivite sonuçları

Bitki türleri	Minimum İnhibisyon Konsantrasyonları (µg/ml)					
	S. aureus ATCC 29213	*E. faecalis* ATCC 29212	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 13883	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
C. maritimum	500	500	250	500	125	250
C. erythraea ssp. rhodense	500	500	250	500	125	125
C. tenuiflorum ssp. acutiflorum	250	250	250	500	125	250
C. spicatum	500	500	500	500	125	250
Siprofloksasin	0,312	0,312	0,0097	0,039	0,625	-
Mikonazol	-	-	-	-	-	1,56

ÇIKAR ÇATIŞMASI

Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

KAYNAKLAR

1. Carmona F., Pereira A.M.S. (2013). Herbal medicines: old and new concepts, truths and misunderstandings, *Braz J Pharmacogn*, *23*(2): 379-385.
2. Shakya A.K. (2016). Medicinal plants: Future source of new drugs, International Journal of *Herbal Medicine*, *4*(4): 59-64.
3. Srivastava J., Lambert J., Vietmeyer N. (1996). Medicinal plants: An expanding role in development. World Bank Technical Paper Number 320.
4. Mahesh B., Satish S. (2008). Antimicrobial Activity of Some Important Medicinal Plant Against Plant and Human Pathogens. *World J. Agric. Sci, 4* (S): 839-843.
5. Compean K.L., Ynalvez R.A. (2014), Antimicrobial Activity of Plant Secondary Metabolites: A Review. *Research Journal of Medicinal Plants, 8*: 204-213.
6. Gokhale M., Wadhwani M. (2015). Antimicrobial Activity of Secondary Metabolites from Plants - A Review. *International Journal of Pharmacognosy*, *2*(2): 60-65.
7. Davis P.H. (1978). Flora of Turkey and the East Aegean Islands, Edinburgh Uninersity Press, vol. 6. Edinburgh,178-180.
8. Güner A. (2012). Türkiye Bitkileri Listesi (Damarlı Bitkiler) Nezhat Gökyiğit Botanik Bahçesi Yayınları Flora Dizisi, İstanbul, 512.
9. Baytop T. (1999). Türkiye'de Bitkiler ile Tedavi (Geçmişte ve Bugün), İlaveli 2. Baskı, Nobel Tıp Kitabevleri, İstanbul: 243.

10. Newall C.A., Anderson L.A. (1994). Herbal Medicines-Guide for Healthcare Professionals. London: Pharmaceutical Press, 6.

11. Iordanov D. (1970). Fitoterapiíà: Lechenie Lekarstvennymi Travami, Meditsina iFizkul'tura, Sofia,

12. Tucakov J. (1990). Healing with plants. Beograd: Rad, 24-37.

13. Zlatković B.K., Bogosavljević S.S., Radivojević A.R., Pavlović M.A. (2014). Traditional use of the native medicinal plant resource of Mt. Rtanj (Eastern Serbia): Ethnobotanical evaluation and comparison. *J Ethnopharmacol*, *151*(1), 704-713.

14. Pieroni A., Quave C.L., Santoro R.F. (2004), Folk Pharmaceutical Knowledge in the Territory of the Dolomiti Lucane, *Inland Southern Italy, 95,* 373–384.

15. Bojan K. Zlatkovi, Bogosavljevi Stefan S., Radivojevi Aleksandar R., Pavlovi Mila A. (2014). Traditional use of the native medicinal plant resource of Mt. Rtanj (Eastern Serbia): Ethnobotanical evaluation and comparison, *Journal of Ethnopharmacology, 151,* 704-713.

16. Hansel R., Keller K., Rimpler H., Schneider G. (Hrsg.). (1994). Hagers Handbuch der Pharmazeutischen Praxis, 5. Aufl., Bde 4-6 (Drogen), Springer Verlag Berlin, Heidelberg, New York.

17. Berkan T., Üstünes L., Lermioglu F., Özer A. (1991). Antiinflammatory, analgesic and antipyretic effects of an aqueous extract of *Erythraea centaurium*. *Planta Med*. *57,* 34–3.

18. Dweck A.C. (1997). Research Director, Peter Black Medicare Ltd., Trowbridge, Wilts., Ethnobotanıcal Use Of Plants Part 4, *The Amerıcan Contınent* .

19. Bisset N.G., Max Wichtl's (1994).Herbal Drugs and Phytopharmaceuticals, A handbook for practice on a scientific basis, Scientific Publisher, Stuttgar, 134-136.

20. Walther H.J. (2004) Tausendguldenkraut-Heilpflanze des Jahres, Pharmaceutische Zeitung, 15(30).

21. Polat R., Satı F., (2012). An ethnobotanical survey of medicinal plants in Edremit Gulf (Balıkesir– Turkey) *Journal of Ethnopharmacology*, *139,* 629-641,

22. Tuzlacı E. (2006). Şifa Niyetine, Türkiye'nin Bitkisel Halk İlaçları, Alfa Yayınları, İstanbul, 68.

23. Orhan I.E., Senol F.S., Haznedaroglu MZ, Koyu H, Erdem SA, Yılmaz G., Çicek M., Yaprak A.E., Arı E., Küçükboyacı N., Toker G. (2017). Neurobiological evaluation of thirty-one medicinal plant extracts using microtiter enzyme assays. *Clinical Phytoscience, 2*(1), 9.

24. Yılmaz, G. (2017). Centaurium erythraea, In.Demirezer,L.Ö.,(Eds). FFD MonografларıBitkiler ve Etkileri, Akademisyen Kitabevi, Ankara, 235-242.

25. NCCLS. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; (2002). Approved Standard—Second Edition. NCCLS document M27-A2 [ISBN 1-56238-469-4]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA,

26. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically (2012). Approved Standard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

27. Morales G, Paredes A, Sierra P, Loyola LA, (2008). Antimicrobial Activity of Three Baccharis Species Used in the Traditional Medicine of Northern Chile. Molecules, 13, 790-794.

28. Kırbağ S., Zengin F., Kurşat M. (2009). Antimicrobial activities of extracts of some plants. *Pakistan Journal of Botany, 41*(4): 2067-2070.

29. Šiler B., Z˘ivkovic S., Banjanac T., Cvetkovic´ J., ´ Z˘ivkovic´ J.N., C´ Iric A., Sokovic´ M., Mišic D. (2014). *Centauries* asunderestimated food additives: Antioxidant and antimicrobial potential. *Food Chemistry, 147,* 367-376.

30. Bouyahya A., Belmehdi O., El Jemli M., Marmouzi I., Bourais I., Abrini J., Faouzi My El A., Dakka N., Bakri Y. (2019). Chemical variability of Centaurium erythraea essential oils at three developmental stages and investigation of their in vitro antioxidant, antidiabetic, dermatoprotective and antibacterial activities. *Industrial Crops & Products, 132,* 111–117.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

KUERSETİN, KURKUMİN VE KOMBİNASYONLARININ MEME KANSERİ HÜCRE HATLARI ÜZERİNDEKİ ANTİPROLİFERATİF ETKİLERİNİN ARAŞTIRILMASI

THE INVESTIGATION OF ANTIPROLIFERATIVE EFFECTS OF QUERCETIN, CURCUMIN AND THEIR COMBINATIONS ON BREAST CANCER CELL LINES

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ÖZ

Amaç: *Meme kanseri, kadınlarda malignite ve mortalite oranı yüksek olan ve Doğu Akdeniz bölgesinde sıklıkla görülen bir kanser türüdür. Kuersetin ve kurkumin, kanser hücrelerinin büyümesini inhibe etme özelliği olduğu bilinen flavonoid türleridir. Bu çalışmanın amacı, antikanser özelliğe sahip olduğu bilinen kuersetin ve kurkumin flavonoidlerinin tek başına ve kombine kullanımının metastatik ve metastatik olmayan meme kanser hücre hatlarındaki antiproliferatif etkisinin belirlenmesidir.*

Gereç ve Yöntem: *Bu çalışmada, kuersetin, kurkumin ve kombinasyonları M4A4 ve MCF-7 meme kanseri hücrelerine uygulandı. Hem M4A4 hem de MCF-7 hücreleri üzerindeki antiproliferatif etkinin belirlenmesi için MTT yöntemi kullanıldı. Combosyn programı kullanılarak %50'sini inhibe eden (IC50) ve kombinasyon indeks (CI) değerleri belirlendi.*

Sonuç ve Tartışma: *M4A4 hücre popülasyonunun IC50 etkin kuersetin dozu 72 saat sonucunda 49 µM olarak, MCF-7 hücre popülasyonu için ise 72 saat sonunda 120 µM olarak hesaplandı. M4A4 ve MCF-7 hücrelerine uygulanan kurkumin polifenolü için IC50 dozları sırasıyla 27 µM ve 54 µM olarak belirlendi. MCF-7 hücrelerine uygulanan kuersetin:kurkumin kombinasyonlarının sinerjistik etki gösterdiği, M4A4 hücreleri için ise antagonistik etki gösterdiği belirlendi. Hücrelere farklı doz ve zamanlarda kuersetin ve kurkumin uygulanmasının sonucunda, her iki polifenolün ayrı ayrı hücre canlılığını inhibe ettiği bulundu. Elde edilen bulgular, kuersetin ve kurkumin polifenoliyle birlikte kombinasyonun metastatik olmayan meme kanserinde kullanılabileceğini gösterirken, metastatik meme kanseri için farklı kombinasyon çalışmalarına ihtiyaç duyulduğunu göstermektedir.*

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Anahtar Kelimeler: *Antiproliferatif etki, kuersetin, kurkumin, meme kanseri*

ABSTRACT

Objective: *Breast cancer is a type of cancer that has high malignancy and mortality rates in women and is frequently seen in the Eastern Mediterranean region. Flavonoids such as quercetin and curcumin are known to have an ability to inhibit the growth of cancer cells. The aim of this study was to determine the antiproliferative effect of quercetin and curcumin which are flavonoids known to have anticancer properties on human metastatic and non metastatic breast cancer cell lines of alone and in combination usage.*

Material and Method: *Cell growth and cytotoxicity of quercetin, curcumin and quercetin:curcumin combinations were measured with MTT assay both on M4A4 and MCF-7 breast cancer cell lines. Combosyn program was used to determine IC50 and combination index (CI) values.*

Result and Discussion: *Half-maximal inhibitory concentrations (IC50) of quercetin for 72 hours were 49 µM and 120 µM on M4A4 and MCF-7 cells, respectively. IC50 doses for curcumin on M4A4 and MCF-7 cells were determined as 27 µM and 54 µM, respectively. It was determined that quercetin:curcumin combinations applied were shown a synergistic and antagonistic effect on MCF-7 and M4A4 cells. It was found that both quercetin and curcumin treatment inhibit cell viability in a dose and time-dependent manner. Findings show that combination with quercetin and curcumin polyphenol can be used in non-metastatic breast cancer, while different combination studies are needed for metastatic breast cancer.*

Keywords: *Antiproliferative effect, quercetin, curcumin, breast cancer*

GİRİŞ

Küresel bir halk sağlığı sorunu haline gelen meme kanseri, kadınlarda yüksek malignite ve mortaliteye sahip önemli bir kanser türüdür [1]. Kadınlarda ortaya çıkan her dört kanser türünden birinin meme kanseri olduğu, bunun yanında daha az sıklıkta erkek bireylerde de görüldüğü bildirilmiştir [2]. Meme kanserinde hormon durumu sadece önemli prognostik bilgileri için değil, aynı zamanda neo-adjuvan ve adjuvan tedavi kararları için de önem taşımaktadır [3]. Meme kanserinde östrojen reseptör (ER) durumu tümör derecesi ve histolojisi ile yakından ilişkilidir. Vakalarının %70'inin östrojen reseptör pozitif (ER+) olduğu rapor edilmiştir [4, 5]. Tamamlanan klinik çalışmalar, genç kadınlarda meme kanserinin, yaşlı kadın popülasyonuna kıyasla daha yüksek histolojik dereceye, olumsuz hormonal duruma ve genel olarak daha yüksek ölüm oranına sahip olduğunu doğrulamıştır [6]. Bu bağlamda meme kanserini tedavi etmeye yönelik olarak cerrahi rezeksiyon, kemoterapi ve radyoterapi yöntemleri kullanılmakla beraber günümüzde mortalite oranı halen yüksek oranlarda seyretmektedir. Bu süreçte, iyileşme potansiyeli yüksek olan meme kanserini tedavi etmeye yönelik yeni terapi ve tedavi seçeneklerinin oluşturulması için çalışmalar devam etmektedir. Bu amaçla, kanser hücrelerinin büyümesini etkileyebildikleri tespit edilen polifenoller yoğun ilgi görmekte ve önemli fitokimyasal grup olarak karşımıza çıkmaktadır [7]. Flavonoidler, birçok bitkide bulunan düşük moleküler ağırlığa sahip olan polifenolik bileşiklerdir [8]. Flavonoidler; soğan, elma, çay, şarap ve zeytinyağı gibi birçok meyve ve sebzede büyük ölçüde bulunmaktadırlar [9, 10]. Kuersetin, diyetle alınabilen ve kanser tedavisinde kullanılan ilgi çekici bir polifenoldur. Biyolojik ve farmakolojik çalışmalar kuersetinin antioksidan, antienflamatuvar, antiproliferatif ve antikanser etkileri olduğunu göstermiştir [11, 12]. Sarı renkli bir polifenol olan kurkumin ise anti-tümör, antioksidan ve antienflamatuar aktiviteler gibi biyofonksiyonel

özellikleri nedeniyle son yıllarda büyük ilgi gören diğer bir polifenoldür [13]. Kurkuminin kanser tedavisinde birçok hücresel yolak üzerine etkisi olduğu rapor edilmiştir [14]. Kurkuminin antikanser aktivitesinin sergilediği ana etki mekanizmaları arasında apoptozu indükleme ve çeşitli hücresel sinyal yollarını etkileyerek tümör proliferasyonunu önleme etkisi yer almaktadır [15]. MCF-7 klinik öncesi laboratuvar deneylerinde sıklıkla kullanılan bir meme kanseri hücre hattıdır [16]. MCF-7 hücreleri, östrojen reseptörü (ER) pozitif meme kanseri hücre deneyleri ve birçok alt klonun farklı ER sınıflarını temsil ettiği araştırmalarda yaygın olarak kullanılmakta ve değişen nükleer reseptör ekspresyon seviyeleri ile farklı (ER+) tümör sınıflarını temsil etmektedir [17]. M4A4 (insan meme duktal karsinomu) meme kanseri hücre hattı ise MDA-MB-435 meme kanseri hücre hattından türetilmiş olan östrojen negatif (ER-), yüksek metastatik özelliğe sahip bir hücre hattıdır [18, 19]. Daha önce farklı hücre hatları üzerinde sinerjistik etkinliği tanımlanmış olan kuersetin ve kurkumin kombinasyonunun M4A4 hücreleri üzerine olan etkisi henüz bilinmemektedir [20]. Bu çalışma kapsamında polifenol olarak bilinen kuersetin ve kurkuminin ayrı ayrı ve kombine dozlarının MCF-7 ve M4A4 meme kanseri hücre hatları üzerindeki antikanser aktiviteleri belirlenmiş ve karşılaştırmalı olarak incelenmiştir.

GEREÇ VE YÖNTEM

Hücre Kültürü: M4A4 [ER(-)] (CRL-2914) ve MCF-7 [pozitif, ER(+)] (HTB-22) hücreleri Amerikan Tipi Kültür Koleksiyonu'ndan (ATCC) temin edildi. M4A4 ve MCF-7 hücreleri 25 cm^2'lik flasklarda, %10 Fetal sığır serumu (Capricorn Scientific, FBS-11B), %1 Penisilin/Streptomisin (Biochrom, A2213) ve %1 L-glutamin (EMD Millipore, K0282) içeren RPMI-1640 (Roswell Park Memorial Institute-1640, Biochrom, Germany) kültür vasatı kullanılarak, 37^{o}C, %5 kısmi CO_2 basıncı ve nemli ortam içeren inkübatörler içerisinde yetiştirildi. Hücreler hem makroskopik hem de mikroskobik olarak düzenli aralıklarla kontrol edildi.

Sitotoksisite Deneyi: Hücrelerin yaşaması ve proliferasyonu MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Glentham Life Sciences, GC4568) testi ile kantitatif ve kolorimetrik olarak belirlendi. MTT testinin yapılması için MCF-7 ve M4A4 hücreleri 96 kuyucuklu plaklar içerisine 24 saat önceden 5000 hücre/kuyu olacak şekilde 100 µL besi ortamı içerisinde ekildi. Kuersetin için 5, 10, 25, 50 ve 100 µM konsantrasyonlar, kurkumin için ise 1, 5, 10, 25 ve 50 µM konsantrasyonlar 96 kuyucuklu plaklar içerisinde yer alan hücrelere uygulanarak 24, 48 ve 72 saat boyunca 37^{o}C, %5 kısmi CO_2 basıncı ve nemli ortam içeren inkübatörler içerisinde inkübe edildi. Ardından her bir kuyucuğa 10 µL MTT solüsyonu ilave edildi. Plaklar MTT solüsyonu ilavesini takiben 3 saat inkübe edildi. İnkübasyonun ardından besi ortamı uzaklaştırılarak kuyucuklara 100 µL DMSO ilave edildi ve 20 dk. daha inkübasyona bırakıldı. Ardından mikroplak okuyucu spektrofotometre ile (Varioskan Flash Multimode Microplate Reader, Skanlt Software 2.4.5, Thermo Scientific) 540 nm

dalga boyunda ölçüm yapıldı. Kuersetin ve kurkumin için her bir konsantrasyon 3 tekrarlı olarak çalışıldı. Elde edilen veriler aşağıda belirtilen formül kullanılarak her bir grup için hücre canlığının %50 inhibe olduğu dozlar (IC50) kuersetin ve kurkumin uygulanmış olan hücrelerde ayrı ayrı belirlendi.

% Hücre canlılık oranı =(Atest-Ablank/Akontrol-Ablank)x100

CompuSyn Kombinasyon Analizi: Kombinasyon analizi için uygun dozların belirlenmesinde kuersetin ve kurkumin polifenollerinin tekil olarak uygulandığı hücre sitotoksisite testi sonuçları baz alındı. CompuSyn bilgisayar yazılımı kullanılarak her iki polifenolün IC50 değerlerinin birbirine oranına göre etkin doz ve süresi belirlendi. Belirlenen doz ve sürede tekrar MTT deneyi yapıldı. Buradan elde edilen absorbans değerlerinden kombinasyon indeks değerleri CompuSyn bilgisayar programı ile belirlendi. Sinerjik etkinin belirlenmesinde CompuSyn programı ile çizilen Fraksiyonel etki (Fa) ve kombinasyon indeksi (CI) grafiği (Fa-CI) ile y ekseninde bulunan 1 değerinin altında kalan değerler sinerjistik etki, 0.5 değerinin altında kalanlar ise güçlü sinerjistik etki gösteren değerler olarak belirlendi [21].

İstatistiksel Analiz: Deneyler sonucunda elde edilen verilerin istatistiksel analizinin gerçekleştirilmesinde Graphpad Prism V-8 bilgisayar yazılımı kullanıldı. Kuersetin ve kurkumin uygulanmış hücre grupları ile kontrol grupları arasında hücre canlılığının inhibisyonu açısından istatistiksel olarak anlamlı bir farklılık olup olmadığı 2-way ANOVA, Tukey's test ile belirlendi. Anlamlılık değerleri; $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$ olarak değerlendirildi.

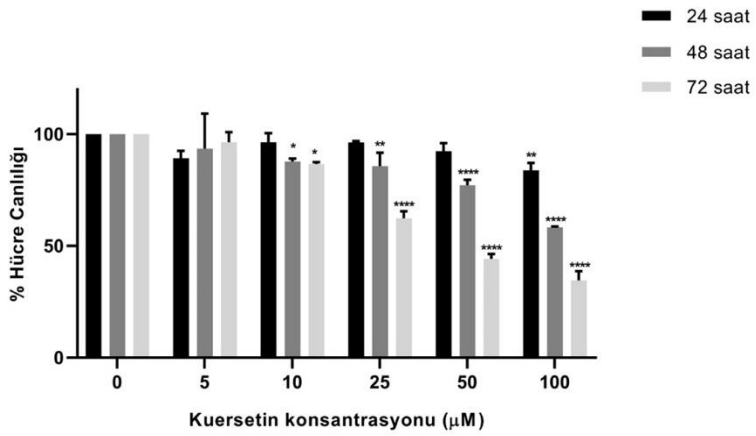
SONUÇ VE TARTIŞMA

Kuersetin ve kurkumin polifenollerinin metastatik (M4A4) ve metastatik olmayan (MCF-7) meme kanseri hücrelerinde hücre canlılığı üzerine olan etkileri MTT testi ile belirlendi. M4A4 ve MCF-7 hücrelerine kuersetin uygulaması 5, 10, 25, 50 ve 100 µM konsantrasyonlarında, kurkumin uygulaması ise 1, 5, 10, 25 ve 50 µM konsantrasyonlarında olacak şekilde 5 farklı dozda 24, 48 ve 72 saat aralıklarında yapıldı. MTT uygulamasından sonra spektrofotometrik olarak elde edilen absorbans verileri istatistiksel olarak değerlendirildi. Buna göre M4A4 hücrelerinin kuersetin uygulamasından 24 saat sonra sadece 100 µM doz uygulanan hücre grubunda %83.89 oranında anlamlı bir canlılık gözlemlenirken, 48 ve 72 saat sonra 10, 25, 50 ve 100 µM konsantrasyonlarda doza ve zamana bağımlı bir şekilde hücre canlılığının istatistiksel olarak anlamlı bir şekilde inhibe olduğu gözlemlendi. 10, 25, 50 ve 100 µM konsantrasyonlarında kuersetin uygulanmasıyla, M4A4 hücrelerinde 48 saat sonra sırasıyla %87.71, %85.72, %77.11, %58.33 canlılık gözlemlenirken, 72 saat sonucunda ise sırasıyla %86.63, %62.31, %44.33 ve %35.35 oranında canlılık gözlemlendi (Şekil 1a). Buna göre hücre

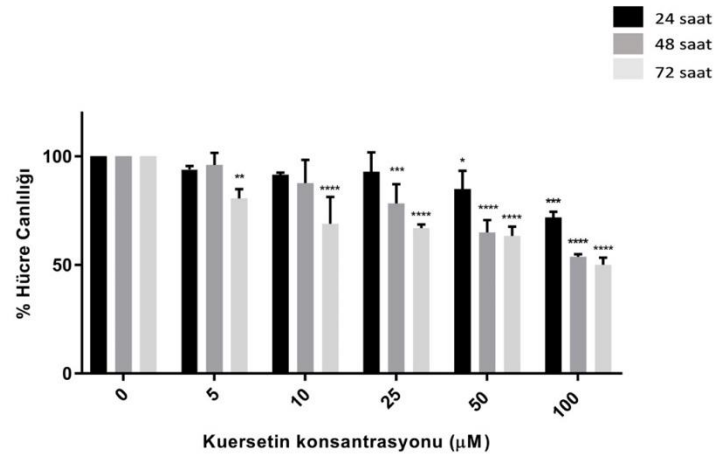
popülasyonunun %50'sini inhibe eden etkin doz M4A4 hücreleri için 72 saat sonucunda 49 µM olarak hesaplandı**.** MCF-7 hücrelerine ise kuersetin uygulanmasından 24 saat sonra 50 ve 100 µM doz uygulanan grupta sırasıyla %84.97 ve %71.95 oranında hücre canlılığı üzerine anlamlı bir inhibitor etkisi tespit edildi. Kuersetinin 48 saat sonra 25, 50 ve 100 µM konsantrasyonlarda uygulanması sonucunda elde edilen verilere göre hücre canlılığını sırasıyla %78.33, %64.94 ve %53.5 inhibe ettiği görüldü. Bunun yanında 72 saat sonra elde edilen veriler incelendiğinde uygulama yapılan 5, 10, 25, 50, 100 µM'daki konsantrasyonlar için sırasıyla %80.66, %68.92, %66.91, %63.39, %53.75 oranında hücre canlılığını anlamlı bir şekilde doz bağımlı olarak inhibe ettiği bulundu (Şekil 1b). MCF-7 hücre hattında hücre popülasyonunun %50'sini inhibe eden kuersetin konsantrasyonu 72 saat sonunda 120 µM olarak hesaplandı.

M4A4 hücrelerine 1, 5, 10, 25 ve 50 µM kurkumin uygulaması ile 24, 48 ve 72 saat sonucunda elde edilen hücre canlılık grafiğini göstermektedir. Elde edilen sonuçlar incelendiğinde, 24 saat sonucunda sırasıyla %85.35, %79.97, %72.21, %72.41 ve %70.93 oranında anlamlı bir canlılık gözlemlendi. Kurkuminin 10, 25 ve 50 µM dozları için 48 ve 72. saat sonuçları incelendiğinde hücre canlılığı 48. saatte sırasıyla %87.06, %74.62, %51.88 ve 72. saatte sırasıyla %87.25, %36.35 ve %8.59 olarak bulundu (Şekil 2a). MCF-7 hücrelerine 5, 10 ve 50 µM dozlarında kurkumin uygulamasından 24 saat sonra, sırasıyla %93.56 %87.31 ve %86.56 oranlarında anlamlı bir canlılık olduğu görüldü. Bunun yanı sıra, 48 saat sonra elde edilen veriler 5, 10 ve 50 µM konsantrasyonlarda kurkumin uygulanan hücrelerde sırasıyla %91.22, %95.05, %66.41 canlılık olduğunu gösterdi. 72 saat sonra elde edilen veriler incelendiğinde ise, 1, 5, 10, 25 ve 50 µM kurkumin uygulanan MCF-7 hücrelerinde sırasıyla %89.09, %73.04, %70.68, %70.54 ve %43.47 olarak hücre canlılığı tespit edildi. M4A4 ve MCF-7 hücrelerine uygulanan kurkumin polifenolü için IC50 dozları sırasıyla 27 µM ve 54 µM olarak belirlendi.

1a



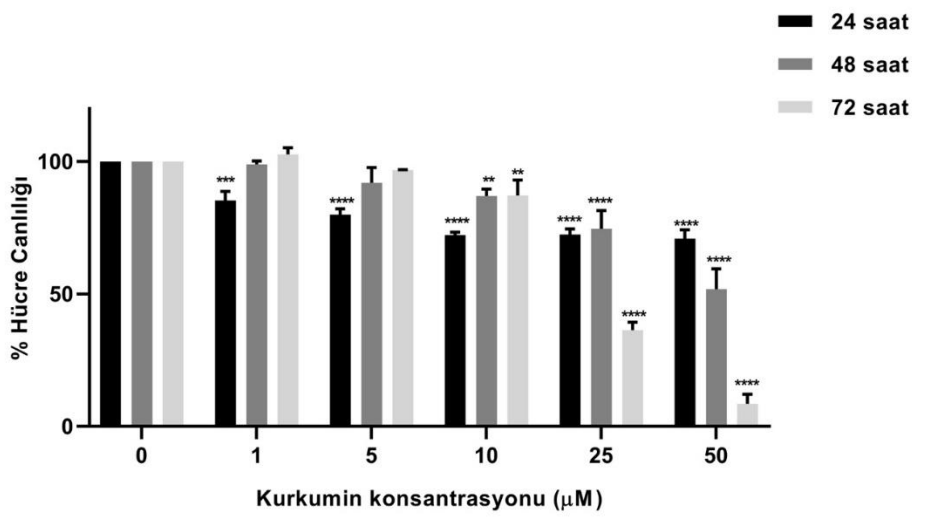
1b



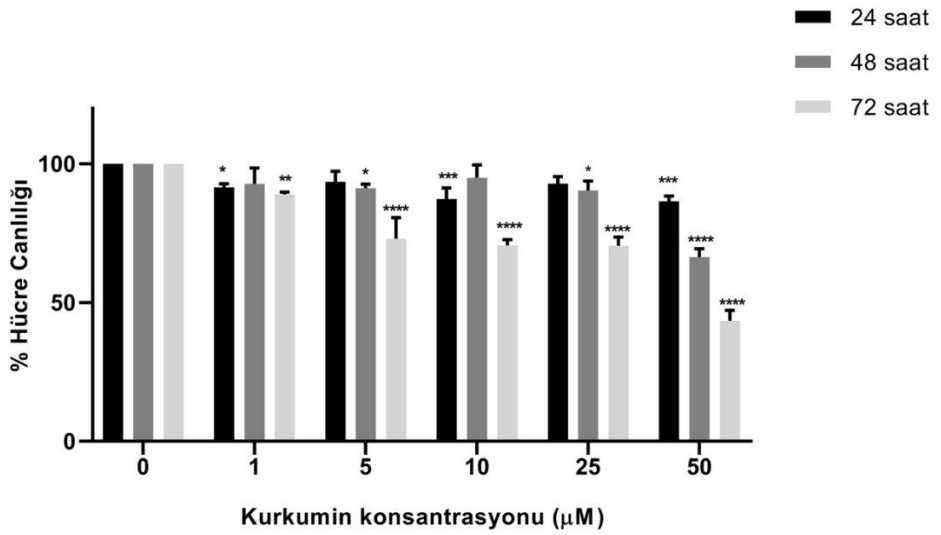
Şekil 1. Kuersetin polifenolünün M4A4 ve MCF-7 hücreleri üzerindeki sitotoksik etkisi. M4A4 meme kanseri hücrelerinin 24, 48 ve 72 saat boyunca farklı konsantrasyonlarda kuersetin ile muamelesi sonucunda elde edilen hücre canlılık grafiği. (Şekil 1a). MCF-7 meme kanseri hücrelerinin 24, 48 ve 72 saat boyunca farklı konsantrasyonlarda kuersetin ile muamelesi sonucunda elde edilen hücre canlılık grafiği (Şekil 1b). Deneyler 3 tekrarlı olarak yapıldı ve anlamlılık değerleri $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$**** olarak değerlendirildi.

M4A4 ve MCF-7 hücre gruplarına ayrı ayrı kuersetin ve kurkumin uygulamalarını takiben her iki polifenolun bir arada kullanıldığı kombinasyon uygulamaları gerçekleştirildi. Kombinasyon dozlarının hazırlanmasında her iki polifenolün IC50 değerlerinin birbirine oranına göre etkin doz ve süre belirlendi. M4A4 hücre grubu için kuersetin:kurkumin konsantrasyon oranı 1.8:1, MCF-7 hücre grubu için ise 2.2:1 olarak belirlendi.

2a



2b



Şekil 2. Kurkumin polifenolünün M4A4 ve MCF-7 hücreleri üzerindeki sitotoksik etkisi. M4A4 meme kanseri hücrelerinin 24, 48 ve 72 saat boyunca farklı konsantrasyonlarda kurkumin ile muamelesi sonucunda elde edilen hücre canlılık grafiği (Şekil 2a). MCF-7 meme kanseri hücrelerinin 24, 48 ve 72 saat boyunca farklı konsantrasyonlarda kurkumin ile muamelesi sonucunda elde edilen hücre canlılık grafiği (Şekil 2b). Deneyler 3 tekrarlı olarak yapıldı ve anlamlılık değerleri $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$**** olarak değerlendirildi.

Buna göre M4A4 hücreleri Tablo 1a'da, MCF-7 hücreleri ise Tablo 1b'de gösterilen kombinasyon dozları ile 72 saat boyunca muamele edildi ve ardından hücre canlılığını belirlemek amacıyla MTT testi yapıldı.

Tablo 1a. M4A4 meme kanseri hücrelerine uygulanan kuersetin:kurkumin kombinasyonlarının konsantrasyonlarını göstermektedir.

Gruplar	Kuersetin Konsantrasyonu	Kurkumin Konsantrasyonu
Konsantrasyon 1 (K-1)	5 µM	2.7 µM
Konsantrasyon 2 (K-2)	10 µM	5.5 µM
Konsantrasyon 3 (K-3)	25 µM	13.8 µM
Konsantrasyon 4 (K-4)	50 µM	27.7 µM
Konsantrasyon 5 (K-5)	100 µM	55.5 µM

Tablo 1b. MCF-7 meme kanseri hücrelerine uygulanan kuersetin:kurkumin kombinasyonlarının konsantrasyonlarını göstermektedir.

Gruplar	Kuersetin Konsantrasyonu	Kurkumin Konsantrasyonu
Konsantrasyon 1 (K-1)	5 µM	2.27 µM
Konsantrasyon 2 (K-2)	10 µM	4.54 µM
Konsantrasyon 3 (K-3)	25 µM	11.3 µM
Konsantrasyon 4 (K-4)	50 µM	22.7 µM
Konsantrasyon 5 (K-5)	100 µM	45.45 µM

M4A4 ve MCF-7 hücre hatları için her iki polifenolün kombine kullanımı sonucunda elde edilen kombinasyon indeksi ve fraksiyonel etki değerleri belirlendi (Tablo 2a ve Tablo 2b).

Tablo 2a. M4A4 meme kanseri hücre hattına uygulanan farklı kombinasyon grupları için elde edilen kombinasyon indeksi ve fraksiyonel etki değerleri.

Gruplar	Kombinasyon İndeksi (CI)	Fraksiyonal Etki (Fa)
K-1	3.34	0.019
K-2	4.18	0.03
K-3	6.26	0.076
K-4	2.73	0.45
K-5	1.19	0.90

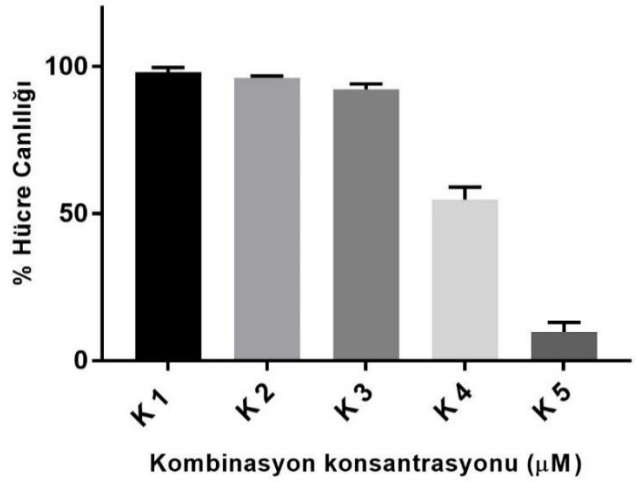
Tablo 2b. MCF-7 meme kanseri hücre hattına uygulanan farklı kombinasyon grupları için elde edilen kombinasyon indeksi ve fraksiyonel etki değerleri.

Gruplar	Kombinasyon İndeksi (CI)	Fraksiyonal Etki (Fa)
K-1	3640	0.01
K-2	57.83	0.07
K-3	5.91	0.23
K-4	0.24	0.64
K-5	0.06	0.82

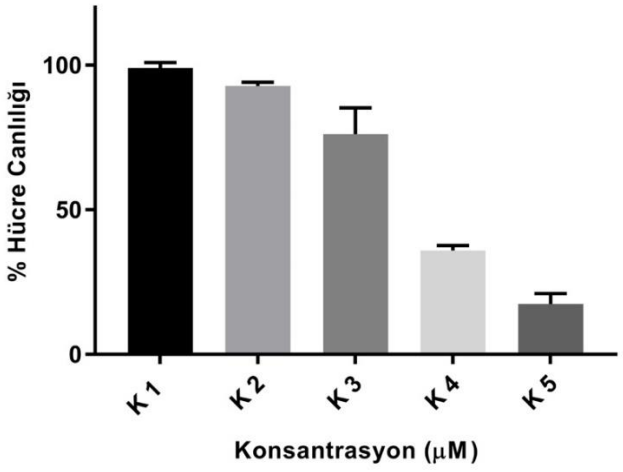
Graphpad Prism V.8 yazılım programı kullanılarak elde edilen absorbans değerlerinden hücre canlılık grafikleri çizildi. Tablo IIa' da belirtilen kombinasyon indeks (CI) değerleri incelendiği zaman M4A4 hücrelerine uygulanmış K1-K5 olmak üzere 5 farklı kombinasyon dozu içerisinde sinerjistik

etkileşim gösteren bir kombinasyon uygulamasının olmadığı görüldü. Bunun yanında uygulanan tüm konsantrasyonların antagonistik etki gösterdiği tespit edildi. Kombinasyon analizinden elde edilen sonuçlara göre M4A4 hücrelerinde antagonizm gözlemlenen K-1, K-2, K-3, K-4 ve K-5 gruplarında kombinasyon uygulamalarının sırasıyla %98.1, %96.28, %92.4, %54.84 ve %9.89 oranında hücre canlılığına neden olduğu belirlendi (Şekil 3a).

3a



3b

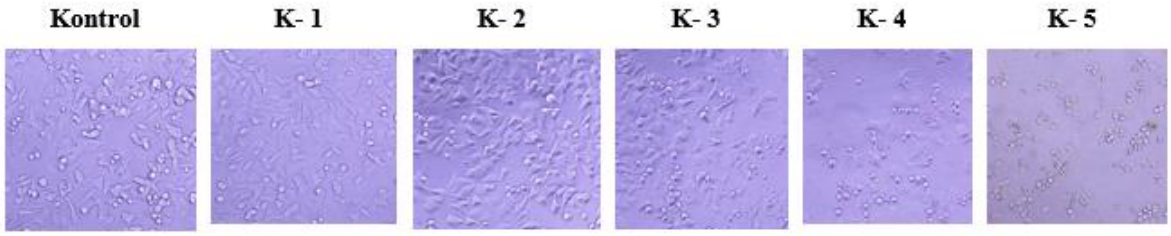


Şekil 3. Kombinasyon gruplarının M4A4 ve MCF-7 hücreleri üzerindeki sitotoksik etkisi. M4A4 hücrelerine uygulanan kombinasyon gruplarına ait hücre canlılık grafiği (Şekil 3a). MCF-7 hücrelerine uygulanan kombinasyon gruplarına ait hücre canlılık grafiği (Şekil 3b).

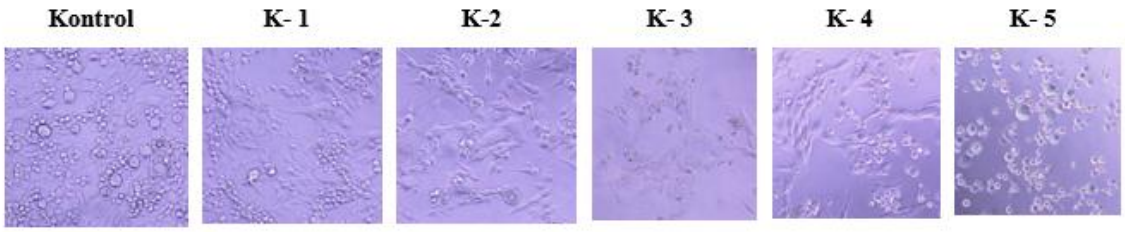
Tablo 2b'de yer alan MCF-7 hücrelerine uygulanmış kombinasyon dozlarına ait kombinasyon indeksi değerleri incelendiği zaman ise K-4 kombinasyonunun 'sinerjizm', K-5 kombinasyonunun ise 'güçlü sinerjizm' etkisi gösterdiği tespit edildi. Kombinasyon analizinden elde edilen sonuçlara göre K-

1, K-2, K-3, K-4 ve K-5 gruplarında kombinasyonların sırasıyla %99, %92.83, %76.14, %35.91 ve %17.47 oranlarında canlılığa yol açtığı belirlendi (Şekil 3b). 72 saat boyunca kombinasyon grupları ile muamele edilen M4A4 ve MCF-7 hücre gruplarına ait MTT uygulaması sonucu elde edilen mikroskop görüntüleri verildi (Şekil 4a, 4b).

4a



4b



Şekil 4. Kombinasyon dozları uygulanan M4A4 (4a) ve MCF-7 (4b) hücrelerinin 72 saat sonucunda MTT ile muamelesi ardından elde edilen mikroskop görüntüleri.

Bu çalışma meme kanseri tedavisinde, yeni tedavi ve terapi yöntemlerinin geliştirilmesine katkı sağlamak amacıyla metastatik ve metastatik olmayan meme kanseri hücre hatlarında *in vitro* şartlar altında yürütülmüştür. Günümüzde büyük ilgi toplayan kuersetin ve kurkumin polifenollerinin antioksidan, antienflamatuvar, antiproliferatif ve antikanser etkileri olduğu daha önce yapılan çalışmalarla gösterilmiştir [11, 12, 13].

Bu çalışmamızda metastatik özelliğe sahip bir meme kanseri hücre hattı olduğu bilinen M4A4 ve metastatik karakterde olmayan MCF-7 meme kanseri hücre hatlarının kuersetin ve kurkumin polifenolleri ile muamelesi sonucunda hücre canlılığında meydana gelen değişiklikler incelenmiştir. Ayrı ayrı ve kombine dozlar şeklinde yapılan uygulamaların neticesinde her iki hücre hattında da hücre canlılığının anlamlı bir şekilde azaldığı tespit edilmiştir. Buna göre M4A4 hücre grubunda kuersetin ve kurkumin uygulamasının hücre canlılığı üzerindeki inhibitor etkisi ilk kez belirlenmiş olup literatüre katkı sağlayacağı düşünülmektedir. M4A4 hücre grubuna uygulanan kuersetinin 72 saat sonunda elde

edilen sonuçlarına bakıldığında, kuersetin ajanının doz bağımlı bir şekilde M4A4 hücrelerinde hücre canlılığını inhibe ettiği bulunmuştur. Aynı şekilde kurkumin uygulaması da M4A4 hücre canlılığını önemli ölçüde inhibe etmiştir. Literatüre göre; M4A4 metastatik meme kanser hattı ile gerçekleştirilmiş herhangi bir sitotoksisite çalışması bulunmamaktadır. Elde edilen sonuçların yeni ve farklı kombinasyon denemeleri yapılması açısından literatüre değerli ve önemli bilgiler sağlayacağı düşünülmektedir. Bunun yanında benzer çalışmalar kuersetin uygulaması yapılmış olan MCF-7 hücrelerinde 48 saat sonunda hücre canlılığında doza bağımlı bir inhibisyon olduğunu daha önce rapor etmiştir [22, 23]. Benzer sonuçlar bu çalışma sonucunda da elde edilmiştir. Kuersetin maruziyetine bırakılan MCF-7 hücrelerinde, 48 ve 72 saat boyunca çeşitli konsantrasyonlarda doza bağımlı bir azalma gözlemlenmiş, 72 saat sonucunda en yüksek doz olan 100 µM konsantrasyonda %50.05 canlılık tespit edilmiştir. Daha önce MCF-7 hücreleri üzerinde kurkumin polifenolünün inhibitor etkisi tanımlanmıştır [24]. Bu çalışma ile de MCF-7 hücreleri üzerinde de doz ve zaman bağımlı bir inhibisyon doğrulanmıştır. Elde edilen veriler 72 saat sonunda 50 µM kurkumin uygulaması yapılmış olan MCF-7 hücrelerinde %43.47 oranında hücre canlılığı olduğunu göstermiştir. Kombinasyon uygulamaları sonucunda elde edilen veriler incelendiği zaman ise K-4 ve K-5 kombinasyonlarının MCF-7 hücrelerinde sinerjistik etkileşim gösterdiği ve sırasıyla %36 ve %17.47 oranlarında hücre canlılığına neden olduğu gösterilmiştir. Bu sonuçlara göre; polifenollerin kombine kullanımının ayrı halde kullanımına göre daha etkin olduğunu doğrulanmaktadır. Kombine kullanım MCF-7 hücre hattında tedavi edici potansiyele sahipken, M4A4 hücrelerinde durum aynı değildir. M4A4 hücrelerinde kombinasyon uygulamaları sonucunda elde edilen hücre canlılığı verileri metastatik karakterde olan bu hücrelerin kuersetin ve kurkuminin ayrı ayrı daha güçlü ve farklı kombinasyonlarının denenmesi gerektiğini göstermektedir. M4A4 hücreleri için her iki ajanın birlikte kullanıldığı kombinasyon gruplarındaki dozlar bu iki polifenolün bu dozlarında antagonist etki yaratmıştır. Her iki hücre hattı için de elde edilen sonuçların altında yatan moleküler mekanizmaların araştırılması için ileri çalışmaların yapılmasına ihtiyaç vardır. Bu çalışma ile elde edilen sonuçlar ileride yapılacak olan çalışmalara ışık tutacaktır.

ÇIKAR ÇATIŞMASI

Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

KAYNAKLAR

1. Akkuzu, M.Z., Küçüköner, M., Irtegun, S, Akdeniz, N., Urakçı, Z., Kaplan, M.A., Büyükbayram, H., Işıkdoğan, A. (2019). Meme Kanserinde Brca-1 ve Brca-2' de Sık Görülen Polimorfizm Mutasyonların Bölgemizde Varlığı. *Dicle Med J, 46,* 623–631.

2. Elbachiri, M., Fatima, S., Bouchbika, Z., Benchekroun, N., Jouhadi, H., Tawfiq, N., Sahraoui, S., Benider, A. (2017). Breast cancer in men: About 40 cases and literature review. *Pan Afr. Med. J, 28,* 287.

3. Foley, N.M., Coll, J.M., Lowery, A.J., Hynes, S.O., Kerin, M.J., Sheehan, M., Brodie, C., Sweeney, K.J. (2018). Re-Appraisal of Estrogen Receptor Negative/Progesterone Receptor Positive (ER−/PR+) Breast Cancer Phenotype: True Subtype or Technical Artefact? Pathol. *Oncol. Res, 24,* 881–884.

4. Anderson, W.F, Chatterjee. N., Ershler, W.B, Brawley, O.W. (2002). Estrogen receptor breast cancer phenotypes in the Surveillance, Epidemiology, and End Results database. *Breast Cancer Res. Treat, 76,* 27–36.

5. Nadji, M., Gomez-Fernandez, C., Ganjei-Azar, P., Morales, A.R. (2005). Immunohistochemistry of Estrogen and Progesterone Receptors Reconsidered. *Am. J. Clin. Pathol, 123,* 21–27.

6. Erić, I., Erić, A.P., Kristek, J., Koprivčić, I., Babić, M. (2018). Breast cancer in young women: Pathologic and immunohistochemical features. *Acta Clin.* Croat, *57,* 497–502.

7. Kampa, M., Nifli, A.P., Notas, G., Castanas, E. (2007). Polyphenols and cancer cell growth. *Rev. Physiol. Biochem. Pharmacol, 159,* 79–113.

8. Birman, H. (2012). Bitkisel Flavonoid Bileşiklerinin Biyoaktiviteleri ve Muhtemel Etki Mekanizmaları. *İstanbul Tıp Fakültesi Derg, 75,* 46–49.

9. Slimestad, R., Fossen, T., Vågen, I.M. (2007). Onions: A source of unique dietary flavonoids. *J Agr Food Chem, 55,* 10067–10080.

10. Spagnuolo, C., Russo, M., Bilotto, S., Tedesco, I., Laratta, B., Russo, G.L. (2012). Dietary polyphenols in cancer prevention: the example of the flavonoid quercetin in leukemia. *Ann. N. Y. Acad. Sci, 1259,* 95–103.

11. Nam, J.S., Sharma, A.R., Nguyen, L.T., Chakraborty, C., Sharma, G., Lee, S. (2016). Application of bioactive quercetin in oncotherapy: From nutrition to nanomedicine. *Molecules, 21(1),* E108.

12. Mutlu Altundağ, E., Yılmaz, A.M., Koctürk, S., Taga, Y., Yalçın, A.S. (2017). Synergistic Induction of Apoptosis by Quercetin and Curcumin in Chronic Myeloid Leukemia (K562) Cells. *Nutr Cancer, 70,* 97-108.

13. Nagahama, K., Utsimi, T., Kumano, T., Maekawa, S., Oyama, N., Kawakami, J. (2016). Discovery of a new function of curcumin which enhances its anticancer therapeutic potency. *Sci. Rep, 6,* 30962.

14. Troselj, K., Kujundzic, R. (2014). Curcumin in Combined Cancer Therapy. *Curr. Pharm. Des, 20,* 6682–6696.

15. Kunnumakkara, A.B., Bordoloi, D., Padmavathi, G., Monisha, J., Roy, N.K., Prasad, S., Aggarwal, B.B. (2017). Curcumin, the golden nutraceutical: multitargeting for multiple chronic diseases. *Br. J. Pharmacol, 174,* 1325–1348.

16. Comşa, Ş., Cîmpean, A.M., Raica, M. (2015). The story of MCF-7 breast cancer cell line: 40 Years of experience in research. *Anticancer Res, 35,* 3147–3154.

17. Sweeney, E.E., McDaniel, R.E., Maximov, P.Y., Fan, P., Craig Jordan, V. (2012). Models and mechanisms of acquired antihormone resistance in breast cancer: Significant clinical progress despite limitations. *Horm. Mol. Biol. Clin. Investig, 9,* 143–163.

18. Urquidi, V., Sioan, D., Kawai, K., Agarwal, D., Woodman, A.C., Tarin, D., Goodison, S. (2002). Contrasting expression of thrombospondin-1 and osteopontin correlates with absence or presence of metastatic phenotype in an isogenic model of spontaneous human breast cancer metastasis. *Clin. Cancer Res, 8,* 61–74.

19. Goodison, S., Viars, C., Grazzini, M., Urquidi, V. (2003). The interrelationship between DRIM gene expression and cytogenetic and phenotypic characteristics in human breast tumor cell lines. *BMC Genomics, 4,* 39.

20. Srivastava, N.S., Srivastava, R.A.K. (2019). Curcumin and quercetin synergistically inhibit cancer cell proliferation in multiple cancer cells and modulate Wnt/β-catenin signaling and apoptotic pathways in A375 cells. *Phytomedicine, 52,* 117–128.

21. Chou, T.C. (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res, 70,* 440–446.

22. Li, X., Zhou, N., Wang, J., Liu, Z., Wang, X. , Zhang, Q., Liu, Q., Gao, L., Wang, R. (2018). Quercetin suppresses breast cancer stem cells (CD44+/CD24−) by inhibiting the PI3K/Akt/mTOR-signaling pathway. *Life Sci, 196,* 56–62.

23. Ranganathan, S., Halagowder, D., Sivasithambaram, N.D. (2015). Quercetin Suppresses Twist to Induce Apoptosis in MCF-7 Breast Cancer Cells. *PLoS One, 10(10),* e0141370.

24. Liu, J.L., Pan, Y.Y., Chen, O., Luan, Y., Xue, X., Zhao, J.-J., Liu, L., Jia, H.-Y. (2017). Curcumin inhibits MCF-7 cells by modulating the NF-κB signaling pathway. *Oncol. Lett, 14,* 5581–5584.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

A QUESTIONNAIRE-BASED STUDY ON MEDICINAL PLANT USE IN RESPIRATORY DISEASES

SOLUNUM YOLU HASTALIKLARINDA TIBBİ BİTKİ KULLANIMI ÜZERİNE ANKET TABANLI BİR ÇALIŞMA

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ABSTRACT

Objective: *We aimed to investigate the rates of medicinal plant use, species of medicinal plants, detailed usage and procurement information, the sociodemographic features affecting the tendency to use plants and the notification status to healthcare professionals in patients with respiratory diseases*.

Material and Method: *This study was carried out between January-April 2018 at the Molla Gurani Family Healthcare Center. An 18-item survey questionnaire was administered to patients suffered from respiratory diseases. Analysis were made in SPSS version 26.0 software using both chi-square analysis and Fisher's Exact test.*

Result and Discussion: *Of the 62 patients, 64.5% had used medicinal plants to treat respiratory diseases. A total of 14 different plants and plant mixture were determined, most commonly used were mint-lemon, licorice, linden and rosehip. Gender (p=0.01) and inhabitation (p=0.007) were significant between the groups that used and did not use plants. The rates of patients' benefiting from plants and recommending plants were also found to be high (87.5%, 90%, respectively). Only 40% of users had notified their doctor, age (p=0.029), educational status (p=0.002) and inhabitation (p=0.015) were statistically significant for notification to healthcare professionals about plant usage. This study has demonstrated the need for further research into commonly used medicinal plants in respiratory diseases.*

Keywords: *Complementary alternative medicine, medicinal plants, respiratory diseases*

ÖZ

Amaç: *Bu çalışmada, solunum yolu hastalıklarında tıbbi bitki kullanım oranının, bitki türlerinin, ayrıntılı kullanım ve satın alma bilgilerinin, bitki kullanma eğilimini etkileyen sosyodemografik özelliklerin ve sağlık profesyonellerine bildirim durumlarının araştırılması amaçlanmıştır.*

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Gereç ve Yöntem: *Araştırma 2018 yılı Ocak-Nisan ayları arasında Molla Gürani Aile Sağlığı Merkezi'nde gerçekleştirildi. Solunum yolu hastalarına 18 maddelik bir anket formu uygulandı. Elde edilen verilerin analizleri SPSS sürüm 26.0 yazılımında Ki-kare ve Fisher's Exact testi kullanılarak yapıldı.*

Sonuç ve Tartışma: *Ankete katılan 62 solunum yolu hastasının 64.5%'inin tıbbi bitkileri kullandığı belirlenmiştir. Toplamda 14 farklı bitki ve bitki karışımının kullanıldığı, bunlardan en yaygın olarak nane-limon, meyan kökü, ıhlamur ve kuşburnu tercih edildiği görülmüştür. Bitki kullanan ve kullanmayan gruplar arasında cinsiyet (p=0.01) ve yerleşim (p=0.007) istatistiksel olarak anlamlıdır. Hastaların bitkilerden yararlanma ve bitki tavsiye etme oranlarının da yüksek olduğu görülmüştür (sırasıyla 87.5%, 90%). Kullanıcıların sadece %40'ı doktorlarına bitki kullanımını bildirirken, yaş (p=0,029), eğitim durumu (p=0,002) ve yerleşim yerinin (p=0,015) istatistiksel olarak anlamlı olduğu bulunmuştur. Çalışma, solunum yolu hastalıklarında yaygın olarak kullanılan tıbbi bitkiler üzerinde daha fazla araştırma yapılması gerektiğini göstermiştir.*

Anahtar Kelimeler: *Geleneksel ve tamamlayıcı tıp, solunum yolu hastalıkları, tıbbi bitkiler*

INTRODUCTION

Several types of respiratory diseases such as asthma, bronchitis, chronic obstructive pulmonary disease (COPD) affect millions of people worldwide and cause an immense health burden in many countries. According to the latest estimates, 334 million people suffer from asthma and about 65 million people have COPD. COPD is assumed as the third leading cause of death worldwide, 3 million of the patients die each year [1,2]. In Europe, respiratory diseases rank as the second most common disease in terms of incidence, prevalence, mortality and economic costs [3]. Among respiratory diseases, COPD and lower respiratory system infections are regarded as leading conditions which can cause a death for all age groups and for both sexes in Turkey [4]. Though there are many risk factors have been identified, Tobacco smoking is the main one which is directly responsible for the morbidity and mortality resulting from respiratory diseases [3,4]. As stated by Turkey Statistical Institute (TUIK) database, respiratory system diseases is the third (10.15%) main cause of death between 2009-2016 in Turkey. The distribution of disorders among respiratory diseases is as follows: COPD, 59.44%; pneumonia, 21.50%; asthma, 4.91%; acute upper respiratory diseases and flu, 0.64%; others, 13.52%, respectively. Death rates from respiratory diseases are higher among male (60%) than among female (40%). It was also found that mortality from COPD and bronchiectasis was 2 times higher in males than in females. Regarding the malignancies, respiratory malignancies were the most common tumors with a rate of 30.6% [5].

Recent years research in respiratory medicine and related technology is in an advanced stage. With modern treatments, symptoms can be controlled and morbidity rate decreases day by day [2,3]. However, maintaining respiratory health is a major issue before illness occurs. Besides various available effective methods in modern/conventional therapy, complementary alternative medicine

(CAM) is also preferred by most people looking for cheaper and traditional different methods to prevent and treat respiratory diseases [6,7].

Current definition of CAM is "any diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine". During the last decades, the popularity of CAM has increased steadily, and these practices have become applicable by healthcare professionals under government supervision in developing countries [8-10]. It is known that in Europe, North America and other industrialized regions, approximately 70% of the population use at least one of the CAM practices. Although CAM has over 150 known treatment modalities (homeopathy, hypnotherapy, energy therapies, Ayurveda, e.g.), herbal medicine is the most common therapy in Turkey [8,11,12].

As a result of floristic richness, Turkey is one of the leading countries in terms of traditional herbal medicine usage. It has more than 11000 plant species with a nearly 34% endemism rate, which is almost equivalent to the entire European continent. Different civilizations settled in Anatolia lands bringing different cultures and customs have constituted an extensive accumulation of knowledge on traditional herbal medicine. Therefore, most people today benefit from medicinal plants for primary healthcare in the light of past knowledge and previous experience [13,14].

The use of medicinal plants has also been widely embraced in many developed countries. About 120000 plant species are used for nutrition or medical purposes all over the world and approximately 25% of today's modern drugs in the world is originated from medicinal plants [15,16]. In recent years, there has been a significant increase in medicinal plant research which evaluate effectiveness and safety of medicinal plants. These studies play an important role in the field of new drugs research and development. On the other hand, the number of companies producing herbal medicine based on quality and standards of synthetic pharmaceutical production has increased [6,11,16,17]. Recent studies revealed that herbal medicines are generally used by patients with common chronic diseases including breast cancer (12%), liver diseases (21%), HIV (22%), asthma (24%) and rheumatologic disorders (26%) [17]. However, information on medicinal plant usage for the treatment of respiratory diseases is limited in Turkey [10,18]. To address this need, we aimed to determine the frequency of medicinal plant use, species of medicinal plants, detailed usage and procurement information, the sociodemographic features affecting the tendency to use plants and the notification status to healthcare professionals in patients with respiratory diseases.

MATERIAL AND METHOD

Participants and questionnaires

A questionnaire-based survey design was used to collect data on use of medicinal plants in respiratory diseases. The study was conducted between January-April 2018 at the Molla Gurani Family Healthcare Centre. Approval was sought from the authorities by the official correspondence between the board of Faculty of Pharmacy and Family Healthcare Center. The questionnaire was filled out by 62 participants (18 years of age or older) diagnosed with respiratory disease. Before performing face-to-face interviews, information was given about the aim of the study and verbal consent was obtained. The questionnaire was designed specifically for this study and consisted of 18 questions. Most of them were multiple-choice questions; however, the participants were able to add other answers. The questionnaire consists of the following main themes: sociodemographic data (age, gender, place of residence, education), medical data (type of respiratory disease), detailed data on usage of medicinal plants (whether they use plants, plant species, reason for using plants, used parts and preparation, advice-benefits, procurement) and the notification status to healthcare professionals (drug or plant usage, response of healthcare professionals).

Statistical analysis

The obtained data were recorded and analyzed using Microsoft Office Excel and Statistical Package for the Social Sciences (SPSS) version 26.0 software. Both chi-square analysis and Fisher's Exact Test was used to determine the statistical significance of differences between groups. A p value less than 0.05 were taken to indicate statistical significance.

RESULT AND DISCUSSION

There were 62 patients included in the study, 36 of which were female and 26 were male. 70% of those who indicated that they had used plants in respiratory diseases was female. Patients came from a wide age range and the majority of patients were between 18-24 years of age (40.3%). Education was on a scale ranging from illiteracy to university graduates. The participants resided either in the urban or in the rural. The detailed sociodemographic features profile of participants who use plants or not are given in Table 1. Gender ($p=0.01$) and inhabitation ($p=0.007$) were statistically significant for plant usage in respiratory diseases. Females and patients who live in urban were more likely to use plants. But no significant difference was determined between plant users and non-users in terms of age and education status ($p>0.05$).

Table 1. Sociodemographic profiles of participants.

		Plant use		
Characteristics		**Yes, n (%)**	**No, n (%)**	**Total, n (%)**
Age p>0.05	18-24	11 (27.5%)	14 (63.6%)	25 (40.3%)
	25-30	11 (27.5%)	4 (18.2%)	15 (24.2%)
	31-40	5 (12.5%)	1 (4.5%)	6 (9.7%)
	41-50	3 (7.5%)	2 (9.1%)	5 (8.1%)
	51-60	6 (15%)	0 (0%)	6 (9.7%)
	61-70	4 (10%)	1 (4.5%)	5 (8.1%)
Gender p=0.01	Female	28 (70%)	8 (36.4%)	36 (58.1%)
	Male	12 (30%)	14 (63.6%)	26 (41.9%)
Education status p>0.05	Illiterate	2 (5%)	0 (0%)	2 (3.2%)
	Primary school	5 (12.5%)	1 (4.5%)	6 (9.7%)
	Middle school	5 (12.5%)	1 (4.5%)	6 (9.7%)
	High school	3 (7.5%)	6 (27.3%)	9 (14.5%)
	University	25 (62.5%)	14 (63.6%)	39 (62.9%)
Inhabitation p=0.007	Urban	26 (65%)	21 (95.5%)	47 (75.8%)
	Rural	14 (35%)	1 (4.5%)	15 (24.2%)

54.8% of the participants used vitamin-mineral at various frequency, whereas 45.2% of the participants did not use. They consumed vitamins-minerals every day, 3-4 times a week or 3-4 times a month. Majority of consumers used only when they feel bad (27.4%). The relation between vitamin-mineral usage and sociodemographic features was examined and no statistical differences were found ($p>0.05$). A total of 8 main respiratory diseases were determined in patients and their prevalance are listed as follows: flu-cold (38.7%), upper respiratory tract infection (14.5%), asthma (12.9%), cough (11.3%), COPD (9.7%), pharyngitis (6.5%), rhinitis (3.2%), tonsillitis (3.2%). Flu-cold was the most common disease that affect female and male patients. In the second place, while females suffered from asthma (19.4%), males suffered from both COPD (15.4%) and upper respiratory tract infection (15.4%). Detailed data is presented in Table 2.

Table 2. The medical data of participants.

Characteristics		**Female, n (%)**	**Male, n (%)**	**Total, n (%)**
Frequency of vitamin-mineral usage	Everyday	4 (11.1%)	1 (3.8%)	5 (8.1%)
	3-4 times a week	4 (11.1%)	3 (11.5%)	7 (11.3%)
	3-4 times a month	4 (11.1%)	1 (3.8%)	5 (8.1%)
	Only when I feel bad	9 (25.0%)	8 (30.8%)	17 (27.4%)
	No	15 (41.7%)	13 (50%)	28 (45.2%)
Respiratory disease	Flu-cold	14 (38.9%)	10 (38.5%)	24 (38.7%)
	Upper respiratory tract infection	5 (13.9%)	4 (15.4%)	9 (14.5%)
	Rhinitis	2 (5.6%)	0 (0%)	2 (3.2%)
	Tonsillitis	1 (2.8%)	1 (3.8%)	2 (3.2%)
	Asthma	7 (19.4%)	1 (3.8%)	8 (12.9%)
	Pharyngitis	1 (2.8%)	3 (11.5%)	4 (6.5%)
	Cough	4 (11.1%)	3 (11.5%)	7 (11.3%)
	Chronic obstructive pulmonary disease	2 (5.6%)	4 (15.4%)	6 (9.7%)

A total of 40 participants used plants for the treatment of respiratory diseases, as can be seen in Figure 1. A wide range of herbs were used for respiratory diseases; patients reported that they had used 14 different types of plants and plant-mixture to treat different conditions. The most popularly used plants and plant-mixture were mint-lemon, licorice, linden and rosehip, their common names are given (Figure 2). It was found that the leaves were the most frequently used part of the plant, accounting for 30%. Following in this category are fruits (23%), seeds (17%), stem (10%), aerial parts (7.5%), flowers (7.5%) and roots (5%) (Figure 3). Infusion (55%) was the most commonly used preparation method, others are given in Figure 4.

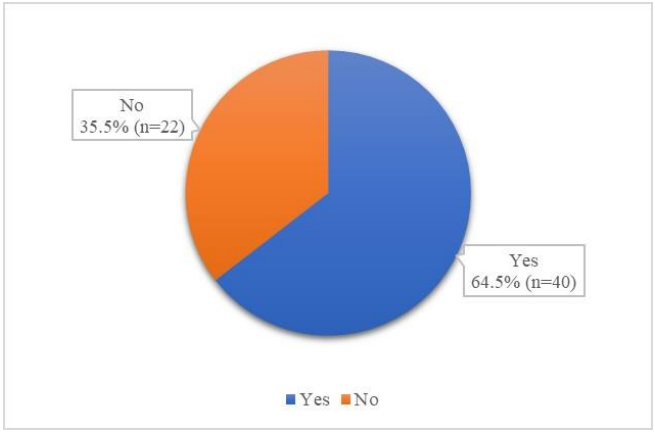

Figure 1. A rate of total plant usage for the treatment of respiratory diseases.

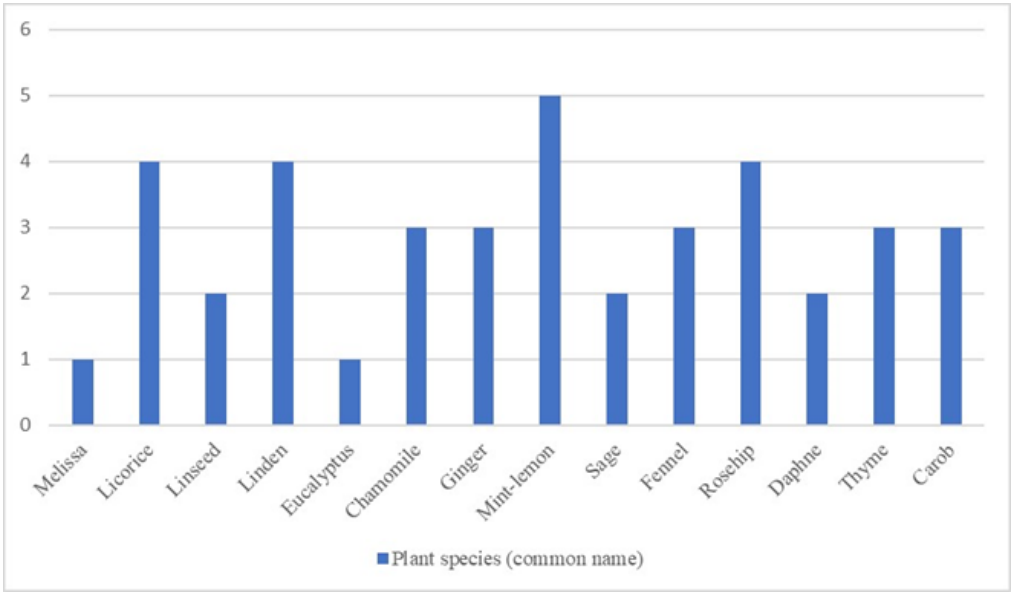

Figure 2. Plants and plant-mixture used for the treatment of respiratory diseases.

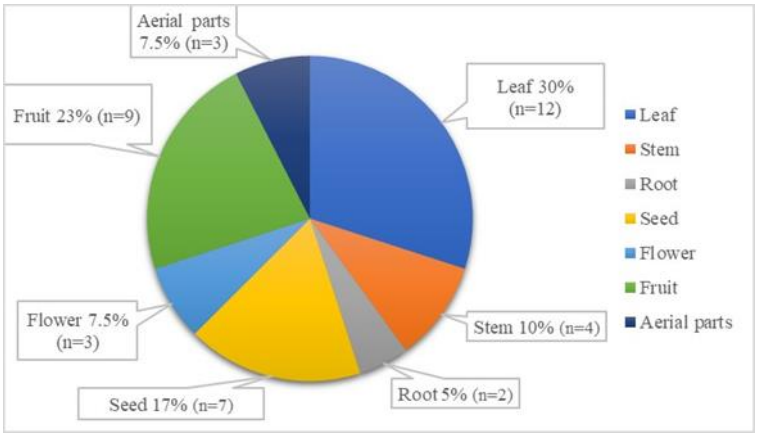

Figure 3. Plant parts used for the treatment of respiratory diseases.

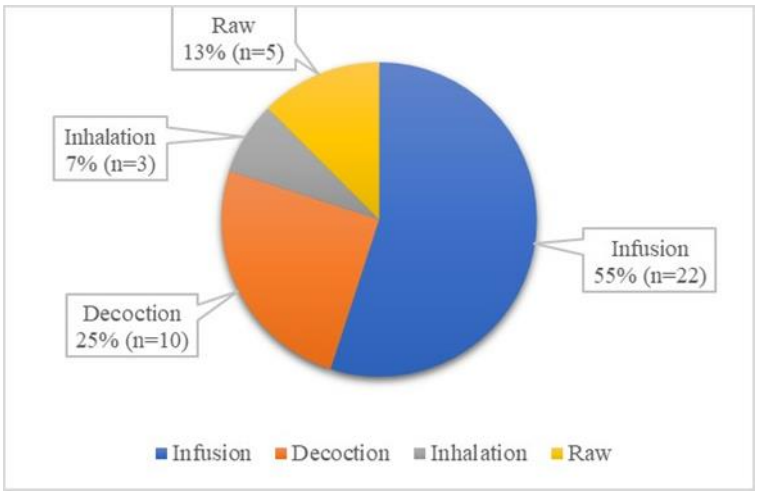

Figure 4. Preparation methods of plants used for the treatment of respiratory diseases.

Patients preferred plants for various reasons, primarily to help treatment (40%). They also used plants to feel good (30%), to treat disease (25%), and small number of patients used to reduce side effects (5%). Participants often purchased plants at a cost of between 0-50 tl (80%). Only 2% stated they bought plants at a cost of 500 TL and over. Participants said that they provided their medicinal plants usually from friends and relatives (37.5%), followed by herbalist (22.5%), other (17.5%) and pharmacist (15%). TV (5%) and Internet (2.5%) were very rarely cited sources. When asked about the effectiveness, most of the participants indicated that medicinal plants were beneficial (87.5%). A large majority of patients recommended the plants to other people (90%). There was no significant difference between sociodemographic features and data on plant usage (reasons, money, sale channel, benefit status, recommendation) ($p>0.05$), except for inhabitation-sale channel preferences

(p=0.014). 30.8% (n=8) of participants who live in city center consulted herbalist to buy plants, whilst who live in village supplied their plants from friends and relatives (64.3%, n=9) (Table 3).

Table 3. Detailed data on plant usage of patients.

Characteristics		n (%)
Reasons for plant use	To treat disease	10 (25%)
	To help treatment	16 (40%)
	To feel good	12 (30%)
	To reduce side effects	2 (5%)
Money paid by patients to supply plants	0-50tl	32 (80%)
	50-100 tl	4 (10%)
	100-500 tl	2 (5%)
	500 tl and over	2 (5%)
Sale channel preferences	Herbalist	9 (22.5%)
	Pharmacist	6 (15%)
	Internet	1 (2.5%)
	Friends and relatives	15 (37.5%)
	TV	2 (5%)
	Other	7 (17.5%)
Benefit status of plants	Beneficial	35 (87.5%)
	Harmful	2 (5%)
	Neutral	3 (7.5%)
Recommendation of plant use	Yes	36 (90%)
	No	4 (10%)

About a third of people said that they got approval from their doctor before taking a drug (33.9%). Additionally, only 40% of participants informed healthcare professionals about plants they had used. More than half of the informers stated that healthcare professionals approved the plant usage. According to the patients, other responses which they got from healthcare professionals were left my choice (18.8%), against the plant usage (12.5%) and no comment (12.5%). Analysis demonstrated that age (p=0.029), educational status (p=0.002) and inhabitation (p=0.015) were statistically significant for notification to healthcare professionals about plant usage. Participants who 18-24 years of age (50%, n=8), graduated from a university (81.3%, n=13) and live in city center (87.5%, n=14) were more likely to inform healthcare professionals about plant usage (Table 4).

Table 4. Notification to healthcare professionals.

Characteristics		n (%)
Taking drug without consulting doctors	Yes	41 (66.1%)
	No	21 (33.9%)
Notification to healthcare professionals about plant usage	Yes	16 (40%)
	No	24 (60%)
Response of healthcare professionals	Against the plant use	2 (12.5%)
	Approved	9 (56.3%)
	Left my choice	3 (18.8%)
	No comment	2 (12.5%)

Turkey is one of the leading country in terms of using herbs as medicines, drawn from a rich inventory of indigenous natural ingredients. The cultural beliefs and attitudes to herbal medicines have a long history and people still prefer herbs for primary healthcare, particularly in rural areas. Although many local studies have been carried out to investigate the prevalence of medicinal plant use in Turkey, few have focused specifically on patients with respiratory diseases [10,18].

This questionnaire-based survey provides detailed information about the use of medicinal plants in respiratory diseases. More than half (64.5%) of the respiratory disease patients reported using some species of plants, with some differences regarding sociodemographic factors. The results indicate that women use plants at greater rates than men do. We also found that people who live in urban areas are more likely to use plants. Many studies conducted worldwide have suggested that women are leading the way in using alternative therapies [19-22].

The most commonly used medicinal plants found in this study were different to previously published studies [20,23-26]. Impact of geographical and cultural variations may also bring out this result. It has been found that there are pharmacological studies supporting the use of these plants in respiratory diseases. While some medicinal plants have more than one medically used species, we researched by the most commonly known scientific names. As a result of our study, the mixture of mint-lemon was determined as most frequently used plant mixture to treat respiratory diseases and related conditions. Lemon (*Citrus limon* L.) has very rich bioactive compounds including citric acid, ascorbic acid, minerals, flavonoids and essential oils. Thanks to the anti-inflammatory, antioxidant and antiallergic effects of the main flavonoids (quercetin, hesperidin, rutin), lemon is thought as beneficial in respiratory diseases such as bronchitis and asthma [27]. *C. limon* is also a rich source of vitamin C, which has an important antioxidant activity [28]. The results from previous study indicated that phenolic and flavonoid compounds of *C. limon* extracts are correlated with a significant antioxidant activity [29]. Furthermore, its essential oil is mainly used to strengthen the immune system [30]. Similar to lemon essential oil, peppermint (*Mentha piperita* L.) oil has various medicinal properties and is used in folk medicine for respiratory diseases as expectorant and anti-congestive. Its leaf is also traditionally used to treat common cold, inflammation of the mouth and pharynx. Antispasmodic activity of peppermint essential oil on rat trachea with the involvement of prostaglandins and nitric oxide synthase has been shown by *in vivo* study [31]. Antioxidant, antimicrobial, antiviral, antiallergic and antiinflammatory activities of *M. piperita* (peppermint) has been proven in diverse studies. In particular, remarkable antimicrobial activity against *Escherichia coli, Streptococcus aureus* and *Pseudomonas aeruginosa* is promising for the usage as a remedy in respiratory diseases [32]. *Glycyrrhiza glabra* L. (licorice) possess important bioactive constituents such as triterpenoid saponin, flavonoids, tannins, alkaloids, phenolic compounds. The licorice

powder and extract are considered to be useful for the treatment of sore throat, cough and bronchial catarrh. It shows anti-tussive and expectorant activity by accelerating tracheal mucus secretion. Glycyrrhizin, an active compound of *G. glabra*, is contributed the demulcent effect. Moreover, antibacterial (against *E. coli*, *P. aeruginosa*, *Salmonella typhimurium*, *S. aureus*), antioxidant, antiviral (Severe Acute Respiratory Syndrome) and immuno-modulator (H1N1 Flue) effects have been demonstrated in several *in vitro* and *in vivo* studies [33,34]. *Tilia platyphyllos* Scop. (linden) is traditionally used in folk medicine. While infusion of flowers is prepared for the treatment of asthma and cough, decoction is used in cold and flu in Turkey [35]. Previous phytochemical and pharmacological investigations revealed antibacterial (against *Klebsiella pneumoniae*, *S. aureus*) and antioxidant activity of *Tilia* sp. owing to its rich chemical composition. The inflorescence of the *Tilia* sp. comprises polysaccharides (mucilaginous), condensed tannins, flavonoids, quercetin glycosides (rutin, quercitrin, and isoquercitrin), kaempferol glycosides and phenolic acids (caffeic, p-coumaric, and chlorogenic acids) [36]. In traditional medicine, *Rosa canina* L. (rosehip) fruits are used to treat various respiratory diseases such as common cold, flu, vitamin C deficiency and lung diseases. Vitamin C, which contributes to its potent antioxidant effect, is highly present in rosehip. Also, tannins and other bioactive compounds might provide its anti-inflammatory potentials [37,38]. Antibacterial activity against Gram positive and Gram negative bacterial species has been observed in various *in vitro* assays [37,39].

Pharmacological effects that can help treat respiratory diseases have been demonstrated in several studies, however, it is substantial to inform patients about the overdose and side effects of some herbs, as well as possible drug interactions. Licorice root can lead to pseudohyperaldosteronism. Patients were reported with long-standing hypokalemia and uncontrolled hypertension related to excessive ingestion of licorice [40]. Combination of ginger (*Zingiber officinale* Roscoe) and blood diluent medicines is not recommended due to strong inhibition effect of ginger on thromboxane synthetase. Long-term use of ginger may prolong bleeding time [41]. Moreover, care should be taken when providing plants that can be replaced with other harmful plant species. For instance, instead of *Matricaria chamomilla* L. (chamomille), similar species such as *Senecio* sp. and *Anthemis cotula* L., which contain toxic or allergic substances, can be taken from improper sale channels [42].

In our study, 87.5% of patients using medicinal plants stated that they benefited from the plants they had used. In addition to the pharmacological effects of these plants, the use of plants may reveal a general well-being of the person as a result of the positive psychological effect in patients. Previous studies reported that people use alternative therapies generally to add benefit to modern therapy or to cure illness [11,19]. In our study, most of the patients had used medicinal plants to help treatment.

There are some studies have emphasized communication gaps between healthcare professionals and patients in herbal therapies [11,19,22]. Patient-doctor communication and patient education about herbalism need to be improved. We detected 60% of the plant users stated that they did not report their plant use to the healthcare professionals. Our research shows that age, place of residence, and educational status have impact on the doctor-patient relationship. The majority of those who reported plant usage to the healthcare professionals stated that the healthcare professionals approved their usage. It seems that there is a prejudice in patients against healthcare professionals, and they do not want to share information about plants. Taking drug without consulting doctors was also found high (66.1%). These results can lead to undesirable consequences such as side effects, drug interactions, therefore healthcare professionals must inquire about plant use while taking an anamnesis.

90% of the plant users reported that they recommend the plants they use to other patients. Biçen et al. [43] reported that the source of information about the herbal product are the neighbor, relative and friend. However, it was observed that plants are generally provided from friends and relatives, and herbalists are preferred as the second option. Doğu et al. [19] stated that cancer patients usually prefer to shop from stores for herbal products. A survey from Western Australia indicated that the majority of the breastfeeding women had purchased their herbal medicines from community pharmacies [44]. According to our results, the place of residence affects the supply of plants. Unconscious consumption of plants that are sold in herbalists, various markets and TV-Internet, may be concluded with serious conditions. Patients should primarily prefer pharmacies to obtain the medicinal plants. Since the information about the use of plants is largely learned by the relatives and friends, it is critical for the public health to use the proper plants under the control of the doctor and the consultancy of the trained pharmacist [42]. Most patients spend money around 0-50 TL for purchase herbs. Supporting this outcome, it is seen that the most frequently used plants in respiratory diseases are everyday household or easily accessible products, which reduces costs.

There are also several limitations that should be mentioned. The survey population was relatively small, study was carried out in a single institution involving generally defined populations of respiratory diseases patients. Healthcare professionals should have been involved in this study. Although we account for several variables in our statistical analyses, other sociodemographic and related factors that were not taken in questionnaire may better account for medicinal plant use. While further studies may be warranted to investigate the attitudes toward medicinal plants in larger populations and with more variables, this present study offer useful information on the overall medicinal plant use in respiratory diseases.

This survey demonstrates a high prevalence of medicinal plant use among patients suffered from respiratory diseases. In total, 14 different plants-plant mixture were determined, most frequently

used were lemon-mint, licorice, linden and rosehip. It is known that medicinal plants are candidate for a source of biological and pharmacological products for the future. Although there is a lack of clinical studies in medicinal plants for many respiratory diseases, the general safety and efficacy data that do exist can lead us in its potential use or further investigation. More pharmacological studies, especially clinical studies regarding the efficacy and safety of medicinal plants are needed. While treatment with plants are even more popular, perceived as moderately effective and inexpensive, doctors should be routinely inquiring into its usage to avoid undesirable results. It is believed that by increasing the knowledge on phytotherapy, the patient-doctor relationship can progress more accurately in terms of treatment compliance and plant usage. This area deserve more careful selection and monitoring due to pharmacological activity, improper use and inability to control their side effects.

REFERENCES

1. Yorgancıoğlu, A. (2019). The global burden of chronic airway diseases. In: I. Agache and P. Hellings (Eds.), Implementing Precision Medicine in Best Practices of Chronic Airway Diseases, (pp. 33-37). Cambridge: Academic Press.

2. Jardins, T.D., Burton, G. (2020). Clinical Manifestations and Assessment of Respiratory Disease, 8th ed, Mosby, London.

3. Brennan, N.J. (2017). Respiratory Diseases: Overview. In: S.R. Quah, (Ed.), International Encyclopedia of Public Health, 2nd ed, (pp. 328-331). Massachusetts: Academic Press.

4. Yorgancıoğlu, A., Türktaş, H., Kalaycı, O., Yardim, N., Buzgan, T., Kocabaş, A., Karlikaya, C., Yildiz, F., Ergün, P., Mungan, D., Kart, L., Göktaş, E., Musaonbaşioğlu, S., Gündoğan, A., Akdağ, R., Akçay, S., Akin, M., Akkurt, I., Altan, P., Altunsu, T., … Bousquet, J. (2009). The WHO global alliance against chronic respiratory diseases in Turkey (GARD Turkey). Tüberküloz ve Toraks 2009, 57(4), 439-452.

5. Akturan, S., Gümüş, B., Özer, Ö., Balandız, H., Erenler, A. (2019). TÜİK verilerine göre Türkiye'de 2009 ve 2016 yılları arasındaki ölüm oranları ve nedenleri. Konuralp Medical Journal, 11(1), 9-16.

6. Asadbeigi, M., Mohammadi, T., Rafieian-Kopaei, M., Saki, K., Bahmani, M., Delfan, M. (2014). Traditional effects of medicinal plants in the treatment of respiratory diseases and disorders: An ethnobotanical study in the Urmia. Asian Pacific Journal of Tropical Medicine, 7, 364-368.

7. Ekor, M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Frontiers in Pharmacology, 4, 1-10.

8. Ernst, E., Fugh-Berman, A. (2002). Complementary and alternative medicine: What is it all about? Occupational and Environmental Medicine, 59(2), 140-144.

9. Algier, L.A., Hanoğlu, Z., Özden, G., Kara, F. (2005). The use of complementary and alternative (non-conventional) medicine in cancer patients in Turkey. European Journal of Oncology Nursing, 9(2), 138–146.

10. Orhan, F., Sekerel, B.E., Kocabaş, C.N., Saçkesen, C., Adalıoğlu, G., Tuncer, A. (2003). Complementary and alternative medicine in children with asthma. Annals of Allergy, Asthma & Immunology, 90(6), 611-615.

11. Özçelik, G., Toprak, D. (2015). Bitkisel tedavi neden tercih ediliyor? Ankara Medical Journal, 15(2), 48-58.

12. Şahin, S. (2017). Geleneksel, tamamlayıcı, alternatif tıp uygulamalarına genel bir bakış. Türkiye Aile Hekimliği Dergisi, 21(4), 159-162.

13. Güner, A., Aslan, S., Ekim, T., Vural, M., Babaç, M.T. (2012). Türkiye Bitkileri Listesi (Damarlı Bitkiler), Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayınları, İstanbul.

14. Erarslan, Z.B., Kültür, Ş. (2019). Ethnoveterinary medicine in Turkey: a comprehensive review. Turkish Journal of Veterinary and Animal Sciences, 43, 555-582.

15. Dutta, T., Paul, A., Majumder, M., Sultan, R.A., Emran, T.B. (2020). Pharmacological evidence for the use of Cissus assamica as a medicinal plant in the management of pain and pyrexia. Biochemistry and Biophysics Reports, 21, 1-8.

16. Yeşilada, E. (2013). An overview of Turkish folk medicine; past and present. Current Drug Delivery, 10, 1-4.

17. Şarışen, O., Çalışkan, D. (2005). Fitoterapi: Bitkilerle Tedaviye Dikkat. Journal of Contemporary Medical Education, 8(14), 182-187.

18. Kalyoncu, A.F., Selçuk, Z.T., İskendarani, A., Coplu, L., Emri, S., Şahin, A.A., Barış, Y.I. (1992). Alternative and complementary medicine for asthma. Thorax, 47, 762.

19. Doğu, G., Kargı, A., Tanrıverdi, Ö., Yaren, A., Demiray, G., Taşköylü, B.Y., Ergin, A. (2014). Complementary/Alternative medicine experience in cancer patients: A questionnaire-based survey. International Journal of Hematology and Oncology, 24, 1-10.

20. Blanc, P.D., Trupin, L., Earnest, G., Katz, P.P., Yelin, E.H., Eisner, M.D. (2001). Alternative therapies among adults with a reported diagnosis of asthma or rhinosinusitis. Chest, 120(5), 1461-1467.

21. Feldman, R.H., Laura, R. (2004). The use of complementary and alternative medicine practices among australian university students. Complementary Health Practice Review, 9(3), 173-179.

22. Chang, K.H., Brodie, R., Choong, M.A., Sweeney, K.J., Kerin, M.J. (2011). Complementary and alternative medicine use in oncology: A questionnaire survey of patients and health care professionals. BMC Cancer, 11(1), 1-9.

23. Chung, Y., Dumont, R.C. (2011). Complementary and alternative therapies: Use in pediatric pulmonary medicine. Pediatric Pulmonology, 46(6), 530-544.

24. Ziment, I., Tashkin, D.P. (2000). Alternative medicine for allergy and asthma. Journal of Allergy and Clinical Immunology, 106(4), 603-614.

25. Sorkness, R.L. (2009). CAM and Respiratory Disease. Nutrition in Clinical Practice, 24(5), 609-615.

26. Akan, H. (2012). Herbal and nonpharmacological therapies in upper respiratory tract infections. The Journal of Turkish Family Physician, 3, 9-15.

27. Dahima, R., Mishra, S., Rathore, D. (2016). In-vitro antioxidant and reducing potential activity of extracts of Citrus limon and Solanum lycopersicum. Der Pharmacia Lettre, 8, 258-269.

28. González-Molina, E., Domínguez-Perles, R., Moreno, D.A., García-Viguera, C. (2010). Natural bioactive compounds of Citrus limon for food and health. Journal of Pharmaceutical and Biomedical Analysis, 51(2), 327-345.

29. Makni, M., Jemai, R., Kriaa, W., Chtourou, Y., Fetoui, H. (2018). *Citrus limon* from Tunisia: Phytochemical and physicochemical properties and biological activities. BioMed Research International, 2018, 1-10.

30. Ali, B., Al-Wabel, N.A., Shams, S., Ahamad, A., Khan, S.A., Anwar, F. (2015). Essential oils used in aromatherapy: A systemic review. Asian Pacific Journal of Tropical Biomedicine, 5(8), 601-611.

31. De Sousa, A.A.S., Soares, P.M.G., De Almeida, A.N.S., Maia, A.R., De Souza, E.P., Assreuy, A.M.S. (2010). Antispasmodic effect of *Mentha piperita* essential oil on tracheal smooth muscle of rats. Journal of Ethnopharmacology, 130(2), 433-436.

32. Trevisan, S.C.C., Menezes, A.P.P., Barbalho, S.M., Guiguer, E.L. (2017). Properties of *Mentha piperita*: A brief review. World Journal of Pharmaceutical and Medical Research, 3(1), 309-313.

33. Sharma, V., Agrawal, R.C. (2013). *Glycyrrhiza glabra* - A plant for the future. Mintage Journal of Pharmaceutical and Medical Sciences, 2(3), 15-20.

34. Shinwari, Z.K., Khan, I., Naz, S., Hussain, A. (2009). Assessment of antibacterial activity of three plants used in Pakistan to cure respiratory diseases. African Journal of Biotechnology, 8(24), 7082-7086.

35. Kültür, Ş. (2007). Medicinal plants used in Kırklareli Province (Turkey). Journal of Ethnopharmacology, 111(2), 341-364.

36. Yayalacı, Y., Çelik, I., Batı, B. (2013). Hepatoprotective and antioxidant activity of linden (*Tilia platyphyllos* L.) ınfusion against ethanol-ınduced oxidative stress in rats. The Journal of Membrane Biology, 247(2), 181-188.

37. Chrubasik, C., Roufogalis, B.D., Müller-Ladner, U., Chrubasik, S. (2008). A systematic review on the Rosa canina effect and efficacy profiles. Phytotherapy Research, 22(6), 725-733.

38. Hamidi, S., Vaez, H., Asgharian, P. (2014). *Rosa canina* as an adjunctive treatment of asthma: A hypothesis. Advances in Bioscience and Clinical Medicine, 3(1), 48-52.

39. Wenzig, E.M., Widowitz, U., Kunert, O., Chrubasik, S., Bucar, F., Knauder, E., Bauer, R. (2008). Phytochemical composition and *in vitro* pharmacological activity of two rose hip (*Rosa canina* L.) preparations. Phytomedicine, 15(10), 826-835.

40. Kılınç, F., Demircan, F., Yıldırım, Y., Yılmaz, Z., Pekkolay, Z., Tuzcu, A.K. (2014). Licorice root (licorice) induced evaluation of the 5 patients presenting with hypertension and hypokalemia. Sakarya Medical Journal, 4(4), 186-190.

41. Moghaddasi, M.S., Kashani, H.H. (2012). Ginger (*Zingiber officinale*): A review. Journal of Medicinal Plants Research, 6(26), 4255-4258.

42. Uzun, M.B., Aykaç, G., Özçelikay, G. (2014). Bitkisel ürünlerin yanlış kullanımı ve zararları. Lokman Hekim Dergisi, 4(3), 1-5.

43. Biçen, C., Elver, Ö., Erdem, E., Kaya, C., Karataş, A., Dilek, M., Akpolat, T. (2012). Hipertansiyon hastalarında bitkisel ürün kullanımı. Journal of Experimental and Clinical Medicine, 29(2), 109-112.

44. Sim, T.F., Sherriff, J., Hattingh, H.L., Parsons, R., Tee, L.B. (2013). The use of herbal medicines during breastfeeding: a population-based survey in Western Australia. BMC Complementary Medicine and Therapies, 13, 1-10.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

PROTECTIVE ROLE OF *CRATAEGUS MONOGYNA* ON SPERM QUALITY AND TESTIS OXIDATIVE STRESS AGAINST COPPER-INDUCED TOXICITY

CRATAEGUS MONOGYNA'NIN, BAKIR KAYNAKLI TOKSİSİTEYE KARŞI SPERM KALİTESİ VE TESTİS OKSİDATİF STRESİ ÜZERİNDEKİ KORUYUCU ROLÜ

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ABSTRACT

Objective: *The purpose of this study was to investigate the possible beneficial use of common hawthorn Crataegus monogyna aqueous extract at 1.5g/Kg bw/day against chronic copper sulfate intoxication (100mg/Kg bw) in Wistar rats.*

Material and Method: *Animals were divided into 6 groups; the untreated control (C), 2 positive controls treated respectively with hawthorn fruits (F) and leaves (L), 1 group treated with copper (Cu) and 2 combined treatment groups treated with Cu and hawthorn-fruits (CuF) and Cu and hawthorn-leaves (CuL). After 30 days of oral administration, testis weight and plasma testosterone levels were evaluated, in addition to the epididymal sperm concentration, motility, vitality, velocity (VCL, VSL and VAP), the amplitude of lateral head displacement (ALH), and the beat cross frequency (BCF). Testicular glutathione (GSH), malondialdehyde (MDA), and glutathione peroxidase (GPx) were also evaluated.*

Result and Discussion: *Cu exposure reduced testosterone, sperm concentration, live sperm, VCL, VSL, VAP, ALH, BCF, GSH, and GPx levels compared to control groups. Dead sperm and MDA levels were increased in rats of Cu group compared to the untreated control. When compared to the Cu group, levels of testosterone, sperm concentration, sperm motility, live sperm, VCL, VSL, VAP, ALH, BCF, GSH, and GPx were much higher in the CuF and CuL groups, along with a significantly lower MDA concentration. In conclusion, hawthorn, when co-administered as an aqueous extract with Cu, protected most biological markers against copper toxicit, while positive control (s) boosted sperm concentration and velocity (VCL and VAP).*

Keywords: *C. monogyna, CASA, Copper, sperm, testosterone*

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ÖZ

Amaç: *Bu çalışmanın amacı, Wistar sıçanlarında, kronik bakır sülfat zehirlenmesine (100mg / Kg bw) karşı yaygın alıç Crataegus monogyna sulu ekstraktının (1.5g / Kg bw/ gün) olası faydalı kullanımını araştırmaktır.*

Gereç ve Yöntem: *Hayvanlar, tedavi edilmemiş kontrol (C), sırasıyla alıç meyveleri (F) ve yaprakları (L) ile muamele edilmiş 2 pozitif kontrol, bakır (Cu) ile muamele edilmiş grup ve Cu ve alıç meyveleri (CuF) ve Cu ve alıç yaprakları (CuL) ile muamele edilmiş 2 kombine tedavi grubu olmak üzere 6 gruba ayrılmıştır. 30 günlük oral uygulamadan sonra, epididimal sperm konsantrasyonu, motilite, canlılık, hız (VCL, VSL ve VAP), dış yana baş deplasmanının amplitüdü (ALH) ve sperm kuyruğu vuruş sıklığının (BCF) yanısıra testis ağırlığı ve plazma testosteron seviyeleri değerlendirilmiştir. Ayrıca, testis glutatyonu (GSH), malondialdehid (MDA) ve glutatyonperoksidaz (GPx) da değerlendirilmiştir.*

Sonuç ve Tartışma: *Cu maruziyeti, kontrol gruplarına kıyasla testosteron, sperm konsantrasyonu, canlı sperm, VCL, VSL, VAP, ALH, BCF, GSH ve GPx seviyelerini azaltmıştır. Cu grubunun sıçanlarında ölü sperm ve MDA seviyeleri, tedavi edilmeyen kontrole kıyasla artmıştır. Cu grubuyla karşılaştırıldığında, testosteron seviyeleri, sperm konsantrasyonu, sperm motilitesi, canlı sperm, VCL, VSL, VAP, ALH, BCF, GSH ve GPx seviyeleri CuF ve CuL gruplarında çok daha yüksek ve MDA konsantrasyonları ise anlamlı olarak düşüktür. Sonuç olarak, alıç, Cu ile sulu bir özüt olarak uygulandığında, bakır toksisitesine karşı çoğu biyolojik belirteci korumuş ve sperm konsantrasyonunu ve hızını (VCL ve VAP) artırmıştır.*

Anahtar Kelimeler: *Bakır, C. monogyna, CASA, sperm, testosteron*

INTRODUCTION

Copper is a trace element and important component of numerous metalloenzymes that are involved in energy and antioxidant metabolisms. However, some of copper's chemical forms, such as copper sulfate are very toxic [1]. Copper binds to binding proteins in the bloodstream and, distributes to all tissues especially the brain and the liver, which can secrete the excess of the metal into bile. Hypercupremia may cause several oxidation reactions, inflammations, and tissues damage by inducing free radical generations [2]. Copper can enter the body orally through food, by inhalation into the lungs or through the skin by direct contact. Copper is used in agriculture as a fungicide, herbicide, and insecticide [3]. Also, it is used as an electrical conductor in several industries, it has many chemical applications, and is known as a coinage metal. In nature, copper exposure may be caused by clouds of dust, volcanoes and forest fires. Copper dyshomeostasis has been linked to a variety of disorders. For example, ATP7A and ATP7B are both involved in copper metabolism; mutations of the former lead to Menkes' disease, but that of the latter causes Wilson's disease [4].

Previous studies showed that human spermatogenesis has decreased by up to 60% after 40 years [5]. In addition, many couples today are infertile [6]; this male sexual disorder may be related to pollutants [7]. The discharge of heavy metals into the environment has led to a harmful deterioration of the ecosystems [8]. Copper can cause several problems both in excess and deficiency. Copper imbalance in both men and women might affect reproduction, and women are said to be copper-dominant and men are zinc-dominant [1]. Excess copper harms male reproduction; metal

contamination provokes certain pathophysiological alterations in humans and animals, affecting sperm quality and causing infertility [9]. In its ionic form, copper is toxic to a variety of cells, including human spermatozoa [10]. According to Wong et al. [11], a positive correlation between blood Cu concentration and sperm motility dysfunction was found. However, cytosolic Cu is mainly bound to metallothioneins that may reduce its toxicity to some extent. The hydroxyl free radicals induced by the Fenton reaction of copper are very destructive to tissues [12] of the testis and epididymis, and also reduce antioxidant biomarkers such as catalase, superoxide dismutase, glutathione, and glutathione peroxidase. This oxidation can change sperm quality by modifying spermatozoa shape and movement. In addition, copper's effect on the pituitary receptor can provoke hormonal imbalance. Copper exposure causes several symptoms such as erectile dysfunction, anxiety, and testicular pain [1]. Furthermore, high copper levels can lead to a decrease in sperm concentration, motility, and vitality [13].

Today, numerous plants are used as remedies in the treatment of many ailments [14]. Hawthorn *Crataegus monogyna* is a very common shrub plant in the Mediterranean basin that is used by the local population in North Africa in certain traditional therapeutic applications including for hypertension, heart disorders, diabetes, anxiety, cancer, and some abdominal symptoms. The Crataegus spp have been used in medicinal treatments and as food [15]. Many studies conducted in Europe, Asia, and the USA showed that hawthorn has a high content of phenolic compounds that show antioxidant activity by scavenging superoxide anions, hydroxyl radicals, hydrogen peroxides, and by reducing lipid peroxidation [16].

This study aims to investigate the possible protective role of common hawthorn *Crataegus monogyna* aqueous extract against the induced toxicity of copper sulfate in Wistar rats by measuring certain reproductive and oxidative stress markers.

MATERIAL AND METHOD

Plant preparation

Fruits and leaves of the common hawthorn Crataegus monogyna were harvested every 3 days from the region of Annaba in the northeast of Algeria in November. Each of the 2 aqueous extracts of fruits (F) and leaves (L) was prepared daily by crushing 1.5g/kg bw (fruits and leaves) in an appropriate volume of distilled water and letting the mixtures streep overnight (12 hours) at room temperature in order to obtain 10 ml of filtered solutions of each of fruits and leaves in the morning. The aqueous extracts were administrated to rats per os daily for a period of 30 consecutive days.

Preparation of copper solution

Copper sulfate powder (Cu) was freshly dissolved in distilled water; this solution was administered to animals by gavage at a dose of 100mg/kg bw/day for 30 days.

Experimental design

Wistar rats were purchased from the Pasteur Institute (Algiers), each weighing 196±8 g. Thirty-six males were divided into 6 equal groups: the control (C), the copper group (Cu) which received 100 mg/kg bw/day, the fruits group (F) which received an aqueous extract of 1.5 g fruits/kg bw/day, the leaves group (L) which recieved an aqueous extract of 1.5g leaves/kg bw/day, and 2 other groups that were treated with a combination of copper and fruits (CuF) or copper and leaves (CuL). Rats received tap water and standard diet ad libitum. After 30 days of continuous treatment, animals were sacrificed by decapitation; the blood was collected in heparinized tubes and then centrifuged at 3000 rpm for 10min. The plasma obtained was stored at -20 °C along with the testes, which had already been weighed, till further analysis. Animals' treatments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba, before starting the experimental work.

Measurement of testosterone

The Ultrasensitive TESTOSTERONE ELISA test (DRG instrument GnbH) is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a district antigenic determinant on the intact testosterone molecule. Mouse monoclonal anti-testosterone antibody was used for solid phase (microtiter wells) immobilization, and goat anti-testosterone antibody was used in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the antibodies. After 2 hours of incubation at room temperature with shaking, the solid phase and enzyme were washed with distilled water to remove unbound labeled antibodies. A solution of tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of a blue color. The color development was stopped with the addition of 1N HCl, and the resulting yellow color was measured using a spectrophotometer at 450 nm. The concentration of testosterone was directly proportional to the color intensity of the sample.

Semen analysis

Semen analysis was realized using the Computer-Assisted Sperm Analysis Method (CASA) using Sperm Class Analysis (SCA®, Microptic, Barcelona, Spain). The epididymal semen was

obtained immediately after sacrifice, and then a drop of semen (about 1µl) was diluted with a physiological solution of NaCl 0.09%, and 5µL of the mixture was placed in an empty chamber slide (GoldCyto model). The slide was then placed on a Nikon Eclipse (Nikon E200-LED) microscope at the phase objective (x4). The sperm markers of concentration, motility, vitality linearity (VCL, VSL), velocity (VAP), the amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF) were automatically calculated.

Hypo-osmotic swelling (HOS) test

The HOST test was used to evaluate the integrity of sperm by exposing a drop of sperm, derived from the epididymis cauda, to the hypo-osmotic solution composed of fructose and sodium citrate [17], after which 100 spermatozoa were observed and the number of live sperms (as shown by an inflation of the tail) were counted.

Measurement of oxidative stress parameters

Frozen stored samples of testis were thawed and 100mg of each sample was taken and transferred to test tubes for the determination of glutathione (GSH) using the method of Cory and Weckbecker [18]. The testicular total proteins were quantified according to the colorimetric method of Bradford [19] by using the Coomassie Brilliant Blue G-250. Malondialdehyde (MDA) was estimated by using the method of Ohkawa et al. [20]. The measurement of glutathione peroxidase (GPx) was realized by the method of Flohe and Günzler [21].

The dosage of glutathione (GSH) was carried out according to the method of Wekbeker and Cory (1988). The principle of this assay is based on the measurement of the optical absorbance of the acid 2-nitro-5-mercapturic. The latter results from the reduction of 5,5'-dithio-bis-2- acid nitrobenzoïque (Ellman's reagent, DTNB) by groups (-SH) of glutathione. For this deproteinization of the homogenate is essential in order to keep only specific thiol groups of glutathione.

The tissues proteins were quantified according to the colorimetric method of Bradford (1967) who uses Gloss Blue Coomassie G250 (BBC) as a reagent and the serum albumin of breef (BSA) as standard. The BBC reacts with the amino groups (-NH2) for protein to form a complex of blue color. The emergence of this color reflects the degree of ionization of the acid and intensity established the concentration of protein which is measured spectrophotometrically at 595nm.

MDA is a product of lipid peroxidation reactions that forms during the attack of polyunsaturated lipids by reactive oxygen species generated by certain contaminants. In our study, testicular MDA levels were assessed using the method of Ohkawa et al (1979). The dosage is based on the formation in an acidic and hot environment (100 ° C) between MDA and thiobarbituric acid

(TBA) of a colored pigment absorbing at 530 nm, extractable by organic solvents like butanol.

The enzymatic activity of glutathione peroxidase (GPx) was measured by the method of Flohe and Gunzler (1984). This method is based on the reduction of hydrogen peroxide (H 2O2) in the presence of reduced glutathione (GSH), the latter is transformed into (GSSG) under the influence of the GPx.

Statistical analysis

Statistics was realized using (MINITAB 18 Software ANOVA Tukey). Results are expressed as mean ±standard deviation. The significant test was considered at $p<0.05$.

RESULT AND DISCUSSION

Testicular weights and testosterone

Results presented in Table 1 showed that differences between the absolute testicular weights of all groups were statistically non-significant when compared to the control. Testosterone concentration (Table 1) was significantly lower in Cu group compared to the untreated control. When compared to the Cu group, testosterone was significantly higher in the CuF and CuL groups. Testosterone levels of the positive control F and L groups were significantly lower compared to the control.

Table 1. Mean testicular absolute weights (g), and testosterone level (ng/ml) in wistar rats treated with copper sulphate and *C. monogyna* leaves and fruits extracts for one month. Results are expressed as mean±SD.

Groups	Testis (g)	Testosterone (ng/ml)
Control	1.67±0.31[a]	5.80±0.007[a]
Cu	1.67±0.015[a]	1.32±0.150[f]
F	1.67±0.01[a]	5.55±0.121[b]
L	1.69±0.02[a]	5.23±0.012[c]
CuF	1.67±0.012[a]	3.10±0.008[e]
CuL	1.67±0.01[a]	4.10±0.003[d]

Means that do not share the same letter are significantly different at $p<0.05$.

Sperm concentration

Results of semen analysis indicated a significant lower sperm concentration in the Cu group compared to the control, Sperm concentration showed no significant difference between the F group and the control but was significantly higher in the L groupthan in the control, and significantlyhigher in the CuF and CuL groups compared to the Cu group (Figure 1).

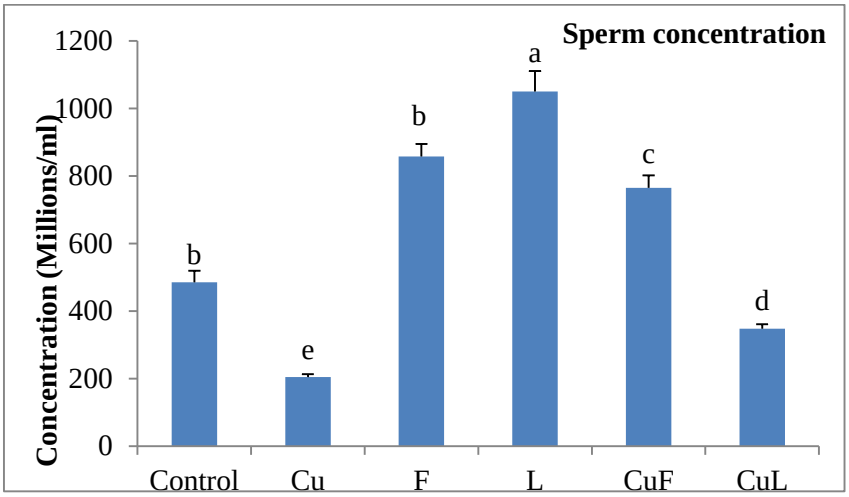

Figure 1. Evaluation of sperm concentration (Millions/ml) of Wistar rats exposed to copper sulphate and *C. monogyna* leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Sperm motility

Sperm motility was significantly lower in both the Cu group and the F group compared to the untreated control. Motility was also significantly lower in the CuF and CuL groups compared to the control but higher than the Cu group (Figure 2).

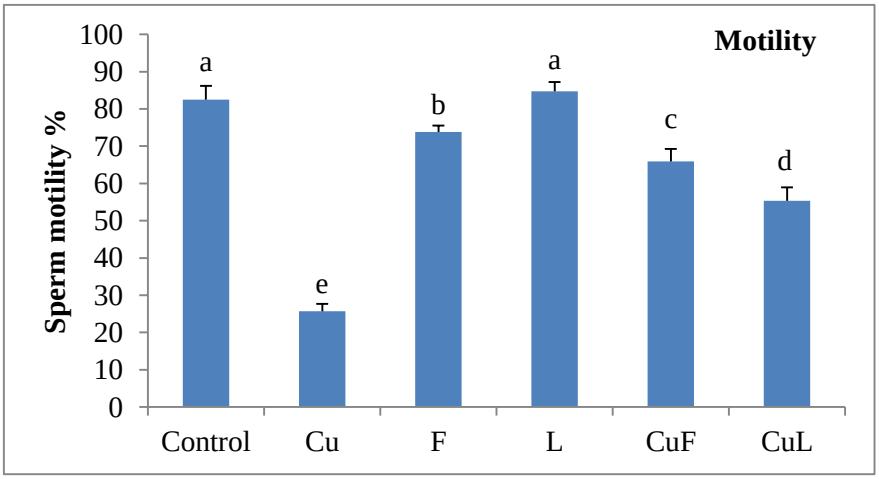

Figure 2. Evaluation ofsperm motility percentage (%) of Wistar rats treated with copper sulphate and *C. monogyna* leaves and fruits for one month. Results are expressed as mean ± SD.Means that do not share the same letter are significantly different at $p<0.05$.

Dead and live sperm

Percentage of dead sperm in the Cu group was significantly higher compared to the control, while F, L, CuF, and CuL group showed levels close to that of the control (Figure 3). On the other hand, percentage of dead sperm was remarkably lower in the combined treatments of CuF and CuL groups compared to the Cu exposed group. Live sperm of the Cu group was slightly decreased when compared to the control, with a weak reduction in the CuF and CuL groups, whereas F, CuF and CuL have kept in close percentage as that of the control (Figure 3).

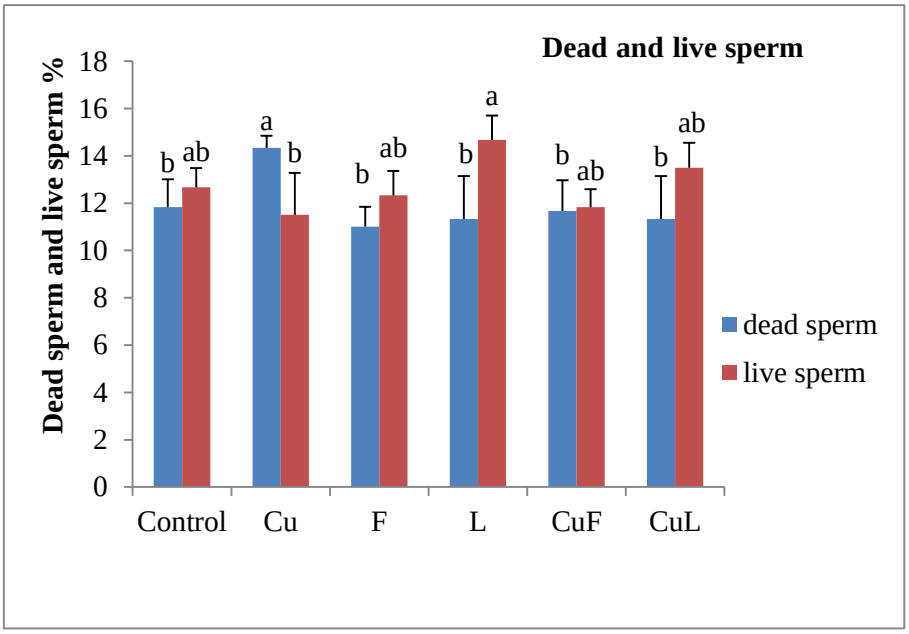

Figure 3. Evaluation of sperm vitality (dead sperm and live sperm percentage %) of Wistar rats exposed to copper sulphate and *C. monogyna* leaves and fruits for one month. Results are expressed as mean ± SD. Means that do not share the same letter are significantly different at $p<0.05$.

Sperm velocity

The VCL, VSL, and VAP of spermfrom the Cu group were significantly lower compared to the control. VCL, VSL, and VAP of spermin the CuF and CuL groups were significantly higher than the Cu group, and not statistically different fromthe control group (Figure 4). The VCL and the VAP of theL positive control were significantly higher than that of the control group.

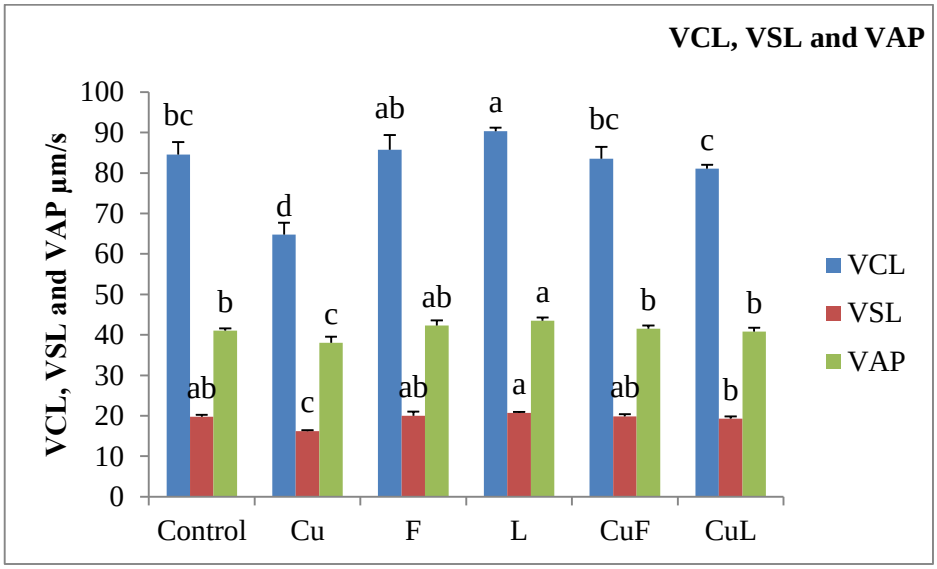

Figure 4. Evaluation of sperm velocity (VCL, VSL and VAP) of Wistar rats exposed to copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Amplitude of lateral head displacement

The sperm ALH was significantly lower in the Cu group compared to the control (Figure 5). Sperm ALH in the other groups were not significantly different from that of the control.

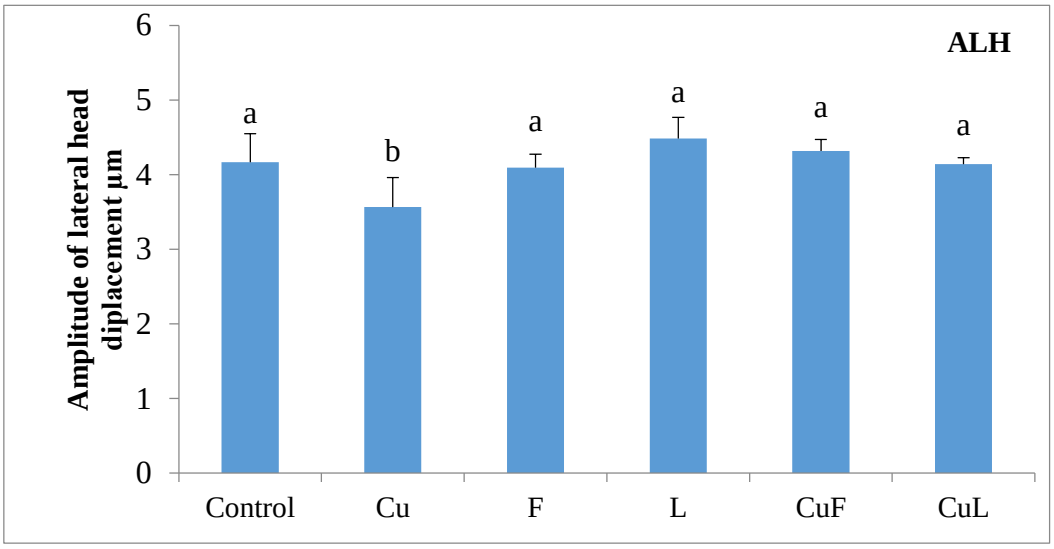

Figure 5. Evaluation of sperm amplitude lateral head displacement (ALH) of Wistar rats exposed to copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Beat cross frequency

The BCF was lower in the Cu group compared to the control, while F, L, CuF and CuL groups did not differ significantly from the control (Figure 6).

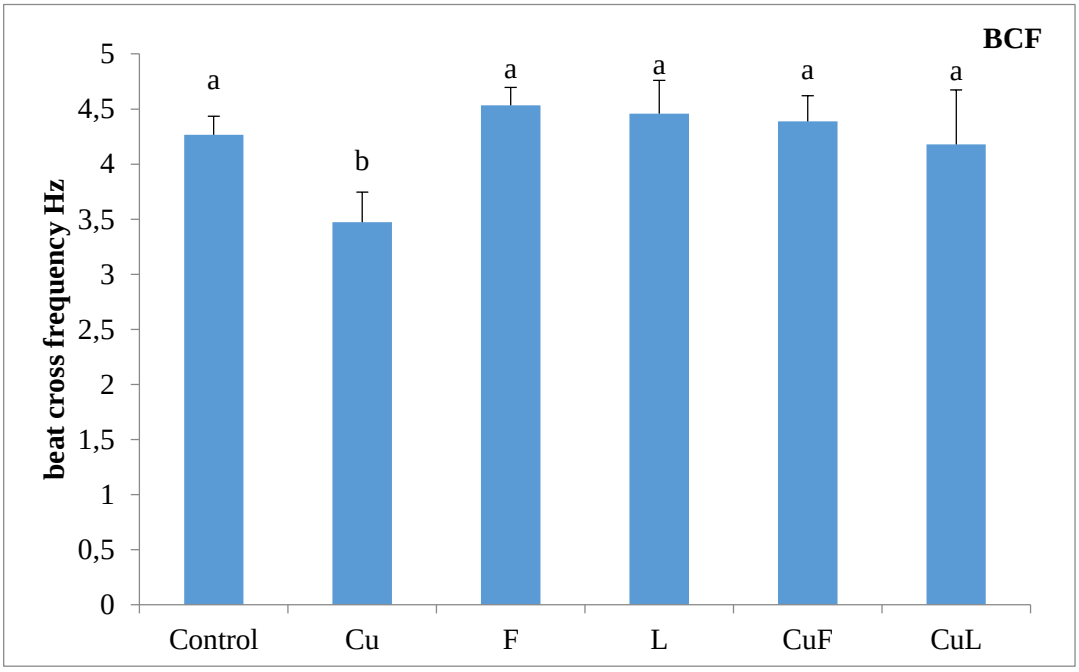

Figure 6. Evaluation of sperm beat cross frequency (BCF) of Wistar rats exposed to copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Oxidative stress markers

The MDA level was significantly higher in Cu group compared to the control group, but it was significantly lower in the CuF and CuL groups compared to the Cu group (Table 2). The CuF group showed significantly higher levels than the control, while the CuL group was not significantly different from the control.

In contrast, the GSH and GPx levels were significantly lower in the Cu group compared to the control. These levels were significantly higher in the CuF and CuL groups compared to both the Cu groupand the control (Table 2). There were no differences in the MDA, GSH and GPx of the positive controls F and L and that of the control.

Table 2. Mean testicular levels of MDA (nmol/g tissue), GSH (nmol/mg proteins), and GPx (µmol GSH/mg proteins) of wistar rats treated with copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD.

	MDA (nmol/g tissue)	**GSH (nmol/mg proteins)**	**GPx (µmol GSH/mg proteins)**
	Testis	Testis	Testis
Control	0.015±0.001^{c}	0.206±0.005^{ab}	0.067±0.007^{a}
Cu	0.018±0.002^{a}	0.066±0.001^{e}	0.026±0.002^{d}
F	0.015±0.005^{c}	0.209±0.001^{a}	0.071±0.001^{a}
L	0.015±0.002^{c}	0.203±0.001^{b}	0.070±0.009^{a}
CuF	0.016±0.002^{b}	0.110±0.001^{d}	0.057±0.008^{b}
CuL	0.015±0.002^{c}	0.179±0.002^{c}	0.035±0.008^{c}

Means that do not share the same letter are significantly different at $p<0.05$.

Results indicated a no difference in the absolute testicular weights of rats exposed to Cu for one month compared to the control group, which agrees with the finding of Chattopadhyay et al. [22]. Other researchers showed a slight decrease of rat testicular weight, in addition to a degenerative and necrotic effect of Cu on the seminiferous tubules and loss of spermatid from the center of the tubules [23]. In humans, Cu can produce adverse effects, including prostate enlargement, prostate infections, erectile dysfunction, depression, anxiety, testicular pain, and testicular cancer [1].

In this study, Cu was associated with lower testosterone concentration in rats; this result is in line with previous research showing that overdose of copper may affect the pituitary receptors, causing a reduction in the concentrations of LH and FSH, the key hormones in controlling testosterone release [24]. As a copper an antagonist of zinc, high copper levels can reduce zinc concentration; notably, zinc plays an important role in the formation of androgens [25]. The accumulation of heavy metals in the testis may lead to the inhibition of steroidogenesis [26].

In this investigation, rats exposed to copper had poorer sperm motility, velocity (VCL, VSL, and VAP), lateral head displacement, beat cross frequency, and vitality. This suggest that accumulation of copper in the epididymis, prostate, and seminal vesicle may inhibit sperm motility, BCF, and ALH, possibly by affecting the spermatozoa energy source, and by modifying the sperm tail shape, which can explain the decrease of the velocity measures VCL, VSL, and VAP. Moreover, Cu ions may accumulate in sperm mitochondria due to attraction to sulfhydryl groups, causing less ATP production, which can reduce sperm motility [27,28]. A high copper level in the reproductive organs is probably responsible for lowering the pH of seminal plasma, which was reported to

decrease sperm motility and the percentage of live sperm [13]. Other authors have found that a pH between 6.2 and 5.2 decreased sperm concentration, movement, and velocity measures VCL, VSL and VAP [29] by deregulating the Na/K ATPase activity [30] that blocks the entrance of calcium, which is responsible for improving the amplitude of flagellar beat [13]. Moreover, as copper is an antagonist of zinc, the deficiency of the latter may lead to low sperm concentration. Compared to other cells, spermatozoa contain less cytoplasm. As the latter is where the antioxidants system is found, having less cytoplasm makes spermatozoa susceptible to oxidative stress through lipid peroxidation [31], which affects the acrosome reactivity reactivity, and increases DNA damage [32]. This may explain the increase in dead sperm observed in the copper treated-group.

In the Fenton reaction, the cupric ions Cu+2 is reduced to cuprous Cu+; the latter is able to catalyze the hydroxyl radical, which is highly reactive, and form lipid radicals from fatty acids [12]. In our study, the group treated with Cu showed higher MDA levels, suggesting that copper ions inhibited the antioxidant reactions [33]. Copper as a destructive metal to tissues can cause lipid peroxidation, which can increase the testicular MDA levels [34].

We expected that high levels of copper in different tissues could produce reactive oxygen species (ROS), DNA damage, and lipid peroxidation [35]. In the present study, the observed low levels of GSH and GPx in the Cu exposed rats were likely to be caused by ROS production [36], and by cellular usage of GSH and GPx in the metabolism and detoxification of copper [23]. In addition, GPx is involved in scavenging hydrogen peroxide and lipid peroxidase caused by copper ions [37].

Although the hawthorn has been reported to treat sexual weakness in Noth Africa [38,39], there are not many studies about the effect of this plant on male fertility. The positive control of L showed higher sperm concentration and VCL in rats after a one-month treatment. As a powerful antioxidant, the fruits of hawthorn (Crataegus spp) may protect Sertoli cells from oxidative stress and may improve sperm quality due to the presence of phenolic compounds, oils, and vitamins. Previous findings showed the existence of vitamin C in the hawthorn and established the role of vitamin C on sperm characteristics [40], and the improvement of sperm concentration in male rabbits [41]. Similarly, our results showed an improvement in sperm quality in the CuL and CuF groups over the Cu group. The vitamin C in Crataegus spp. and in Rosaceae plants in general [40] helps in neutralizing hydroxyl, superoxide, and hydrogen peroxide radicals to prevent sperm agglutination [42]. In addition, vitamin C can both inhibit copper intestinal absorption, and increase its excretion [43]. The ability of vitamin C to scavenge free radicals could explain the remarkable lower levels of MDA observed in theCuL and CuF groups.

Furthermore, the augmentation in sperm motility in the CuL group is in line with the finding of Hu and Xiong [44] who observed that sperm from patients with asthenospermia showed increased

motility when co-incubated with extracts from the genus hawthorn (Crataegus spp). Studies on the chemical composition of C. monogyna revealed the presence of vitamins, flavonoids, and oils [40,45,46] that can act as a source of energy to boost sperm movement, increasing VSL and VAP [46]. This is probably why, in this study, the velocity measures (VCL, VSL, and VAP), ALH, and BCR were higher in the groups administered with C. monogyna extract.

Researchers previously found an effective role of C. monogyna extracts as an antioxidant [47,48] through the scavenging of free radicals and the inhibition of LDL oxidation [49]. Furthermore, the vitamin E in C. monogyna [45] can act as chain-breaking antioxidant [50], and prevents lipid peroxidation and tissue damage [42]. The occurrence of linoleic acid (omega-6); oleic acid (omega-9); oxalic acid bis (trimethylsilyl) ester; palmitic acid; and tetramethyl-cyclodecasiloxane that show powerful antioxidant activity [46] in C. monoyna might explain the lower levels of MDA and the higher levels of both GSH and GPx in the CuF and CuL groups. Previous results showed that omega-6 fatty acids can improve sperm motility [51], which may have contributed to the improvement of VCL, VSL, VAP, ALH and BCF levels in this study.

Quercetin was found in hawthorn, has been reported to act as a growth inhibitor for several malignant tumor cell lines, such as human epididymal cancer [52] by scavenging free radicals and chelating divalent cations [53]. Such a compound might have a role in reducing hydrogen peroxide, increasing sperm antioxidant defenses and preventing DNA damage induced by oxidative stress [54]. Moreover, quercetin may improve sperm quality by preserving the sex organs' functions [55]. Similarly, catechin polyphenols from C. monogyna, on the other side, were shown to reduce ROS by quenching free radicals and chelating transition metals [56], while catechins from green tea were shown to boost reproductive parameters [57]. This may explain the observed improvement of sperm concentration, motility, live sperm, velocity, ALH and BCF in the present study, especially in the CuL group.

In conclusion, copper induced oxidative stress, affecting testicular MDA, GSH, and GPx levels, and sperm quality parameters. The co-administration of extracts of C. monogyna fruits and leaves kept oxidative stress markers to almost their normal physiological ranges and improved semen quality, perhaps by mitigating the copper toxicity.

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REFERENCES

1. Badiye, A., Kapoor, N., Khajuria, H. (2013). Copper Toxicity: A Comprehensive Study. *Research Journal of Recent Sciences, 2,* 58-67.

2. Sinkovic, A., Strdin, A., Svensek F. (2008). Severe acute copper sulphate poisoning: a case report. *ArhHig Rada Toksikol, 59(1),* 31-5.

3. Blundell, S., Curtin, J., Fitzgerald, D. (2003). Blue lips, coma and haemolysis. Journal of Paediatrics and Child Health, 39(1), 67-8.

4. Mario, M. (2014). Abnormal Copper Homeostasis: Mechanisms and Roles in Neurodegeneration, *Toxics, 2,* 327-345.

5. Hagai, L., Niel, J., Anderson, M.A., Jaime, M., Dan, W.D., Irina, M., Rachel, P., Swan, H. (2017). Temporal trends in sperm counts: a systematic review and meta-regression analysis. *Human Reproduction Updates, 23(6),* 646-659.

6. Bamidele, R., Pelumi, O. (2017). Childlessness and Its Socio-Cultural Implication on Married Couples within Some Selected Yoruba Communities in South-West Nigeria. *International Journal of Innovative Social Sciences & Humanities Research, 5(1),* 42-54.

7. Chen, L., Shi, G.R., Huang, D.D., Li, Y., Ma, C.C., Shia, M., Su, B.X., Shi, G.J. (2019). Male sexual dysfunction: A review of literature on its pathological mechanisms, potential risk factors, and herbal drug intervention. *Biomedicine and Pharmacotherapy, 112,* 108-585.

8. Duruibe, J., Ogwuegbu, M.O.C., Egwurugwu, J. (2007). Heavy Metal Pollution and Human Biotoxic Effects. *International Journal of Physical Sciences, 2(5),* 112–118.

9. Swan, H.S., Elkin, E.P., Fenster, L. (2000). The question of declining sperm density revisited: an analysis of 101 studies published 1934–1996. *Environmental Health Perspectives, 108(10),* 961–966.

10. Liu, J.Y., Yang, X.F., Sun, X., Zhuang, C., Xu, F., Li, Y. (2016). Suppressive Effects of Copper Sulfate Accumulation on the Spermatogenesis of Rats. *Biological Trace Element Research, 174,* 356–361.

11. Wong, W.Y., Flik, G., Groenen, P.M.W., Swinkels, D.W., Thomas, C.M.G., Copius-Peereboom, J.H.J., Merkus, H.M.W.M., Steegers-Theunissen, R.P.M. (2001). The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. *Reproductive Toxicology, 15,* 131–136.

12. Moriwaki, H., Osborne, M.R., Phillips, D.H. (2008). Effect of mixing metal ions on oxidative DNA damage mediated by a Fentom-type reduction. *Toxicology in Vitro, 22(1),* 36-44.

13. Eidi, M., Eidi, A., Pouyan, O., Shahmohammadi, P., Fazaeli, R., Bahar, M. (2011). Seminal plasma levels of copper and its relationship with seminal parameters. *Iranian Journal of Reproductive Medicine, 8(2),* 60-65.

14. JibrinBala, Y., Meriçli, A.H. (2016). Pharmacological activities of Crataegus species: A review. *International Journal of Multidisciplinary Research and Development, 3,* 73-76.

15. Fong, H.H., Bauman, J.L. (2002). Hawthorn. Journal of Cardiovascular Nursing, *16,* 1–8.

16. Rice-Evans, C. (2004). Flavonoids and isoflavones: absorption, metabolism, and bioactivity. *Free Radical Biology and Medicine, 36(7),* 827-8.

17. Jeyendran, R.S., Vander-Van, H.H., Perez-Peleaz, M., Crabo, B.G., Zanevld, L.J.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characters. *Journal of Reproducion and Fertility, 70(1),* 219-228.

18. Weckbercker, G., Cory, J.G. (1988). Ribonucleotide reductase activity and growth of glutathione depended mouse leukaemia L 1210 cells in vitro. *Cancer Letters, 40,* 257-264.

19. Bradford, M.M. (1976). Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry, 72,* 248-254.

20. Ohkawa, H., Ohishi, N., Yagi, K. (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry, 95,* 351-358.

21. Flohe, L., Gunzler, W.A. (1984). Analysis of glutathione peroxidase. *Methods of Enzymology, 105,* 114-121.

22. Chattopadhyay, A., Sarkar, M., Biswas, N.M. (2005). Dose-dependent effect of copper chloride on male reproductive function in immature rats. *Kathmandu University Medical Journal, 3(4),* 392-400.

23. Mandil, R., Rahal, A., Parkash, A., Gangwar, S.K. (2016). Ameliorative potential of αtocophenol against flubendiamide and copper-induced testicular-insult in wistar rats. *Chemico-bioloical Interactions, 260,* 91-101.

24. Chang, CS., Choi, J.B., Kim, H.J., Park, S.B. (2011). Correlation between serum testosterone level and concentration of copper and zink in hair tissue. *Bioloical Trace Element Research, 144(1-3),* 264-271.

25. Bedwal, R.S., Bahuguna, A. (1994). Zinc, copper and selenium in reproduction. *Experientia, 50(7),* 626–40.

26. Wiebe, J.P., Salhanick, A.I., Myer, K.I. (1983). On the mechanism of action of lead in the testis: in vitro suppression of FSH receptors, cyclic AMP and steroidogenesis. *Life Science, 32(17),* 1997-2005.

27. Krumschnabel, G., Manzal, C., Berger, C., Hofer, B. (2005). Oxidative stress, mitochondiral permeability transition, and cell death in Cu-exposed trout hepatocytes. *Toxical Toxicology and Applied Pharmacology*, 209(1), 62-73.

28. Knazicka, Z., Tvrda, E., Bardos, L., Lukac, N. (2012). Dose- and time- dependent effect of copper ions on the viability of bull spermatozoa in different media. *Journal of Environmental Science and Health Part A Toxic/Hazardous Substances and Environmental Engineering, 47(9),* 1294-300.

29. Zhou, J., Chen, L., Li, J., Li, H., Hong, Z., Xie, M., Chen, S., Yao, B. (2015). The Semen pH Affects Sperm Motility and Capacitation. *PLoS ONE, 10(7),* e0132974.

30. Jimenez, T., Sanchez, G., Wertheimer, E.V., Blanco, G. (2010). Activity of the Na,K-ATPase alpha4 isoform is impor- tant for membrane potential, intracellular Ca2+, and pH to maintain motility in rat spermatozoa. *Reproduction, 139(5),* 835–45.

31. Olivari, F.A., Hernåndez, P.P., Allende, M.L. (2008). Acute copper exposure induces oxidative stress and cell death in lateral line hair cells of zebrafish larvea. *Brain Research, 1244,* 1-12.

32. Sadek, A.S., Abd El Shakour, A., Zaki A., Ibrahim, A.M., Mahmoud, I.S., Mostafa, T.B., (2011). Sperm chromatin condensation in infertile men with varicocele before and after surgical repair. *Fertility and Sterility, 95(5),* 1705-8.

33. Dupont, C.L., Grass, G., Rensing, C. (2011). Copper toxicity and the origin of bacterial resistance-new insights and applications. *Metallomics, 3(11),* 1109–1118.

34. Witting, P.K., Bowry, V.W., Stocker, R. (1995). Inverse deuterium kinetic isotope effect for peroxidation in human low-density lipoprotein (LDL): a simple test for tocopherol- mediated peroxidation of LDL lipids. *FEBS Letters, 375(1-2),* 45-49.

35. Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z. (2011). Metals as a cause of oxidative stress in fish: a review. *Veterinárnímedicína, 56(11),* 537-546.

36. Jiang, W.D., Liu, Y., Kai, H., Jiang, J., Li, S.H., Feng, L., Zhou, X.Q. (2014). Copper exposure induces oxidative injury, disturbs the antioxidant system and changes the Nrf/ARE (CuZnSOD) signaling in the fish brain: protective effect of myo-inositol. *Aquatic Toxicology, 155C,* 301-313.

37. Danyelle, T.M., Kanneth, T.D. (2003). The role of glutathion S-transferase in anti-cancer drug resistance. *Oncogene, 22(47),* 7369-75.

38. Miller, A.L. (1998). Botanical influences on cardiovascular disease. *Alternative Medicine Review, 3,* 422-431.

39. Ju, L.Y. (2005). Crataegusoxyacantha (aubepine) in the use as herb medicine in France. *ZhongguoZhong Yao ZaZhi, 30(8*), 634-40.

40. Rosario, G.M., Emmanuel, I.E., Raúl, N.A. (2013). Antioxidant compounds in hawthorn fruits (Crataegus spp.) of Mexico. *Revista Mexicana de Biodiversidad, 84(4),* 1298-1304.

41. Yousef, M.I., Abdullah, G.A., Kamel, I. (2003). Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Animal Reproduction Science, 76(1-2), 99-111.

42. Agarwal, A., Nallella, K.P., Allamaneni, S.S. (2004). Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reproductive Biomedicine Online, 8(6),* 616-27.

43. Frieden, E. (1986). Perspectives on copper biochemistry. *Clinical Physiology and Biochemistry, 4(1),* 11-9.

44. Hu, L., Xiong, C.L. (2006). The influence of medicated serum with root of Crataeguscuneata on human sperm motility parameters in vitro. *ZhongguoZhong Yao ZaZhi. 31(4),* 333-5.

45. Keser, S., Celik, S., Turkoglu, S., Yilmaa, Ö.,Turkoglu, I. (2015). Vitamin, Sterol and Fatty Acid Contents of Some Edible and Medicinal Plants from East and Southeast Anatolia (Turkey). *Turkish Journal of Pharmaceutical Sciences, 12(2),* 46-59.

46. Bechkri, S., Berrehal, D., Semra, Z., Bachari, K., Kabouche, A., Kabouche, Z. (2017). Composition and biological activities of seeds oils of two Crataegus species growing in Algeria. . *Journal of Materials and Environmental Sciences, 8(3),* 1023-1028.

47. Froehlicher, T., Hennebelle, T., Martin-Nizard, F., Cleenewerck, P., Hilbert, J.L., Trotin, F., Grec, S. (2009). Phenolic profiles and antioxidative effect of hawthorn cell suspension, fresh fruits, and medicinal dried parts. *Food Chemistry, 115(3),* 897-903.

48. Masteikova, R., Muselik, J., Bernatonienė, J., Bernatonienė, R. (2007). Antioxidative activity of Ginkgo, Echinacea, and Ginseng tinctures. *Medicina (Kaunas), 43(4),* 306-9.

49. Liu, T., Cao, Y., Zhao, M. (2010). Extraction optimization, purification and antioxidant activity of procyanidins from hawthorn (C. pinnatifidaBge. var. major) fruits. *Food Chemistry, 119(4),* 1656-1662.

50. Hwang, K. and Dolores J.b. (2013). Molecular mechanisms of antioxidants in male infertility. In: S.J. Parekattil and A. Agarwal (eds.), Antioxidants in male infertility: A guide for clinicians and researchers, (pp 93). New York Heidelberg Dordrecht London: springer.

51. Putri, N.D., Lipoeto, N.I., Reza, M. (2018). Effects of Omega 3 on Testosterone Hormone Levels and Quality of Spermatozoa in Obese RattusNorvegicusWistar Albino Strai. *International Biological and Biomedical Journal Summer, 4(3),* 156-163.

52. Kanadaswami, C., Lee, L.T., Lee, P.P.H., Hwang, J.J., Ke, F.C., Huang, Y.T., Lee, M.T. (2005). *The antitumor activities of flavonoids. In vivo, 19(5),* 895-909.

53. Seddiki, Y., Helena, M., Fernando, M. (2017). Antioxidant Properties of Polyphenols and Their Potential Use in Improvement of Male Fertility: A Review. Biomedical Journal of Scientific and *Technical Research, 1(3),* 2574-1241.

54. Zribi, N., Chakroun, FN., Ben Abdallah, F., Elleuch, H., Sellami, A., Jalel, G., Rebai, T., Fakhfakh, F., Ammar-Keskes, L. (2012). Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity. *Cryobiology, 65(3),* 326-331.

55. Taepongsorat, L., Tangpraprutgul, P., Noppadon, K., Malaivijitnond, S. (2008). Stimulating effects of quercetin on sperm quality and reproductive organs in adult male rats, *Asian Journal of Androloy, 10(2),* 249–258.

56. Nabavi, S.F., Habtemariam, S., Touqeer, A., Antoni, S., Daglia, M., Eduardo, S., Nabavi, S.M. (2015). Polyphenolic Composition of Crataegusmonogyna Jacq: From Chemistry to Medical Applications. *Nutrients, 7,* 7708-7728.

57. Sajid, U., Yingying, H., Lei, Z., Shibin, F., Ibrar, M.K., Jinjie, W., Yu, L., Xichun, W. (2018). Therapeutic Role of Green Tea Polyphenols in Improving Fertility: A Review. *Nutrients, 10(7),* 834.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

MOLECULAR DOCKING STUDIES ON SOME BENZAMIDE DERIVATIVES AS TOPOISOMERASE INHIBITORS

TOPOİZOMERAZ İNHİBİTÖRLERİ OLARAK BAZI BENZAMİD TÜREVLERİ ÜZERİNDE MOLEKÜLER DOKİNG ÇALIŞMALARI

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ABSTRACT

Objective: *In order to examine the interactions of some benzamide derivatives, which are thought to exhibit anti-cancer activity, with human Topo I and IIα enzymes at the molecular level, docking studies were carried out on these enzymes.*

Material and Method: *In conducting the docking studies, the protein was selected from the protein data bank for Topo I (1K4T) and for Topo IIα (5GWK). Doking was performed with the CDocker method using the Discovery studio 3.5 program, and the binding energies of benzamide derivatives to enzymes were calculated and their molecular interactions were revealed.*

Result and Discussion: *As a result of the docking process on Topo I and IIα, it was found that benzamide derivative compounds have higher affinity for Topo IIα enzyme. For Topo I compounds 4N6, 5N5; for Topo IIa compounds 5N3, 5N7 have been identified as promising compounds in terms of anticancer activity.*

Keywords: *Anticancer, Benzamide, Docking, Topoisomerase I, Topoisomerase IIα*

ÖZ

Amaç: *Antikanser aktivite göstereceği düşünülen Bazı benzamid türevlerinin insan Topo I ve IIα enzimleri ile moleküler düzeydeki etkileşimlerinin incelenmesi amacıyla bu enzimler üzerinden doking çalışmaları gerçekleştirilmiştir.*

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Gereç ve Yöntem: *Doking çalışmalarının gerçekleştirilmesinde protein veri bankasından Topo I için (1K4T) ve Topo IIα için (5GWK) seçilmiştir, Discovery studio 3.5 programı kullanılarak CDocker yöntemiyle doking işlemi yapılmış ve benzamid türevlerinin enzimlere bağlanma enerjileri hesaplanmış ve moleküler etkileşimleri ortaya çıkartılmıştır.*

Sonuç ve Tartışma: *Topo I ve IIα üzerinden yapılan docking işlemi sonucunda benzamid türevi bileşiklerin Topo IIα enzimine afinitesinin daha yüksek olduğu bulunmuştur. 4N6, 5N5 bileşikleri Topo I; 5N3, 5N7 bileşikleri de Topo IIα inhbitörleri olarak antikanser aktivite göstermesi açısından umut verici bileşikler olarak belirlenmiştir.*

Anahtar Kelimeler: *Antikanser, Benzamid, Doking, Topoizomeraz I, Topoizomeraz IIα*

INTRODUCTION

DNA topoisomerases are the enzymes which play key roles on cellular processes such as replication, transcription, recombination and repair, and chromatin assembly by solving these topological problems of genomic DNA[1-7]. Because of their essential functions in cell cycle, they are significant targets for killing cancer cells or pathogenic bacteria. DNA topoisomerases are classified into two classes as Topo I and Topo II, depending on the number of broken strands of DNA by the enzymes in one reaction cycle. All type of topoisomerases indicates their biochemical functions by catalyzing DNA cleavage and relegation [8].

Topo I functions by generating transient single-stranded cuts in DNA supercoils relaxing torsional strain that has accumulated during DNA replication and transcription [9, 10]. Intracellular levels of Topo I are upraised in some human solid tumors, relative to the corresponding normal tissues, suggesting that variations in Topo I levels are specific to the type of tumor [11-13]. DNA Topo I inhibitors, have recently emerged as a prominent class of anticancer agents with a novel mechanism of action, potent antiproliferative activity on a widespectrum of tumor cells including multidrug-resistant lines, and fascinating activity in xenograft models [14]. At first, camptothecin was discovered as a Topo I inhibitor in 1966, but could not be used in the clinic due to unpredictable and severe myelo suppression, gastrointestinal toxicity, and hemorrhagiccystitis [15]. Afterwards, it was found that the FDA approved anticancer agents topotecan and irinotecan, which are the analogue of camptothecin, inhibited the Topo I activity by intercalating into the cleavage complex and preventing the religation step of the catalytic cycle [16, 17].

Topo II cuts both strands of DNA by the enzymes in one reaction circle. Human Topo II have two available isoforms as α and β. Both of them sharing a similar tertiary structure and primary sequence, and perform similar functions but their levels differ depending on the replicative activity and type of tissue [18-20]. They also show various cellular functions, Topo IIα overexpressed in proliferating cells and generally located in the nuclearplasma. Topo IIβ plays apparent roles in transcriptional regulation, cell development, and differentiation, but not essential for cell

proliferation and survival. Although human Topo IIα relaxes negatively supercoiled plasmid slower than positively supercoiled plasmids, but Topo IIβ is not. Thus selective Topo IIα inhibitors have been of particular interest in cancer therapy, as they may represent a more targeted approach to highly proliferative cells [21-24]. Doxorubicin and Etoposide, classified as DNA Topo II inhibitors, have recently emerged as a prominent class of anticancer agents. Topo II inhibitors prevents re-ligation of the DNA strands, and breaks the DNA strands. Cancer cells depend on this enzyme more than healthy cells, for that they divide more rapidly. Therefore, this generates errors in DNA synthesis and promotes apoptosis of the cancer cell [10, 25].

Recently, amide derivatives received significant attention for their antitumor properties, especially the compounds which containing benzamide pharmacophore. The benzamide derivatives have been reported for their wide range of pharmacological activities including antitumor [26], histone deacetylase inhibition [27] and CYP24A1 inhibitory activity [28]. In addition to these activities some benzamide derivatives were used as HDAC inhibitors [29], glucokinase activators [30], antiprion agents [31] and topoisomerase inhibitors [32, 33] etc.

Recent developments in the field of cell biology want to introduce selective anticancer agents with low side effects to the pharmaceutical market, and the promising bioactive diversity of benzamide derivatives made us think that these derivatives will act as topoisomerase inhibitors, and in this study, the docking studies were performed to elucidate the interactions between various previously synthesized benzamide derivatives [34] and human Topo I and IIα enzymes and were aimed to identify a new type of anticancer drug candidates which have suitable properties to be promising oral human Topo I and IIα inhibitors.

MATERIAL AND METHOD

Preparation of the enzyme

Human Topo I has monomer structure and composed of 765 amino acids and human Topo IIα has a homo dimer structure and its monomer is composed of 1531 amino acids including four sections DNA-gate, Ngate, C-gate, and CTD [35]. The X-ray crystallographic structure of Topo I (PDB: 1K4T) and Topo IIα (PDB: 5GWK) are available in Protein Data Bank and further modified for docking calculations [36]. For preparation of protein Discovery Studio 3.5 software [37] was used. The target proteins were taken, hydrogens were added and their positions were optimized using the all atom CHARMm [38] force field and the Adopted Basis set Newton Raphson (ABNR) method [39] available in the D.S 3.5 protocol until the root mean square deviation (RMSD) gradient was <0.05 kcal/mol Å2. The minimized protein was defined as the receptor using the binding site module.

The binding site was defined from current selection around the ligand inside. The binding sphere were selected for 1K4T 6.12, 47.51, 26.54, 14.67 **(Figure 1A)** and for 5GWK 31.34, -23.16, -57.75, 10.32 **(Figure 1B)** from the active site using the binding site tools.

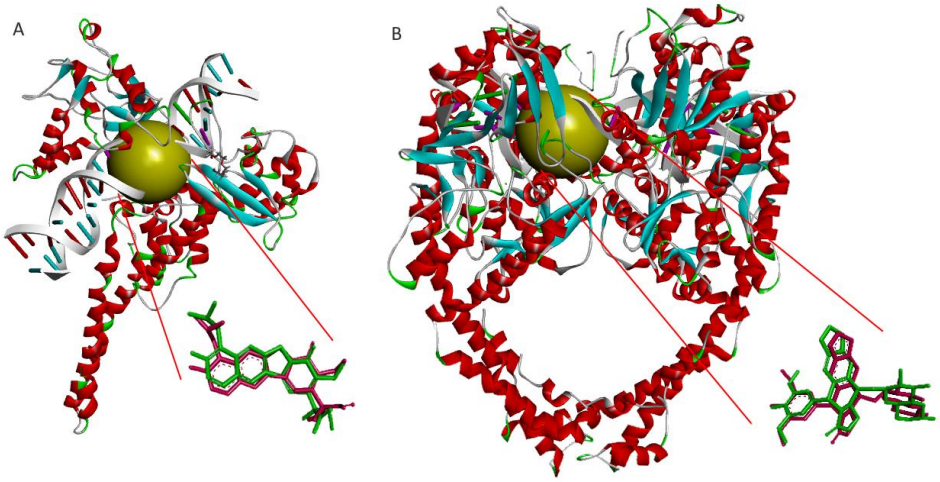
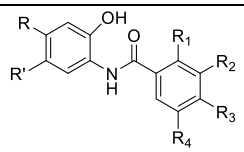


Figure 1. A. Topo I (pdb:1K4T) enzyme, the active site is located inside the sphere marked in yellow, superimpose position of Camptothecin with RMSD:1.2424**. B.** Topo IIα (pdb:5GWK) enzyme, the active site is located inside the sphere marked in yellow, superimpose position of Etoposide with RMSD:1.7219.

Preparation of ligands

Benzamide derivatives [34] given in **Table 1**, selected inhibitors Camptothecin and Etoposide were sketched with ChemDraw Professional; all-atom CHARMm force field parameterization was assigned and then minimized using the ABNR method as described above.

Table 1. Benzamide derivatives tested in molecular docking process



COMPOUND	R	R'	R_1	R_2	R_3	R_4
4N1	NO_2	H	H	H	C_4H_9	H
4N2	NO_2	H	H	H	$C(CH_3)_3$	H
4N3	NO_2	H	H	H	OC_2H_5	H
4N4	NO_2	H	H	H	OC_4H_9	H
4N5	NO_2	H	H	CH_3	H	CH_3
4N6	NO_2	H	H	OCH_3	H	OCH_3
5N1	H	NO_2	H	H	C_2H_5	H
5N2	H	NO_2	H	H	$C(CH_3)_3$	H
5N3	H	NO_2	H	H	OC_2H_5	H
5N4	H	NO_2	H	H	OC_4H_9	H
5N5	H	NO_2	CH_3	H	CH_3	H
5N6	H	NO_2	H	CH_3	H	CH_3
5N7	H	NO_2	H	OCH_3	H	OCH_3

Validation of Docking Process

In order to validate the accuracy of the process, docking studies were performed using the CDOCKER method [40] to the region determined on the proteins of the ligands carried by the enzymes. RMSD values were calculated by overlapping the obtained poses with the ligand found in the X-ray crystallography of the protein. The RMSD values expressing the difference between the optimal conformation of the ligand and X-ray crystallography were found to be 1. 2424 **(Figure 1A)** and 1.7219 **(Figure 1B)** for 1K4T and 5GWK, respectively.

Molecular Docking

Docking process was performed using the CDOCKER method in which the ligand moves flexibly while keeping the receptor stable. Ligands were interacted in 3000 different conformations in the active site of the enzyme. After the validation step, docking processes of benzamide derivatives and selected inhibitors were performed. Among the poses obtained as a result of these processes, the most suitable ones were determined, and their binding energies were calculated.

RESULT AND DISCUSSION

The interactions of benzamide derivatives with Topo I and IIα enzymes have been elucidated by applying molecular docking processes, and it has been found that the compounds generally show a better interaction with the Topo IIα enzyme. When benzamide derivatives and Camptothecin were docked in the active site of the 1K4T enzyme selected from pdb as the Topo I enzyme, they show various interactions with residues DT10, DG12, DA113, DC112 and TGP11 of DNA and amino acids ASN352, GLU356, ARG364, TRP416, LYS425 and THR718 of enzyme as given in **Table 2** and the binding energies of these compounds also range between -57,7457 and 97.388 kcal/mol. The interactions of Camptothecin, Topo I enzyme inhibitor, were examined, it was observed that it binds to the enzyme with -16,5852 kcal/mol binding energy and interacted with LYS425 amino acid and DT10, DA113, DC112, TGP11 residues, as given **Figure 2A**. Compounds 4N6, 5N5, 4N2, 5N4, 4N4, 4N3 and 5N3 were interacted with lower binding energies than Camptothecin to the enzyme respectively, while other compounds exhibited positive binding energies. The compound 4N6 gave the best binding energy (-57,7457 kcal/mol) with Topo I enzyme and interacted with GLU356, TGP11, DC112, DA113 residues through phenyl and hydroxyl groups in the molecule, as given **Figure 2B**. The compound 5N5 showed a good interaction with Topo I enzyme with its binding energy of -50,3612 kcal/mol and made H bond to DA113 residue with its hydroxyl group and showed

pi interactions between phenyl rings and DT10, TGP11, DC112, DA113 residues, as given **Figure 2C**.

Table 2. Interaction properties of benzamide derivatives with Topo I

Compound	binding energy (kcal/mol)	conventional Hydrogen Bond	carbon Hydrogen bond	Pi Interactions
4N1	97.388	THR718, TGP11	DG12	DC112, DA113
4N2	-37,6963	-	DA113	TGP11, DA113, LYS425
4N3	-23,5498	ASN352	DT10	DA113, TGP11,
4N4	-32,0942	ASN352	DT10	DA113
4N5	30,0267	DC112, DA113, ARG364	TGP11	DT10
4N6	-57,7457	-	GLU356, DC112, DA113	TGP11
5N1	-19,2227	TGP11	-	TRP416, LYS425
5N2	0,04961	TGP11	-	DA113, TRP416, LYS425
5N3	-22,824	DT10	-	DC112, DA113
5N4	-35,4994	ASN352	DT10	GLU356, TGP11, DC112, DA113
5N5	-50,3612	DA113	-	DT10, TGP11, DC112
5N6	15,7991	-	TGP11	TGP11, DC112, DA113
5N7	2,02614	ASN352	DT10, DA113, TGP11	-
Camptothecine	-16,5852	-	LYS425	DT10, TGP11, DC112, DA113

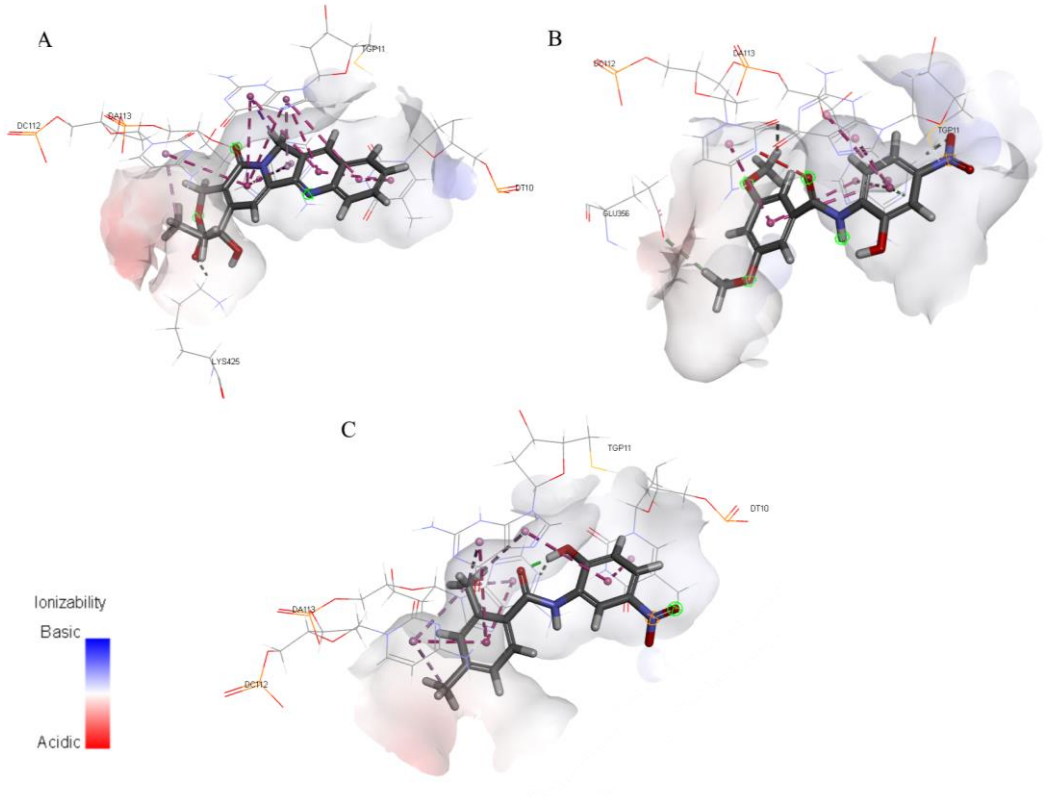


Figure 2. Molecular interactions of the Topo I enzyme **A.** Docked pose of Camptothecine, **B.** Docked pose of compound 4N6, **C.** Docked pose of compound 5N5.

When benzamide derivatives and Etoposide were docked in the active site of the 5GWK enzyme selected from pdb as the Topo IIα enzyme, they show various interactions with residues DC8, DT9, DG10, DC11, DA12, DG13 and DC14 of DNA and amino acids GLY462, ARG487, GLY760, MET762 and TYR805 of enzyme as given in **Table 3** and the binding energies of these compounds also range between -114,71 and -60,1444 kcal/mol. The interactions of Etoposide, Topo IIα enzyme inhibitor, were examined, it was observed that it binds to the enzyme with -114,71 kcal/mol binding energy and made H bond to DG13 residue with its hydroxyl group. It also interacted with LYS440, ARG487 amino acids and DT9, DA12, DG13, ARG487 residues, as given **Figure 3A**. The binding energies of benzamide derivatives were higher than etoposide, but it was observed that all molecules interacted with the enzyme with low binding energies. Compounds 5N3 and 5N7 indicated good interactions with the Topo IIα enzyme with binding energies of -94,3762 and -92,0598 kcal/mol, respectively. The compound 5N3 made H bond to DG13 residue with its nitrogen atom in amide group and also interacted with ARG487, DC8, DT9 residues, as given **Figure 3B**. The compound 5N7 made H bond to ARG487 with its methoxy group and showed pi interactions with DC8, DT9, DA12, DG13 residues, as given **Figure 3C**.

Table 3. Interaction properties of benzamide derivatives with Topo IIα

Compound	binding energy (kcal/mol)	conventional Hydrogen Bond	carbon Hydrogen bond	Pi Interactions
4N1	-74,4069	DT9, ARG487	DG13	DC8, DA12
4N2	-74,456	TYR805	GLY462	ARG487, DG13
4N3	-65,2691	DT9	DG13	ARG487, MET762, DG8, DG13
4N4	-88,0887	DT9	DG13	DC8, DA12
4N5	-62,9599	DG13	GLY760	DA12
4N6	-65,1616	DT9	DG13	DC8, DA12
5N1	-75,7866	DT9, ARG487		MET762, DC8, DT9, DG13
5N2	-61,5032			DC8, DT9
5N3	-94,3762	DG13	ARG487	DC8, DT9
5N4	-90,2323	DT9, DC14		DC8, DT9, ARG487
5N5	-60,1444	DG13	GLY760	ARG487, DC8, DT9
5N6	-87,8215	DG13		DC8, DT9
5N7	-92,0598	ARG487		DC8, DT9, DA12, DG13
Etoposide	-114,71	DG13	DG10, DC11, DA12, LYS440, ARG487	DT9, DA12, DG13, ARG487

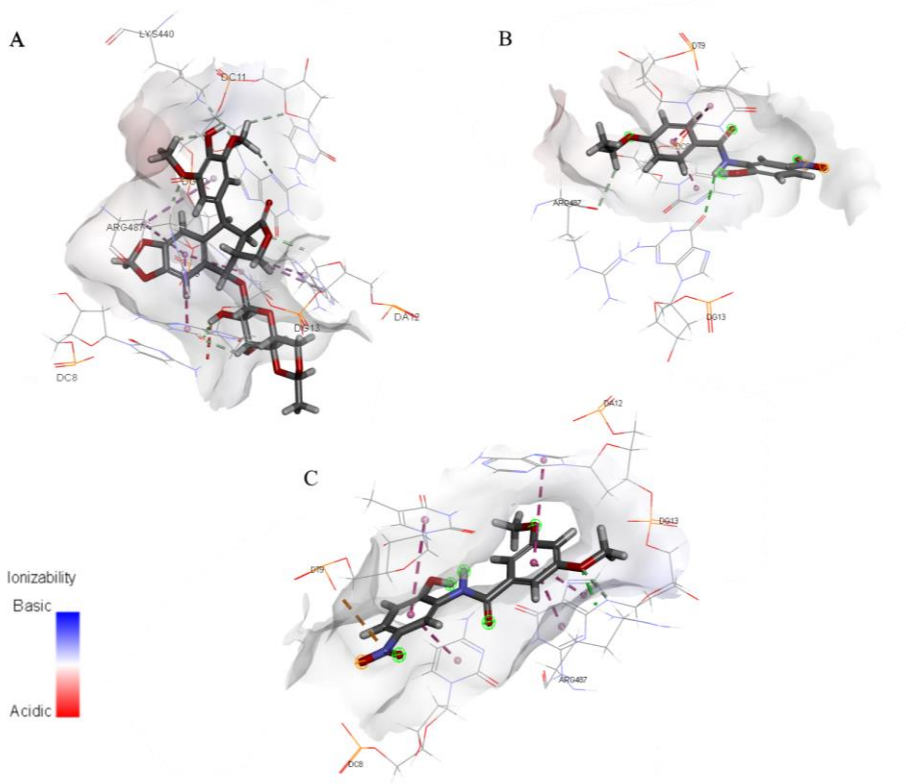


Figure 3. Molecular interactions of the Topo IIα enzyme **A.** Docked pose of Etoposide, **B.** Docked pose of compound 5N3, **C.** Docked pose of compound 5N7.

As a result of the docking studies on Topo I and IIα enzymes of benzamide derivatives, which are thought to have anticancer activity as topoisomerase inhibitors, it has been shown that the compounds have higher affinity for the Topo IIα enzyme, but have a lower effect than the reference compound. However, most of the compounds docked on Topo I enzyme were performed better results than the reference molecule. The performed docking studies should be supported by experimental results, but its clear that the accompanying results represent that compounds are promising inhibitors for Topo I and IIα enzymes.

REFERENCES

1. Holden, J.A. (2001). DNA Topoisomerases as anticancer drug targets from the laboratory to the clinic. *Current Medicinal Chemistry-Anti-Cancer Agents*. *1*(1), 1-25.

2. Topcu, Z. (2001). DNA topoisomerases as targets for anticancer drugs. *Journal of clinical pharmacy and therapeutics*. *26*(6), 405-416.

3. Wang, J.C. (1996). DNA topoisomerases. *Annual review of biochemistry*. *65*(1), 635-692.

4. Berger, J.M. (1998). Structure of DNA topoisomerases. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*. *1400* (1-3), 3-18.

5. Kaufmann, S.H. (1998). Cell death induced by topoisomerase-targeted drugs: more questions than answers. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*. *1400* (1-3), 195-211.

6. Pommier, Y. (1998). Diversity of DNA topoisomerases I and inhibitors. *Biochimie*. *80*(3), 255-270.

7. Redinbo, M.R., Stewart, L., Kuhn, P., Champoux, J.J., Hol, W.G. (1998). Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. *Science*. *279* (5356), 1504-1513.

8. Cowell, I.G., Sondka, Z., Smith, K., Lee, K.C., Manville, C.M., Sidorczuk-Lesthuruge, M. , Rance, H.A., Padget, K., Jackson, G.H., Adachi, N. (2012). Model for MLL translocations in therapy-related leukemia involving topoisomerase IIβ-mediated DNA strand breaks and gene proximity. *Proceedings of the National Academy of Sciences*. *109*(23), 8989-8994.

9. Wang, J.C.. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. *Nature reviews Molecular cell biology*. *3*(6), 430-440.

10. Pommier, Y., Leo, E., Zhang, H., Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chemistry & biology*. *17*(5), 421-433.

11. Giovanella, B.C., Stehlin, J.S., Wall, M.E., Wani, M.C., Nicholas, A.W., Liu, L.F., Silber, R., Potmesil, M. (1989). DNA topoisomerase I--targeted chemotherapy of human colon cancer in xenografts. *Science*. *246*(4933),1046-1048.

12. Husain, I., Mohler, J.L., Seigler, H.F., Besterman, J.M. (1994). Elevation of topoisomerase I messenger RNA, protein, and catalytic activity in human tumors: demonstration of tumor-type specificity and implications for cancer chemotherapy. *Cancer research*. *54*(2), 539-546.

13. Lima, C.D., Wang, J.C., Mondragón, A. (1994). Three-dimensional structure of the 67K N-terminal fragment of E. coli DNA topoisomerase I. *Nature*. *367*(6459), 138-146.

14. Garcia-Carbonero, R., Supko, J.G. (2002). Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clinical Cancer Research*. *8*(3), 641-661.

15. Wall, M.E., Wani, M.C., Cook, C., Palmer, K.H., McPhail, A.A., Sim, G. (1966). Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminata1, 2. *Journal of the American Chemical Society*. *88*(16), 3888-3890.

16. Thomas, C.J., Rahier, N.J., Hecht, S.M. (2004). Camptothecin: current perspectives. *Bioorganic & medicinal chemistry*. *12*(7), 1585-1604.

17. Teicher, B.A. (2008). Next generation topoisomerase I inhibitors: Rationale and biomarker strategies. *Biochemical pharmacology*. *75*(6), 1262-1271.

18. Nitiss, J.L. (2009). DNA topoisomerase II and its growing repertoire of biological functions. *Nature Reviews Cancer*. *9*(5), 327-337.

19. Pogorelcnik, B., Perdih, A., Solmajer, T. (2013). Recent advances in the development of catalytic inhibitors of human DNA topoisomerase IIα as novel anticancer agents. *Current medicinal chemistry*. *20*, 694-709.

20. Schmidt, B.H., Osheroff, N., Berger, J.M. (2012). Structure of a topoisomerase II–DNA–nucleotide complex reveals a new control mechanism for ATPase activity. *Nature structural & molecular biology*. *19*, 1147.

21. Larsen, A.K., Escargueil, A.E., Skladanowski, A. (2003). Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacology & therapeutics*. *99*, 167-181.

22. Farr, C.J., Antoniou-Kourounioti, M., Mimmack, M.L., Volkov, A., Porter, A.C. (2014). The α isoform of topoisomerase II is required for hypercompaction of mitotic chromosomes in human cells. *Nucleic acids research*. *42*, 4414-4426.

23. Arcy, D. N., Gabrielli, B. (2017). Topoisomerase II inhibitors and poisons, and the influence of cell cycle checkpoints. *Current Medicinal Chemistry*. *24*, 1504-1519.

24. McClendon, A.K., Rodriguez, A.C., Osheroff, N. (2005). Human topoisomerase IIα rapidly relaxes positively supercoiled DNA implications for enzyme action ahead of replication forks. *Journal of Biological Chemistry*. *280*, 39337-39345.

25. Hande, K. (1998). Etoposide: four decades of development of a topoisomerase II inhibitor. *European journal of cancer*. *34*, 1514-1521.

26. Zhang, J., Yang, P.L., Gray, N.S. (2009). Targeting cancer with small molecule kinase inhibitors. *Nature Reviews Cancer*. *9*, 28-39.

27. Lu, A., Luo, H., Shi, M., Wu, G., Yuan, Y., Liu, J., Tang, F. (2011). Design, synthesis and docking studies on benzamide derivatives as histone deacetylase inhibitors. *Bioorganic & medicinal chemistry letters*. *21*, 4924-4927.

28. Aboraia, A.S., Yee, S.W., Gomaa, M.S., Shah, N., Robotham, A.C., Makowski, B., Prosser, D., Brancale, A., Jones, G., Simons, C. (2010). Synthesis and CYP24A1 inhibitory activity of N-(2-(1H-imidazol-1-yl)-2-phenylethyl) arylamides. *Bioorganic & medicinal chemistry*. *18*, 4939-4946.

29. Li, Y., Zhou, Y., Qian, P., Wang, Y., Jiang, F., Yao, Z., Hu, W., Zhao, Y., Li, S. (2013). Design, synthesis and bioevalution of novel benzamides derivatives as HDAC inhibitors. *Bioorganic & medicinal chemistry letters*. *23*, 179-182.

30. Mao, W., Ning, M., Liu, Z., Zhu, Q., Leng, Y., Zhang, A. (2012). Design, synthesis, and pharmacological evaluation of benzamide derivatives as glucokinase activators. *Bioorganic & medicinal chemistry*. *20*, 2982-2991.

31. Fiorino, F., Eiden, M., Giese, A., Severino, B., Esposito, A., Groschup, M.H., Perissutti, E., Magli, E., Incisivo, G.M., Ciano, A. (2012). Synthesis of benzamide derivatives and their evaluation as antiprion agents. *Bioorganic & medicinal chemistry*. *20*, 5001-5011.

32. Kamal, A., Suresh, P., Ramaiah, M.J., Srinivasa Reddy, T., Kapavarapu, R.K., Rao, B.N., Imthiajali, S., Lakshminarayan Reddy, T., Pushpavalli, S.N.C.V.L., Shankaraiah, N., Pal-Bhadra, M. (2013). 4β-[4′-(1-(Aryl)ureido)benzamide] podophyllotoxins as DNA topoisomerase I and IIα inhibitors and apoptosis inducing agents. *Bioorganic & Medicinal Chemistry*. *21*, 5198-5208.

33. Kamal, A., Suresh, P., Ramaiah, M.J., Mallareddy, A., Imthiajali, S., Pushpavalli, S.N.C.V.L., Lavanya, A., Pal-Bhadra, M. (2012). Synthesis and biological evaluation of 4β-sulphonamido and 4β-[(4′-sulphonamido)benzamide]podophyllotoxins as DNA topoisomerase-IIα and apoptosis inducing agents. *Bioorganic & Medicinal Chemistry*. *20*, 2054-2066.

34. Acar, C., Yalcin, G., Ertan-Bolelli, T., Onurdag, F.K., Okten, S., Sener, F., Yildiz, I. (2020). Synthesis and molecular docking studies of some novel antimicrobial benzamides. *Bioorganic Chemistry*. *94*, 103368.

35. Wendorff, T.J., Schmidt, B.H., Heslop, P., Austin, C.A., Berger, J.M. (2012). The structure of DNA-bound human topoisomerase II alpha: conformational mechanisms for coordinating inter-subunit interactions with DNA cleavage. *Journal of molecular biology*. *424*, 109-124.

36. Wang, Y.-R., Chen, S.-F., Wu, C.-C., Liao, Y.-W., Lin, T.-S., Liu, K.-T., Chen, Y.-S., Li, T.-K., Chien, T.-C., Chan, N.-L. (2017). Producing irreversible topoisomerase II-mediated DNA breaks by site-specific Pt (II)-methionine coordination chemistry. *Nucleic acids research*. *45*, 10861-10871.

37. Accelrys, *Discovery Studio 3.5* 2012.

38. Momany, F.A., Rone, R. (1992). Validation of the general purpose QUANTA® 3.2/CHARMm® force field. *Journal of Computational Chemistry*. *13*, 888-900.

39. Crisfield, M. (1979). A faster modified Newton-Raphson iteration. *Computer Methods in Applied Mechanics and Engineering*. *20*, 267-278.

40. Wu, G., Robertson, D.H., Brooks III, C.L., Vieth, M. (2003). Detailed analysis of grid-based molecular docking: A case study of CDOCKER—A CHARMm-based MD docking algorithm. *Journal of computational chemistry*. *24*, 1549-1562.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

MANUFACTURE OF SPHERICAL GRANULES OF ISOSORBIDE DINITRATE WITH MODIFIED RELEASE

MODİFİYE SALIMLI İSOSORBİT DİNİTRAT KÜRESEL GRANÜLLERİNİN ÜRETİMİ

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ABSTRACT

Objective: *This study was aimed to obtain spherical matrix pellets of isosorbide dinitrate with different dissolution kinetics to create a multidosage medicinal form with controlled release of the active substance.*

Material and Method: *Spherical matrix granules were obtained by extrusion-spherinization and coated with different amounts of polymer shell. Dissolution profiles of the obtained spheroids were compared with reference drug tablets.*

Result and Discussion: *The results showed that the combination of spherical granules provides an isosorbide dinitrate release profile similar to the dissolution of Cardicet Retard tablets. The target dissolution profile is achieved using a combination of uncoated spherical granules and spheroids with a 10% film coating. The similarity coefficient (f2) is 73.6.*

Keywords: *Isosorbide dinitrate, release profile, spherical matrix pellets*

ÖZ

Amaç: *Bu çalışma, etkin maddenin kontrollü salımını yapan çok dozlu bir tıbbi form oluşturmak için farklı çözünme kinetiklerine sahip izosorbit dinitratın küresel matris pelletlerini elde etmeyi amaçlamıştır.*

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Gereç ve Yöntem: *Küresel matris granüller ekstrüder-sferonizer yöntemi ile elde edilmiş ve farklı miktarlarda polimer kabuk ile kaplanmıştır. Elde edilen kürelerin çözünme profilleri, referans ilaç tabletleri ile karşılaştırılmıştır.*

Sonuç ve Tartışma: *Sonuçlar, küresel granüllerin kombinasyonunun, Cardicet Retard tabletlerinin çözünmesine benzer bir izosorbit dinitrat salım profili sağladığını göstermiştir. Hedef çözünme profili, kaplanmamış küresel granüller ve % 10'luk film kaplı kürelerin bir kombinasyonu kullanılarak elde edilmiştir. Benzerlik katsayısı (f2) 73.6'dır.*

Anahtar Kelimeler: *İsosorbit dinitrat, salım profili, küresel matriks pelletler*

INTRODUCTION

Isosorbide dinitrate belongs to the group of first choice drugs for the treatment of angina pectoris. Prolonged forms of isosorbide dinitrate have high preventive efficacy. Modified-release drugs can combine high pharmacological activity, a long period of therapeutic action, and serious side effects absence, which are characteristic to all nitrate group drugs. The need of up-to-date cardiac drugs with a high level of efficacy and safety necessitates the development of modified-release drugs, primarily for oral administration [1,2].

Multicomponent medicinal forms are becoming more widely used, in comparison with monolithic medicinal forms. They have a number of potential benefits, such as predicted gastrointestinal movement, overdose risk absence, ability to manage the release profile, increased bioavailability, and also less intra-subject and inter-subject variability in pharmacokinetic characteristics [3,4].

Pellets, or spherical granulas, are one of the most popular medicinal multidosage forms. Granulation is an agglomeration process, that converts fine powders or particles of drugs and additional components into small spherical or hemispherical pellets. Pelletizing technologies provide a large corridor of capabilities and can be performed in various required dosages without changing the manufacturing process. Typically, spherical pellets are 0.5 to 1.5 mm in size and are generally used to achieve sustained release. After receiving the drug, the pellets are evenly distributed over a large volume of the gastrointestinal tract, which leads to the release of the active component with reduced risk of local irritation and minimizes the possibility of overdose [4,5].

The purpose of this study is to obtain spherical matrix pellets of isosorbide dinitrate with different dissolution kinetics to create a multidosage medicinal form with controlled release of the active substance. Matrix tablets, despite the simplicity of the technology, have the risk of dose breakthrough and overdose, which is especially critical for substances with a narrow therapeutic range, such as nitrates. Unlike matrix tablets Cardiket Retard, this innovative form will allow you to create the necessary release profile of the active ingredient through the combination of granules

without the risk of possible overdose.

Extrusion-spheronization technology is the most popular method of the pellet production.

The advantages of the extrusion-spheronization in comparison with other methods are [6,7]:

- the possibility of achieving higher content of the active component;
- in the pellets some ratio of several active substances can be combined in;
- the possibility of leveling unwanted physical characteristics of the active ingredients (low bulk density, hygroscopicity) through the use of various inert fillers;
- high density of the obtained pellets and narrow size distribution;
- smoother pellet surface in comparison with other technologies.

For the manufacturing of isosorbide dinitrate pellets, it is advisable to use the extrusion-spheronization method to obtain dense smooth pellets with a high drug content [8].

MATERIAL AND METHOD

Reference drug

In order to determine the reference release profile of isosorbide dinitrate for matrix granules, prolonged-release tablets "Cardiket Retard" 40 mg (Aesica Pharmaceuticals GmbH, Germany) were used.

Excipients, which were used

Isosorbide dinitrate (RPF "MICROKHIM", Ukraine)

Polyacrylate dispersion of Eudragit NE 30D (Evonik, Germany)

Microcrystalline Cellulose HEWETEN 101 (JRS Pharma, USA)

Lactose monohydrate Pharmatose 200M (DFE Pharma, Germany)

Hydroxypropylmethylcellulose Mantrocel E-6 (Mantrose-Haeuser, USA)

Talc (Imifabi, USA)

Obtaining of spherical pellets

Spherical matrix pellets of isosorbide dinitrate were obtained by the extrusion-spheronization, by means of Eudragit NE 30D as a polymer, that provides modified release.

The weighed amounts of the solids were mixed in a drum mixer HSD5-100 (SaintyCo, China). The resulting dry mixture was moistened with an aqueous Eudragit NE 30D dispersion and mixed in a planetary granulator-mixer XF DH-5L (Nantong KMM, China). The resulting plastic mass was extruded on a screw radial extruder YC-910 (Pilotech, China) with a screen diameter of 0.75 mm.

The obtained extrudate was spheronized in a laboratory unit for spheronization and film coating on spherical granules YC-910 (Pilotech, China) equipped with a 250 mm diameter corrugated disc with a 2 mm corrugation step (900 rpm).

After spheronization, the pellets were dried in a shelf oven at 40°C for 24 hours.

Screening of spherical pellets

To determine the particle size composition of the obtained spherical granules and separation them into fractions, a set of stainless steel laboratory sieves with a mesh size of 0.25 mm, 0.5 mm, 0.8 mm and 1.0 mm was used [9].

Obtaining of coated spherical pellets

Coating of spherical pellets with a film sheath was carried out on a laboratory unit for spheronization and film coating on spherical granules YC-910 (Pilotech, China), equipped with a smooth disk (600 rpm) and a top spray nozzle with a nozzle of 1.2 mm.

After coating, the pellets were dried in a shelf oven at 40 ° C for 6 hours.

Quantitative determination

The content of the isosorbide dinitrate in the obtained matrix pellets was determined, by means of their own validated analytical HPLC – technique. The analysis was performed on a Shimadzu LC-20AD XR liquid chromatograph with a diode-array detector under the following conditions: Supelco Discovery C18 chromatographic column (150 × 4.6 mm, 5 μm); mobile phase - water R - buffer solution (pH 4.7) - methanol R2 (35:10:55); elution mode - isocratic; mobile phase velocity - 1.0 ml / min; the detection wavelength is 220 nm [10].

To prepare buffer solution (pH 4.7) 15.4 g of ammonium acetate R was made into a volumetric flask with a capacity of 1000.0 ml, was added 300.0 ml of water R, 11.5 ml of glacial acetic acid R, mixed and adjusted the volume solution with water to the mark. The pH of the solution was adjusted as needed with glacial acetic acid R.

To prepare a comparison solution of 0.120 g (exact portion) CRS isosorbide dinitrate was added to a volumetric flask with a capacity of 500.0 ml, was added 300 ml of methanol R2 and kept in an ultrasonic bath for 10 minutes, bring the volume of the solution to the mark methanol R2 and mixed thoroughly.

To prepare the test solution, about 0.24 g (exact portion) powder of ground matrix granules was made into a volumetric flask with a capacity of 200.0 ml, was added 100 ml of methanol R2 and kept on ultrasound bath for 30 minutes at 40-50ºC, adjusted the volume of the solution to the mark

with methanol R2 and mixed thoroughly. 25.00 ml obtained the solution was transferred to a volumetric flask with a capacity 50.0 ml and adjusted the volume of the solution with the mobile phase to the mark, mixed thoroughly and filtered through the PES syringe filter (d = 25 mm, 0.45 μm) or similar, discarding the first portions of the filter installment [10].

Dissolution of matrix pellets

Dissolution Tester - COPLEY DIS 6000 (Copley, UK).

For testing apparatus 1 - with a basket was used.

The rotation speed of the basket is 100 rpm.

The dissolution medium is water.

The volume of dissolution medium is 500 ml.

The temperature of dissolution medium is 37 ± 0.5 ° C.

The dissolution time is 12 hours.

To test 100 mg of matrix pellets (which approximately corresponds to 40 mg of isosorbide dinitrate) or 1 tablet "Cardiket Retard" 40 mg were placed in baskets. All 6 baskets are lowered into glasses, so the entire volume of the basket, along with the contents, is completely immersed in liquid. After 1 hour of dissolution 10 ml of solution was selected from the center of the glass, filtered through a paper filter "blue ribbon", discarding the first portions of the filtrate. The sample was diluted 1:1 with water R. After 1, 2, 4, 6, 8, 10 and 12 hours from the beginning of the dissolution, the samples were repeated in a similar manner.

Preparation of standard isosorbide dinitrate solution is: a sample of isosorbide dinitrate CRS equivalent to 0.050 g of 100% isosorbide dinitrate is placed into a 50.0 ml volumetric flask, 2/3 flask of methanol R2 is added, it is kept in an ultrasonic bath for 10 minutes, it is brought by the volume of methanol R2 to the mark and stirred. 1 ml of the resulting solution is transferred into a volumetric flask with a capacity of 25.0 ml, bring the volume of the solution with water R to the mark and mix.

Preparation of a buffer solution with a pH of 4.7: 15.4 g of ammonium acetate R is placed in a volumetric flask with a capacity of 1000.0 ml, it is added 300.0 ml of water R, 11.5 ml of glacial acetic acid, mix and bring the volume of the solution water R to the mark. If necessary, the pH of the solution with glacial acetic acid R is adjusted potentiometrically.

50 μl of the test solution and 50 μl of the standard isosorbide dinitrate solution are chromatographed on a liquid chromatograph with a UV detector, receiving at least 3 chromatograms for each of the solutions under the following conditions:

- a column of 150x4,6 mm in size is filled with LS-18 sorbent;
- mobile phase: water R is a buffer solution with pH 4.7 is methanol R2 (350: 100: 550);

- the speed of the mobile phase is 1.0 ml /per min;
- detection at a wavelength is 210 nm.

Comparison of dissolution profiles

To compare the dissolution profiles of matrix granules and tablets, the similarity factor **f2** was calculated according to the formula [11]:

$$f_2 = 50 \cdot \log\left\{\left[1+(1/n)\sum_{i=1}^{n} | R_i - T_i |^2\right]^{-0,5} \cdot 100\right\},$$

where **n** is the number of time points; **Ri** is the amount of active substance transferred to the solution from the comparison drug at the i-th time point (on average, %); **Ti** is the amount of active substance transferred to the solution from the test drug at the i-th time point (on average, %).
The value of **f2**, which is in the range from 50 to 100, indicates a similar dissolution kinetics of drugs.

RESULT AND DISCUSSION

Selection of excipients

As an excipient for the manufacture of granules by extrusion-spheronization is most often used microcrystalline cellulose (MCC). Water is an acceptable moisturizer for use with microcrystalline cellulose. The use of lactose as a filler is unacceptable when moistened with water due to its high solubility. However, binary mixtures of microcrystalline cellulose and lactose retain the absorption and adsorption properties of MCC, which are absent or minimal in the compositions with lactose [7].

Lactose was included into the pellets to provide complete leaching from the matrix of the active component, because isosorbide dinitrate has low solubility in aqueous media, and virtually all other components of the composition are insoluble in water. Lactose is taken in an amount about in 10 times smaller, then microcrystalline cellulose. This approach allows to preserve as much as possible for the mixture the physical and technological properties of the MCC to ensure an efficient process of the extrusion-spheronization.

As the polymer, that modifies the release of the active component, polyacrylate copolymer in the form of an aqueous 30% dispersion of Eudragit NE 30D was selected. This polymer is insoluble in water, has low permeability and its properties are independent of the pH of the medium. The Eudragit NE 30D is also highly ductile and it doesn't require plasticizer. The product does not require

the use in organic solvent technology and it is therefore absolutely safe. These properties make polyacrylate dispersion the most suitable product for both wet granulation and film coating, which regulates the release of the active substance [12].

Low viscosity hydroxypropylmethylcellulose (HPMC) is added to the composition to create stickiness. To ensure the necessary properties, the HPMC was taken in a mass ratio of 1:19 to microcrystalline cellulose [13].

Obtaining of spherical pellets

The number of components per 1 extrusion-spheronization operation is shown in table 1.

Table 1. Downloaded component masses per one extrusion-spheronization operation.

Component	g	%
Isosorbide dinitrate	120	40
Microcrystalline cellulose	114	38
Lactose monohydrate	15	5
Eudragit NE 30D	150 (45 anhydrous)	15
Hydroxypropylmethylcellulose	6	2
Together:	405	100

The resulting spherical pellets were dried, then the combined number of spheroids from 10 operations were scattered on laboratory sieves. The scattering results are shown in table 2.

Table 2. Fractional composition of spherical matrix pellets.

Fraction of pellets , mm	g	%
< 0,25	7,3	0,24
0,25- 0,5	94,3	3,14
0,5 – 0,8	2027,6	67,59
0,8 – 1,0	763,9	25,46
> 1,0	35,4	1,18
Together :	2928,5	97,61

The main part of the obtained spherical matrix pellets, as expected, was represented by a fraction of 0.5 mm to 1.0 mm. Mechanical loss (the "dead" residue in the extruder; the mass adhered to the disk, etc.) was about 2% of the mass. The total yield of fractions 0.5 - 0.8 mm and 0.8 - 1.0 mm is more than 90%. Pellets of these sizes were of interest for further use in modified release medicinal forms.

The release profiles of isosorbide dinitrate from the obtained pellets are presented in table 3 and on the figure 1.

Table 3. Results of matrix pellets' dissolution of isosorbide dinitrate and tablets Cardiket Retard 40 mg

Index	0.5 - 0.8 mm fraction	0.8 – 1.0 mm fraction	Cardiket Retard 40 mg
Quantitative determination, %	39,7	39,5	
Dissolution time, hours	**% released isosorbide dinitrate ± SD**		
0	0,0	0,0	0,0
1	45,22 ± 2,14	39,94 ± 2,13	22,27 ± 2,04
2	60,69 ± 2,23	53,57 ± 2,36	34,08 ± 2,73
4	79,50 ± 2,74	73,19 ± 3,16	53,23 ± 2,56
6	91,24 ± 3,42	84,12 ± 3,21	66,55 ± 3,20
8	97,53 ± 4,03	92,82 ± 3,67	75,92 ± 3,58
10	100,38 ± 4,15	97,83 ± 3,79	83,31 ± 4,07
12	101,41 ± 4,43	99,68 ± 4,29	88,60 ± 4,74

In 12 hours, almost complete dissolution of the tablet Cardiket Retard (about 90%), which is consistent with the instructions for use of the drug (taken 1-2 times a day).

More than 80% of the dose of isosorbide dinitrate is released from the obtained matrix granules in 6 hours. For more effective prolongation on the matrix granules it is necessary to apply a shell that regulates the dissolution rate of the active ingredient.

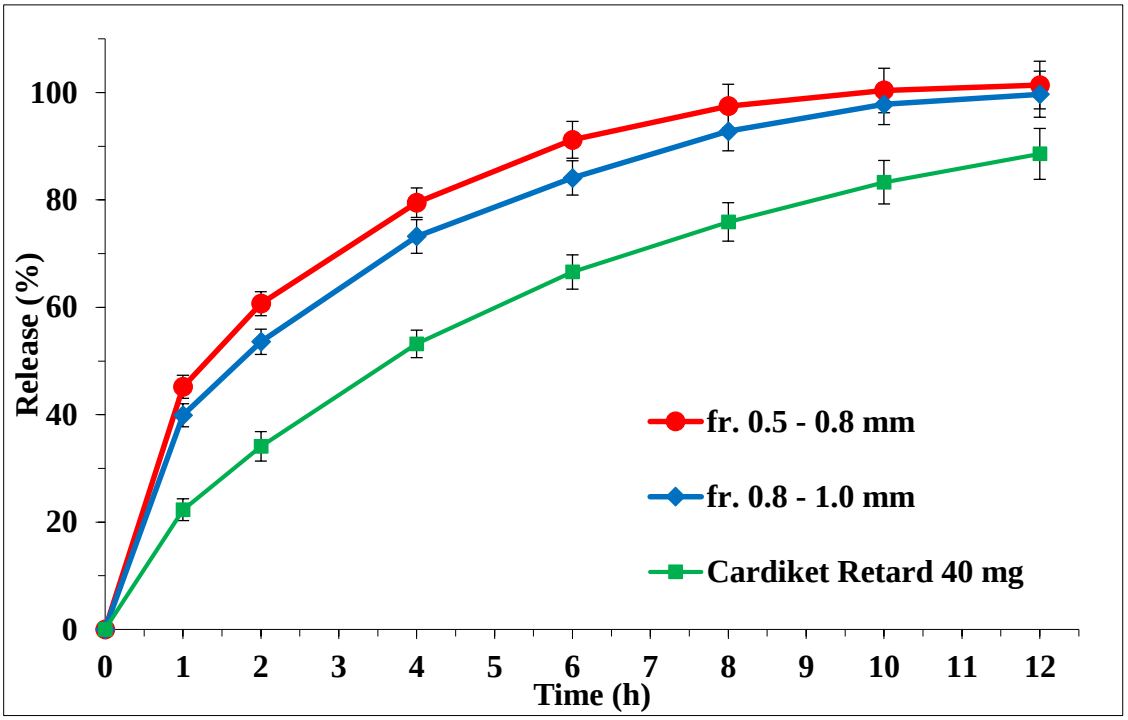

Figure 1. Isosorbide dinitrate release profiles from matrix pellets and tablets Cardiket Retard 40 mg.

Obtaining of coated spherical pellets

Spheroids of 0.5 - 0.8 mm fraction were used to obtain coated matrix granules. Spheroids of the fraction of 0.8 - 1.0 mm can be used without coating for combination with coated granules, as their dissolution profile is slightly lower.

In order to select the optimal amount of coating on the spheroids applied different portions of the polymer shell (5, 10, 15 and 20% by weight of the granules). For each of the variants of the amount of coating did 2 application operations. For 1 operation took 250 g of granules. The composition of the suspension, which was applied to the matrix spheroids, is shown in table 4.

Table 4. The composition of the suspension applied on the matrix pellets

Component	Composition per 1 liter of suspension for coating
Eudragit NE 30D	500 g (150 g of dry polymer)
Talc	150 g
The purified water	up to 1000 ml

The parameters of the film coating process, regulating the release of the active component are shown in table 5.

Table 5. Parameters of the film coating process on spherical matrix pellets

Process parameters	Value
Disk rotation speed	600 rpm
Supplied air temperature	29 – 31 ºC
Air consumption	40 m^3/ h
Outlet air temperature	24 – 26 ºC
The spray pressure on the nozzle	1,7 bar
Consumption of the applied suspension	3 ml / min

The release profiles of isosorbide dinitrate from the obtained coated pellets are presented in table 6 and on the figure 2.

Table 6. The results of coated matrix pellets dissolution of isosorbide dinitrate

Index	coated 5%	coated 10%	coated 15%	coated 20%
Quantitative determination, %	38,2	36,3	34,6	33,1
Dissolution time, hours	**% released isosorbide dinitrate ± SD**			
0	0,0	0,0	0,0	0,0
1	19,76 ± 2,42	11,90 ± 2,73	4,28 ± 2,54	3,26 ± 2,28
2	33,36 ± 2,45	21,12 ± 2,62	9,13 ± 2,09	5,73 ± 2,39
4	55,71 ± 2,88	40,36 ± 2,54	20,74 ± 2,49	14,68 ± 3,36
6	71,66 ± 3,23	53,82 ± 3,09	31,14 ± 3,56	23,29 ± 3,52
8	86,77 ± 3,93	65,17 ± 3,97	40,82 ± 4,12	30,12 ± 3,33
10	96,48 ± 4,50	75,65 ± 4,37	50,73 ± 3,97	39,16 ± 4,54
12	98,13 ± 4,67	86,02 ± 4,18	59,70 ± 5,15	45,85 ± 5,16

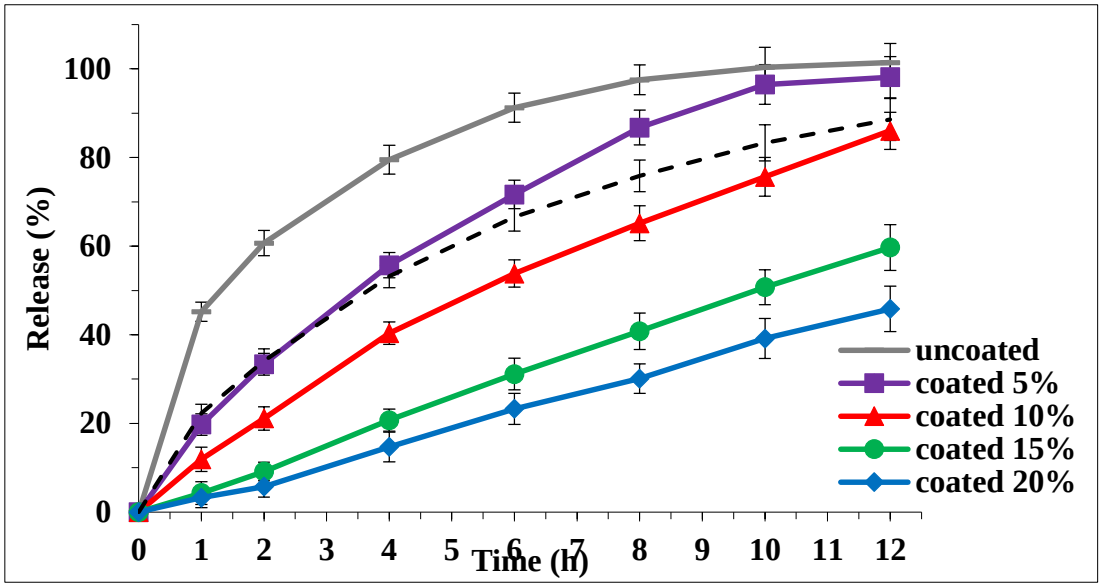

Figure 2. Release profiles of isosorbide dinitrate from coated matrix pellets

Granules with 5% coating provide the closest release profile to the comparison drug. Spheroids with a large amount of applied coating do not have time to release even 90% of the active component in 12 hours.

To reproduce the dissolution of Cardiket Retard tablets, it is advisable to use either spheroids with a 5% coating or a combination of uncoated and coated granules.

Spheroids with 10% film coating were used for combination with uncoated granules (fr. 0.8 - 1.0 mm). The granules were taken in the ratio in which they were obtained after operation (25,4: 67,59).

Comparative profiles of the release of isosorbide dinitrate from different combinations of granules are presented in table 7 and figure 3.

Table 7. Comparative results of dissolution of different combinations of matrix granules

Index	**Combinations of granules**	**Coated granules (5%)**	**Cardiket Retard 40 mg**
Dissolution time, hours	**% released isosorbide dinitrate ± SD**		
0	0,0	0,0	0
1	19,60 ± 2,17	19,76 ± 2,42	22,27 ± 2,04
2	30,04 ± 2,12	33,36 ± 2,45	34,08 ± 2,73
4	49,42 ± 2,54	55,71 ± 2,88	53,23 ± 2,56
6	62,13 ± 3,45	71,66 ± 3,23	66,55 ± 3,20
8	72,79 ± 4,06	86,77 ± 3,93	75,92 ± 3,58
10	81,71 ± 4,59	96,48 ± 4,50	83,31 ± 4,07
12	89,77 ± 4,34	98,13 ± 4,67	88,60 ± 4,74
f2	73,6	55,3	-

The combination of coated and uncoated granules provides a more Cardiket-like release profile than using only coated granules with a 5% shell. The ratio of granules in the test sample corresponds to the fractional distribution of spheroids during their operation.

For further work on the development of a multidose dosage form of isosorbide dinitrate, it was chosen to use uncoated spheroids of the fraction 0.8 - 1.0 mm and spheroids of the fraction 0.5 - 0.8 mm with a coating of 10% by weight of the granules. The use of combination spheroids allows you to create different variations of release profiles within a 12-hour time interval.

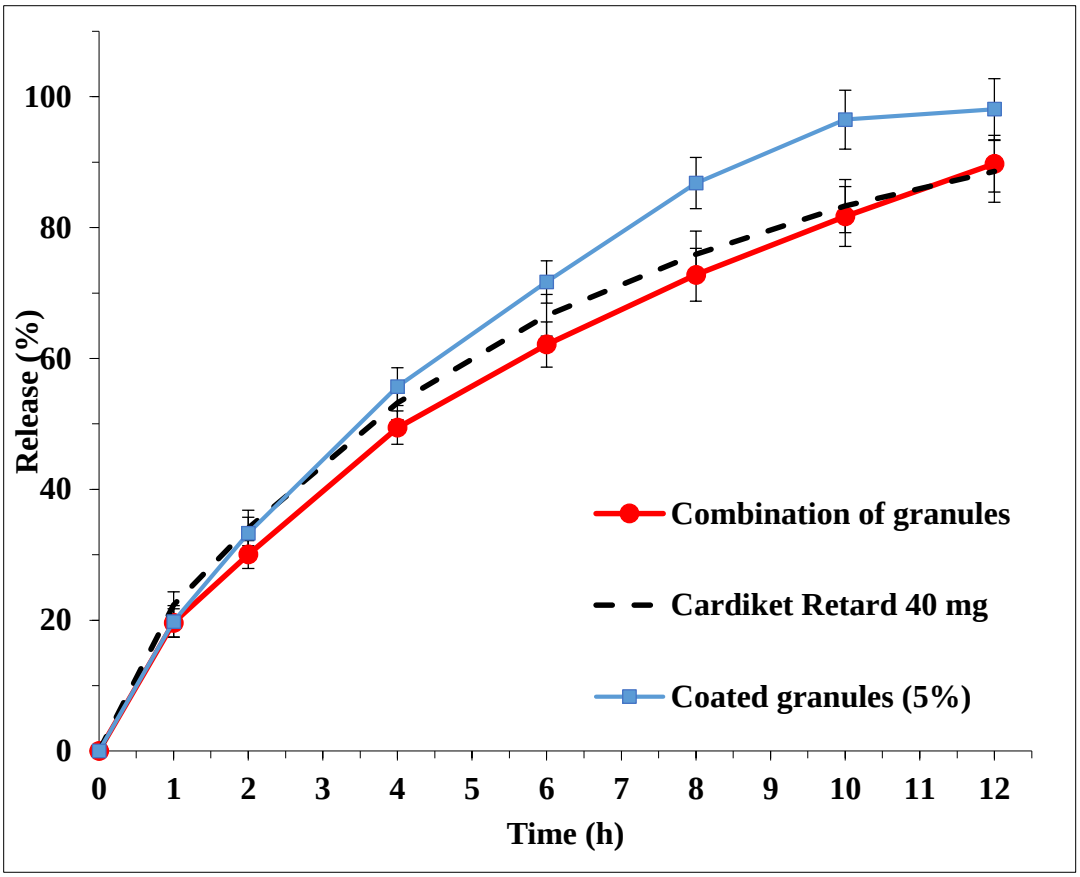

Figure 3. Comparative release profiles from different combinations of granules

Conclusions

The laboratory technology of production of matrix spherical granules of isosorbide dinitrate by extrusion-spheronization method was worked out in the work. The fractional composition of the obtained spherical granules is estimated - more than 90% of spheroids is a fraction from 0.5 mm to 1.0 mm.

The release profiles of the active substance from the obtained matrix granules, uncoated and coated with different amounts of applied polymer shell, were studied.

The use of a combination of uncoated and coated matrix granules makes it possible to achieve the required dissolution profile for multidose dosage forms.

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CONFLICT OF INTEREST

There are no conflicts of interest have been declared.

REFERENCES

1. Kovalenko, V.M., Lutaj, M.I., Bratus', V.V. et. al.; Kovalenkao, V.M. (Ed.) (2008). Rukovodstvo po kardiologii' [Guide for cardiology]. Kyiv: MORION, 1424. http://snsk.az/snsk/file/2013-06-20_09-52-44.pdf

2. Soman, B., Vijayaraghavan, G. (2017). The role of organic nitrates in the optimal medical management of angina. *European Society of Cardiology* 15 (2)

3. Kumar, S., Jeet, K., Baldi, A. (2015). Recent technological advancements in multiparticulate formulations: The smart drug delivery systems. *Asian Journal of Pharmaceutic* 9 (4): 13-25. https://www.researchgate.net/publication/28891779

4. Roy, P., Shahiwala, A. (2009). Multiparticulate formulation approach to pulsatile drug delivery: current perspectives. *J Control Release* 134: 74–80. https://doi.org/10.1016/j.jconrel.2008.11.011

5. Shah, S., Shah, B.P., Patel,M., Patel, M.R. (2015) A review on extended release drug delivery system and multiparticulate system. *World Journal of Pharmaceutical Research* 4 (8):724-747. https://www.researchgate.net/publication/281098252

6. Jadhav, N., Irny, P., Mokashi, A., Souche, P., Paradkar, A. (2012). Pelletization by Extrusion Spheronization Technique: An Excipient Review. Drug Delivery Letters. 2. 132-145. https://www.researchgate.net/publication/284590372

7. Sagar, M., Tanaji, N., Sushilkumar, P. (2016). Extrusion–spheronization a promising pelletization technique: In-depth review. *Asian Journal of Pharmaceutical Sciences II*: 684–699. http://dx.doi.org/10.1016/j.ajps.2016.08.001

8. Oliinykov, D.S., Kaplaushenko, A.G. (2019a). Substantiation of manufacture technology development for the tablets of isosorbide dinitrate with modified release. *Current issues in pharmacy and medicine: science and practice* 12 (2): 160–165. https://doi.org/10.14739/2409-2932.2019.2.170982

9. European Directorate for the Quality of Medicines et al. & HealthCare: *European Pharmacopoeia 9.0.*[Internet]. 2019.

10. Oliinykov, D.S., Kaplaushenko, A.G. (2019b). Development and validation of the HPLC-procedure for the quantitative determination of isosorbide dinitrate in matrix granules. *Journal of Organic and Pharmaceutical Chemistry* 17 (3): 51-58. https://doi.org/10.24959/ophcj.19.174771

11. CPMP/QWP/EWP/1401/98 Rev.1/Corr** *«Guideline on the Investigation of Bioequivalence»*

12. Access mode: https://healthcare.evonik.com/sites/lists/NC/DocumentsHC/Evonik-Eudragit_brochure.pdf

13. Bhaskaran, S., Lakshmi, P.K. (2010). Extrusion Spheronization –A Review. *Int.J. PharmTech Res* 2(4): 2429-2433.

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 44(3): 494-505, 2020
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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF SEVERAL BITLIS HONEYS BASED ON THEIR BOTANIC AND BIOCHEMICAL PROFILES

BİTLİS YÖRESİNDEN ALINAN BAZI BALLARIN BOTANİK VE BİYOKİMYASAL PROFİLİ

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ABSTRACT

Objective: *In this paper, we aimed to evaluate the pollen composition and antioxidant activity of 4 randomly selected honey samples from the Bitlis region in 2017.*

Material and Method: *The melisopalynological analysis was used standard protocol without acetolysis. The antioxidant activity of samples were evaluated using several in vitro methods, 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity (DPPH), hydrogen peroxide scavenging activity (HPSA), Ferrous ions chelating activities (FICA), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid radical cation scavenging assays (ABTS) and the ferric reducing antioxidant power assays (FRAP).*

Result and Discussion: *All the samples were determined as multifloral honey. Outcomes obtained declared that the antioxidant activity changed remarkably from honey to honey. ABTS and FICA of samples differ slightly each other's. DPPH, HPSA, and FRAP were ranged from between 54.45 – 387.60 µg/mL, 212.11 – 246.64 µg/mL and 47.20 – 78.23 %, respectively. In addition, total phenol and total flavonoid contents were determined. Comparatively, Sample 3 showed highest levels of phenolic and flavonoid content (626.48 mg GAE/100 g and 4.4 mg CAE/100 g, respectively) in ethanol extract. Almost all the samples are well source for antioxidants.*

Keywords: *Antioxidant, ethanol, multiflora, palynology*

ÖZ

Amaç: *Bu makalede 2017 yılında Bitlis bölgesinden rastgele seçilen 4 bal örneğinin polen kompozisyonunu ve antioksidan aktivitesini değerlendirmeyi amaçladık.*

Gereç ve Yöntem: *Melisopalinolojik analizde asetolizsiz standart protokol kullanılmıştır. Numunelerin antioksidan aktivitesi, 1,1-difenil-2-pikrilhidrazil serbest radikal giderme aktivitesi (DPPH), hidrojen peroksit giderme aktivitesi (HPSA), demirli iyon şelat aktiviteleri (FICA), 2,2'-azino-bis-3-etilbenztiyazolin-*

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6-sülfonik asit radikal katyon temizleme deneyleri (ABTS) ve ferrik indirgeyici antioksidan güç deneyleri (FRAP) gibi birkaç in vitro yöntem kullanılarak değerlendirilmiştir.

Sonuç ve Tartışma: *4 balın polen kompozisyonu değerlendirildiğinde balların multifloral olduğu görülmüştür. Elde edilen sonuçlar, antioksidan aktivitesinin baldan bala belirgin bir şekilde değiştiğini göstermiştir. ABTS ve FICA örnekleri birbirlerinden biraz farklı, DPPH, HPSA ve FRAP sırasıyla 54.45 - 387.60 ug / mL, 212.11 - 246.64 ug / mL ve % 47.20 - 78.23 arasında bulunmuştur. Ek olarak, toplam fenol ve toplam flavonoid içerikleri belirlenmiştir. Örnekler kıyaslandığında 3, en yüksek fenolik ve flavonoid içeriği (626.48 mg GAE / 100 g ve 4.4 mg CAE / 100 g-etanol) göstermiştir. Hemen hemen tüm numunelerin iyi bir antioksidan kaynağı olduğu tespit edilmiştir.*

Anahtar Kelimeler: *Antioksidan, etanol, multifloral, palinoloji*

INTRODUCTION

Honey is sweet solution that consists of fructose, glucose and sucrose but also contains other natural macro- and micro-nutrients [1]. Each honey has unique different characteristics [2]. The physical and biochemical properties of honey are affected by the plant composition of the region where the hives are located [2-4]. Bitlis is located at the Irano-Turanian floristic region which has plentiful plant biodiversity. Asteraceae, Fabaceae, Poaceae Caryophyllaceae, Ranunculaceae and Brassicaceae in Bitlis are seen most commonly families. In addition, *Alyssum* L., *Centaurea* L., *Astragalus* L., *Trifolium* L., *Vicia* L, *Ranunculus* L., *Veronica* L., *Gypsophylla* L., and *Silene* L. are widely present genus [5-9]. These taxa constitute very important nectar and pollen sources for honey bees of Bitlis region. However, there are few reports about botanic origins on honeys from Bitlis.

Honey is the most widely produced and consumed in the worldwide since ancient times. It has been used different purpose such as therapy and food because of its strong antioxidant capacity [1].

Antioxidant compounds play a crucial factor in the defence act of living against pathogens. Antioxidants protect our body against the harmful effects of free radicals by giving a hydrogen atom and a single electron or with various mechanisms such as metal chelating feature [10]. In this study, in order to show the antioxidant profile of honey samples, DPPH free radical scavenging activity, metal-chelate activity, hydrogen peroxide scavenging activity, ABTS radical cation scavenging activity, ferric reducing antioxidant power, as well as total phenolic and flavonoid substance contents that play a very important role in antioxidant capacity were detected.

Beekeeping in Bitlis is varying from amateur to career, becoming always more creative, technical and generative. For this, there is still a lot to learn and develop related to the properties and structures of bee products. However, there is not enough scientific knowledge about the botanic origin and antioxidant activity of Bitlis honey. The goal of this study is the estimation of botanic origin, total phenolic, total flavonoid content, and antioxidant properties of honey produced in Bitlis.

MATERIAL AND METHOD

Honey samples

The study was done on 4 honeys collected directly from beekeepers of Bitlis in 2017 (Figure 1). The botanical origin of the honey samples was approved by melisopalynological examination.

Figure 1. Location of honey samples collected from Bitlis (Google Earth)

Palynological analysis

A 10 g sample of each honey was done following scientifically accepted method without acetolysis [11]. The botanic classifications of each honey sample were carried out by microscopic pollen analysis. The terms were allocated for frequency classes: predominant pollen (>45%), secondary pollen (16–45%), important minor pollen (3–15%) and trace pollen (<3%) [12]. The following equation (Eq. 1) was used to declare the frequency per taxon.

$$Frequency\ (\%) = \frac{Total\ number\ of\ pollen\ of\ a\ particular\ species}{Total\ number\ of\ observed\ pollen} x100 \quad (1)$$

Antioxidant analyses

A 10 g of honey was added to 50 mL of distilled water and kept in a water bath until the honey dissolved. Finally, total volumes are adjusted to 100 mL by using distilled water. All the reagents and chemicals used in the experiments were of analytical grade.

TFC assays

Total flavonoid contents of samples were done via colorimetric method with minor modifications [13]. In this assay, sample solutions (0.5 mL) were mixed with 1.5 mL of pure ethanol. And then AlCl3.6H20 (0.1 mL, 10.0%) and potassium acetate (0.1 mL, 1.0 mol / L) were added. Distilled water was used for bringing the total volume to 5.0 mL. 30 min later, the absorbance values were read at 415 nm. The found results were calculated as μg catechin equivalent by using catechin standard calibration graphic (R2=0.9979).

TPC assays

Total phenolic content assays were performed according to previous method [14]. For this assay, sample solutions (0.5 mL) were added to distilled water (7.0 mL) and Folin C reagent (0.5 mL), respectively. 3 min later, sodium carbonate solution (3.0 mL, 2.0 %) was mixed with this solution. Colour change expected during 1 h and the absorbance values were recorded at 760 nm. For evidence of the total phenolic contents, standard Gallic acid graphic was used (R2= 0.9995).

DPPH assays

The DPPH radical scavenging activity assays were carried out according to previous method with minor modifications [15]. For this reason, the diluted samples (3.0 mL) at the varied concentrations (10-100 μg/mL) were mixed with stable DPPH radical solutions (1.0 mL, 0.2 mM in ethanol). The mixtures were shaken vigorously and left to stand for 30 min at RT and in an unlit environment. Finally, absorbance values were measured 517 nm. The radical scavenging activity results were evaluated as SC50 (μg/mL).

HPSA assays

The hydrogen peroxide scavenging activities of samples were tested out by using spectrophotometric method described by Ruch et al. (1989) [16]. According to this method, the samples (3.4 mL) were added to hydrogen peroxide (0.6 mL, 40 mM). The results were given as SC50 values (μg/mL) with absorbance values at 230 nm.

FICA assays

Ferrous ions chelating activities were examined by using previous method [17] with slowly modifications. Briefly, the tested solutions (0.4 mL) added to FeCl2 solution (0.05 mL, 2 mM) and kept at the room temperature for 10 min. Finally, ferrozine solution (0.2 mL, 5 mM) and pure ethanol (3.3 mL) were mixed with this solution, respectively and all tubes were vortexed about 5 min. After these mixing processes, absorbances were recorded at 562 nm and FICA values were expressed as

activity (%).

$$\text{Ferrous Ions Chelating Activity (\%)} = [1 - (As/Ac)] \times 100 \qquad (2)$$

ABTS assays

ABTS radical cation scavenging assays were performed via spectrophotometric method [18]. In this experimental method, ABTS (2.0 mM) and potassium persulfate (2.45 mM) was stirred to product ABTS•+. The fresh prepared solution was stand up for 16 h at room temperature and in an unlit environment. The absorbance of this solution was fixed to 0.750 ± 0.020 at 734 nm. Therefore, dilution process was realized by using the PBS (0.1 M pH 7.4). The obtained ABTS radical cation solution is stable for 2 days. For determination of activity, the adjusted ABTS•+ (1.0 mL) was added to serially diluted sample solutions (3.0 mL) at the different concentrations (1-10 µg/mL in PBS). The activities were enounced as SC50 values (µg/mL).

FRAP assays

The ferric reducing antioxidant power assays were exerted according to Oyaizu method [19] with minor modifications. Firstly, PBS (2.5 mL, pH 6.6, 0.2 M) and potassium ferricyanide (2.5 mL, 1.0%) were stirred with samples (2.5 mL), respectively. Later, the coloured mixture solutions were incubated for 20 min at 50◦C. Then, TCA (2.5 mL, 10%) were mixed with this solution. Lastly, this solution (2.5 mL) was stirred with FeCl3 (0.5 mL, 0.1%) and distilled water (2.5 mL). The absorbance value of the obtained solution was recorded at 700 nm. The ferric reducing antioxidant power results were given as %.

$$\text{FRAP (\%)} = (As/Ac) \times 100 \qquad (3)$$

RESULT AND DISCUSSION

The detected pollen types and their taxa were summarised in Table 1. According to the palynological analysis 16 families, 19 genus and 3 pollen types in samples were identified and detected no predominant taxa. *Onobrychis* Adans., *Trifolium* L. and *Vicia* L. were observed as seconder group. In addition, the frequently observed pollens were belonging to Asteraceae, Brassicaceae, Boraginaceae, Cistaceae and Fabaceae families. These families were in agreement with Bitlis vegetation and were generally consist of melliferous plants. The taxa determined in samples match the results declared by other authors for botanic origin of honeys [20-22]

Table 1. Pollen profile in samples and samples having the corresponding frequency class

	Sample 1	Sample 2	Sample 3	Sample 4
Apiaceae				
Pimpinella sp.			M	M
Asteraceae				
Helianthus type	M		M	M
Senecio type	M	M		M
Artemisia type	M	M	M	M
Berberidaceae				
Berberis sp.	T		T	
Betulaceae				
Betula sp.	T	T		
Brassicaceae	M	M	M	M
Boraginaceae	M	M		M
Echium sp.			M	
Cistaceae				
Cistus sp.	M	T	T	T
Cupressaceae				
Juniperus sp.		T		
Fabaceae				
Astragalus sp.			M	
Coronilla sp.				M
Glycyrrhiza sp.				T
Onobrychis sp.	S	M	S	S
Trifolium sp.	S	S	M	S
Vicia sp.	M	S	M	M
Fagaceae				
Alnus sp.				T
Moraceae				
Morus sp.	T			
Lamiaceae (6-colpate)		M	M	
Juglandaceae				
Juglans sp.	T			
Primulaceae				
Primula sp.	T	T		
Ranunculaceae				
Ranunculus sp.	M			
Thalictrum sp.		M	T	
Rosaceae	M	M	T	
Sanguisorba sp.	T			
Undetermined		T	T	T

In the present investigation, the commonly accepted assays HPSA, FICA, ABTS, FRAP and DPPH were used for the evaluation of antioxidant power of samples. Moreover, TPC and TFC were determined (Table 2). FICA and ABTS activity results of the samples are very close to each other. Therefore, comparisons between these activities of the samples were not considered appropriate. ABTS of the samples varied from 20.05 to 20.12 µg/mL. In literature, a research group investigated the ABTS radical removal activities of Saudi Arabian honey samples as 0.36 – 1.2 µg / mL [23]. Their ABTS radical cation scavenging activity is higher than our result. In another study, ABTS radical cation scavenging activities of honey samples were determined in terms of SC50 as 54.33 - 99.40 µg / mL and 10.33 - 41.20 µg / mL [24]. Alzahrani et al. (2012) investigated three types honeys and results ranged from 43.25-202.26 µg / mL [25]. These results can be seen to be quite low compared to the results of our samples. ABTS radical cation removal activities of the standards we use are too small to be compared with our samples.

The FICA of samples was closely values, between 93.38 and 94.03%. Temizer et al. (2018) were carried out three chestnut honeys' antioxidant activity. Their FICA results were determined as 36.7 – 36.9 % [26]. Sherin et al. (2015) determined the metal chelation activities for honey samples as 54.2% [27]. These results are considerably lower than the FICA values of our samples. FICA results of the standard antioxidant substances we use were found lower than our samples.

The antioxidant capacity of ethanoic extracts of honey samples reacted with DPPH radical and was highly diverse SC50, from 54.45 to 387.60 (µg/mL). Salgueiro et al. (2014) found DPPH radical removal activities of eleven different honey samples in the range of 278.61-1601.8 µg / mL [28]. Pontis et al. (2014) expressed DPPH radical scavenging activity of honey samples as 3170 – 8790 µg / mL [29]. DPPH radical removal activities were found as 2.15 - 3.68 µg / mL in the study done in Saudi Arabian honey samples [30]. In another study, DPPH radical removal activities of honey samples were determined in the range of 29.388 - 458.450 µg / mL. Yegin et al. (2018) found DPPH radical removal activities in the range of 76.50 – 275.48 µg / mL in nine multifloral honey samples. [31]. In another study, DPPH free radical removal activities of honey samples were determined in terms of SC50 as 54.33 - 99.40 µg / mL [24]. Sample 3 showed a DPPH radical scavenging activity at a level comparable to these results. DPPH radical removal activities of our standards were determined higher than our samples.

Table 2. Biochemical profile of samples ([1]mg GAE/100 g, [2]mg CAE/100 g, [3]%, [4]SC_{50} µg/mL)

	TPC[1]	TFC[2]	FICA[3]	HPSA[4]	ABTS[4]	DPPH[4]	FRAP [3]
Sample 1	485.89	1.13	93.38	229.12	20.12	373.83	53.01
Sample 2	370.96	3.30	93.86	213.06	20.05	387.60	47.20
Sample 3	626.48	4.40	93.53	212.11	20.09	54.45	78.23
Sample 4	225.24	1.26	94.03	246.64	20.12	330.58	54.15
BHA			84.25	72.05	193.27	8.42	8.47
RUT			86.80	92.66	122.98	15.54	16.90
TRO			69.44	53.04	445.06	4.18	26.74

The results get from FRAP indicated large variability, from 47.2 to 78.23. In the literature, FRAP activities of three chestnut honey samples were determined between 71.30 – 73.70 % [26]. In another study, FRAP activities were determined in a study with multifloral honey samples [31].When we compare the FRAP activities of our standards with our examples, they were found quite low.

The HPSA of samples were slightly variety in terms of SC50, between 212.11-246.64 µg/mL. In the previously research, hydrogen peroxide scavenging activities of nine multifloral honey samples were found between 57.70 – 197.24 µg/mL [31]. In another literature, HPSA of three chestnut honey samples were determined as 252.0 – 258.6 µg/mL [26] According to these results, the HPSA values of our sample and the literature data are compatible. HPSA values of our samples are lower than the standards when compared with the standard antioxidant substances used.

The lowest phenolic content was detected in sample 4 while the highest in sample 3 (225.24 and 626.48 mg GAE/100 g, respectively). Silici and Ülgen (2019) determined TPC of eighteen honey samples as 70.60 – 212.06 mg GAE/100 g honey [32]. TPC of honey samples from Brazil were found as 25.00 – 54.80 mg GAE/100 g [29]. Temizer et al. (2018) found TPC of honey samples between 93.80 – 173.20 mg GAE/100g [26]. Juszcak et al. (2016) argued that TPC of honey samples range from 21.73 to 50.12 mg GAE/100g [33]. Dżugan et al.,(2018) found TPC of samples 23.94 – 187.58 mg GAE/100 g [34]. Yegin et al. (2018) determined TPC values between 26.57 – 184.85 mg GAE/100 g [31]. Stagos et al. (2018) determined TPC of the honey samples between 55 – 92 mg GAE/100 g [35]. TFC of ten monofloral honey samples obtained from Bangladesh ranged from 1.146 to 11.67mg CAE/100 g [36] . Temizer et al. (2019) determined that total phenolic and total flavonoid amounts of Ordu honeys varied from 32.5-171.05 mg GAE/100g [2]. Nayık and Nanda (2016) declared that TPC of Indian honey samples between 37 – 117 mg GAE/100 g for 37 samples [37]. The mean value of TFC ranged from 1.13 to 4.4 mg CAE/100g of honey (Table 2). Yegin et al.

(2018) declared that TFC of nine multifloral honey samples were 3.13 – 20.26 mg CAE/100 g[31]. In another study, TFC of honey samples were found as 8 – 17 mg QE/100 g [37]. Temizer et al. determined TFC of Ordu honeys as 1.65 – 38.75 mg CAE/100g [2]. Temizer et al. (2018) found TFC of honey samples between 5.5 – 8.3 mg CAE/100 g [38]. The TPC and TFC values obtained from our honey samples are compatible with the total phenolic and flavonoid substance contents of the honey samples studied in the literature. Temizer et al. (2020) studied five honey samples to determine the antioxidant activity and botanic origin. The antioxidant activity results of honey samples are compatible with our Bitlis honey samples [39].

As a result of the pollen analysis of honey samples, it was determined that Fabacae and Asteraceae families in the region are important food for bees. According to antioxidant test results, 4 honey samples showed different antioxidant activity due to their pollen components. Bitlis honey samples can be consumed to protect our health from endogenous and exogenous reactive species in normally produced in daily metabolic functions.

REFERENCES

1. Pasupuleti, V. R., Sammugam, L., Ramesh, N., Gan, S. H. (2017). Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits. Oxidative medicine and cellular longevity, 1259510-1259510.

2. Temizer, İ. K., Türkmen, Z., Güder, A. (2019). Assessment of Palynological Characterization and Total Phenol-Flavonoid Content of Some Honeys from Ordu in Turkey. 12(3), 1275-1282.

3. Dżugan, M., Sowa, P., Kwaśniewska, M., Wesołowska, M., Czernicka, M. (2017). Physicochemical Parameters and Antioxidant Activity of Bee Honey Enriched With Herbs. Plant Foods for Human Nutrition, 72(1), 74-81.

4. Temizer, İ. K., Güder, A., Çelemli, Ö. G. (2016). Botanic Origin, Various Physicochemical and Antioxidant Properties of Honey Samples From Giresun, Turkey. Hacettepe Journal of Biology and Chemistry, 44(3), 209-215.

5. Behçet, L. (1991). Süphan Dağı (Bitlis) Florası. Yüzüncü Yıl Üniversitesi, Fen Bilimleri Enstitüsü Dergisi, 1(1), 29-38.

6. Altan, Y. Behçet, L. (1995). Hizan (Bitlis) Florası. Turk Journal of Botany, 19 (3), 331-344.

7. Altıok, A. (2004). Bitlis Çayı Havzası Florası, in Fen Bilimleri Enstitüsü. Yüzüncü Yıl Üniversitesi.

8. Çelik, T. (2006). Kesan Deresi (Bitlis) Florası, in Fen Bilimleri Enstitüsü., Yüzüncü Yıl Üniversitesi.

9. Keser, A. M. Özgökçe, F. (2019). The Flora of Karz (Garez) Mountain (Tatvan, Bitlis/ Turkey). Biological Diversity and Conservation, 12(2), 78-91.

10. Santos-Sánchez, N. F., Salas-Coronado, R., Villanueva-Cañongo, C., Hernández-Carlos, B., Antioxidant Compounds and Their Antioxidant Mechanism, in Antioxidants, E. Shalaby, Editor. 2019, IntechOpen: London.

11. Louveaux, J., Maurizio, A., Vorwohl, G. (1978). Methods of Melissopalynology. Bee World, 59(4), 139-157.

12. Song, X.-Y., Yao, Y.-F., Yang, W.-D. (2012). Pollen Analysis of Natural Honeys from the Central Region of Shanxi, North China. PLOS ONE, 7(11), e49545.

13. Gür, M., Güder, A., Verep, D., Güney, K., Özkan, O. E., Seki, N., Kandemirli, F. (2018). Some Important Plants for Epilepsy Treatment: Antioxidant Activity and Flavonoid Compositions. Iranian Journal of Science and Technology, Transactions A: Science, 42(4), 1847-1857.

14. Ateş, S., Gür, M., Özkan, O. E., Akça, M., Olgun, Ç., Güder, A. (2015). Chemical Contents and Antifungal Activity of Some Durable Wood Extractives vs. Pleurotus ostreatus. 2015, 10(2), 11.

15. Blois, M. S. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. Nature, 181(4617), 1199-1200.

16. Ruch, R. J., Cheng, S.-j., Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis, 10(6), 1003-1008.

17. Dinis, T. C. P., Madeira, V. M. C., Almeida, L. M. (1994). Action of Phenolic Derivatives (Acetaminophen, Salicylate, and 5-Aminosalicylate) as Inhibitors of Membrane Lipid Peroxidation and as Peroxyl Radical Scavengers. Archives of Biochemistry and Biophysics, 315(1), 161-169.

18. Gökce, H., Alpaslan, Y. B., Zeyrek, C. T., Ağar, E., Güder, A., Özdemir, N., Alpaslan, G. (2019). Structural, spectroscopic, radical scavenging activity, molecular docking and DFT studies of a synthesized Schiff base compound. Journal of Molecular Structure, 1179, 205-215.

19. Oyaizu, M. (1986). Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese Journal of Nutrition and Dietetics, 44(6), 307-315.

20. Antonie, I. (2014). The biodiversity of the melliferous plants in the surroundings of the town sebes (alba county) and their economical importance Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development 14(4), 13-18.

21. Farkas, A. Zajacz, E. (2007). Nectar production for the Hungarian honey industry. The European Journal Plant Science and Biotechnology, 1(2), 125-151.

22. Çelemli, Ö.G., Özenirler, Ç., Bayram, N. E., Zare, G., Sorkun, K. (2018). Melissopalynological analysis for geographical marking of Kars honey. Kafkas Universitesi Veteriner Fakultesi Dergisi, 24(1), 53-59.

23. Al-Hindi, R. R., Shehata, A. (2014). Evaluation of antioxidant and antibacterial activities and essential elements content of locally produced honey in Saudi Arabia. Life Science Journal, 11, 175-185.

24. Kıvrak, Ş., Kıvrak, İ. (2017). Assessment of phenolic profile of Turkish honeys. International Journal of Food Properties, 20(4), 864-876.

25. Alzahrani, H. A., Boukraa, L., Bellik, Y., Abdellah, F., Bakhotmah, B. A., Kolayli, S., Sahin, H. (2012). Evaluation of the antioxidant activity of three varieties of honey from different botanical and geographical origins. Global journal of health science, 4(6), 191-196.

26. Temizer, İ. K., Güder, A., Temel, F. A., Cüce, H. (2018). Antioxidant activities and heavy metal contents of Castanea sativa honey. Global NEST Journal, 20(3), 541-550.

27. Sherin, M. S., Benny, B., Ashadevi, S. (2015). Antioxidant Activity, DNA and Cellular Protective Effect of Honey from Srilanka. Asian Journal of Biochemistry, 10(3), 106-116.

28. Salgueiro, F. B., Lira, A. F., Rumjanek, V. M., Castro, R. N. (2014). Phenolic composition and antioxidant properties of Brazilian honeys. Quím. Nova, 37(5), 821-826.

29. Pontis, J. A., Costa, L. A. M. A. d., Silva, S. J. R. d., Flach, A. (2014). Color, phenolic and flavonoid content, and antioxidant activity of honey from Roraima, Brazil. Food Sci. Technol (Campinas), 34(1), 69-73.

30. Al-Hindi, R. R. Shehata, A. (2014). Evaluation of Antioxidant and Antibacterial Activities and Essential Elements Content of Locally Produced Honey in Saudi Arabia. 11(5), 175-185.

31. Yegin, S. Ç., Alver, D. O., Çiçek, F., Güder, A. (2018). Investigation of Antioxidant and Antimicrobial Activity of Different Multifloral Honeys. Mellifera, 18(2), 7-14.

32. Silici, S., Ülgen, N. (2019). Bioactive Properties of Blossom and Honeydew Honeys Mellifera, 19(2), 41-52.

33. Juszczak, L., Gałkowska, D., Ostrowska, M., and Socha, R., (2016). Antioxidant activity of honey supplemented with bee products. Natural Product Research, 30(12), 1436-1439.

34. Dżugan, M., Tomczyk, M., Sowa, P., Grabek-Lejko, D. (2018). Antioxidant Activity as Biomarker of Honey Variety. Molecules, 23(8): 2069.

35. Stagos, D., Soulitsiotis, N., Tsadila, C., Papaeconomou, S., Arvanitis, C., Ntontos, A., Karkanta, F., Adamou-Androulaki, S., Petrotos, K., Spandidos, D. A., Kouretas, D., Mossialos, D. (2018). Antibacterial and antioxidant activity of different types of honey derived from Mount Olympus in Greece. International Journal of Molecular Medicine 42(2), 726-734.

36. Moniruzzaman, M., Yung An, C., Rao, P. V., Hawlader, M. N. I., Azlan, S. A. B. M., Sulaiman, S. A., Gan, S. H. (2014). Identification of Phenolic Acids and Flavonoids in Monofloral Honey

from Bangladesh by High Performance Liquid Chromatography: Determination of Antioxidant Capacity. BioMed Research International, 2014, 737490.

37. Nayik, G. A. Nanda, V. (2016). A chemometric approach to evaluate the phenolic compounds, antioxidant activity and mineral content of different unifloral honey types from Kashmir, India. LWT, 74, 504-513.

38. Temizer, İ. K., Güder, A., Temel, F. A., Avcı, E. (2018). A comparison of the antioxidant activities and biomonitoring of heavy metals by pollen in the urban environments. Environmental Monitoring and Assessment, 190(8), 462.

39. Temizer, İ. K., Güder, A., Başer, B. (2020). Botanic Origin and Antioxidant Activity of Some Bitlis Honeys. The Black Sea Journal of Sciences, 10(1), 121-130.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

SIMULTANEOUS QUANTITATIVE RESOLUTION AND VALIDATION OF A BINARY MIXTURE IN A PHARMACEUTICAL DOSAGE FORM BY USING A SPECTROPHOTOMETRIC SIGNAL PROCESSING TECHNIQUE

FARMASÖTİK BİR PREPARATTAKİ İKİLİ KARIŞIMIN SPEKTROFOTOMETRİK SİNYAL İŞLEME TEKNİĞİ KULLANILARAK AYNI ANDA MİKTAR TAYİNİ VE VALİDASYONU

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ABSTRACT

Objective: *In this context, a rapid and powerful signal processing approach, a ratio-derivative spectrophotometry (RDS) method were developed for the simultaneous determination of irbesartan (IRB) and hydrochlorothiazide (HCT) in a tablet.*

Material and Method: *The RDS method was applied to the UV spectra of the IRB and HCT. The calibration equations were obtained by measuring amplitudes at 236.3 nm for the IRB determination and at 260.0 nm for the HCT determination, respectively. The proposed method was validated by using the recovery studies, intra-day and inter-day assays and standard addition technique.*

Result and Discussion: *This study aims to apply a rapid and powerful signal processing method to the simultaneous quantification of IRB and HCT in their synthetic mixtures and tablets. As can be seen, the UV spectra of two drugs overlapped strongly in same spectral region. Direct conventional absorbance measurements do not give expected results for the IRB-HCT tablet analysis mainly due to the spectral interference. However, in order to reach a reasonable spectral analysis outcome, we focused mainly on the application of the ratio-derivative spectra treatment to the quantitative resolution of IRB-HCT mixtures without having to a separation procedure.*

Keywords: *Derivative, quantitative determination, spectrophotomety, signal processing, validation*

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ÖZ

Amaç: *Bu çalışmada, tablette irbesartan (IRB) ve hidroklorotiyazidin (HCT) aynı anda miktar tayini için hızlı ve güçlü bir sinyal işleme yaklaşımı, spektrum oranları türev spektrofotometri (RDS) yöntemi geliştirilmiştir. RDS yöntemi, IRB ve HCT'nin UV spektrumlarına uygulanmıştır.*

Gereç ve Yöntem: *Kalibrasyon denklemleri, sırasıyla IRB tayini için 236.3 nm'de ve HCT tayini için 260.0 nm'de ölçülerek elde edilmiştir. Önerilen yöntem, geri kazanım çalışmaları, gün içi ve günler arası testler ve standart ekleme tekniği kullanılarak doğrulanmıştır.*

Sonuç ve Tartışma: *Bu çalışma, IRB ve HCT'nin sentetik karışımları ve tabletlerinde aynı anda miktar tayini için hızlı ve güçlü bir sinyal işleme yöntemi uygulamayı amaçlamaktadır. Görülebileceği gibi, iki ilacın UV spektrumları aynı spektral bölgede güçlü bir şekilde örtüşmektedir. Doğrudan geleneksel absorbans ölçümleri, spektral girişim nedeniyle IRB-HCT tablet analizi için beklenen sonuçları vermemektedir. Bununla birlikte, makul bir spektral analiz sonucuna ulaşmak için, esas olarak spektrum oranları türev spektrofotometri işleminin IRB-HCT karışımlarının kantitatif tayinine bir ayırma prosedürüne gerek kalmadan uygulanmasına odaklanılmıştır.*

Anahtar Kelimeler: *Kantitatif tayin, spektrofotometri, sinyal işleme, türev, validasyon*

INTRODUCTION

New analytical methods or approaches play an increasingly important role in practical applications such as biomedical, clinical, pharmaceutical analysis, environmental monitoring, quality control of industrial manufacturing and forensic science. For these purposes, analytical chemists work to develop more powerful methods and to improve the reliability of existing analytical techniques to meet the demands for better chemical measurements in many fields of science as well as the above mentioned areas [1-3].

As described above, the analytical methods e.g. spectrophotometry [4], mass spectrometry [5], chromatography [6], electrophoresis [7], electrochemistry [8] and their combined devices have been used for analytical aims. For example, the separation techniques, LC and CE combined with various spectroscopic systems (hyphenated techniques namely LC-MS and CE-MS) have been applied to provide additional chemical information and to decrease the complexity of multicomponent mixtures in material analyses. In addition, LC method has been proposed as main or comparison method for the analysis of active compounds in pharmaceutical preparations and other samples. In applications, these chromatographic analysis approaches require a preliminary separation and other tedious analytical processes during analysis for searching optimal separation and other chromatographic conditions. In addition to that, these methods using the combined devices bring high cost and time-consuming for analysis [9-11]. Therefore, the separation methods based on high technology may not provide successful analytical results in some cases.

Due to the mentioned disadvantageous of the above separation techniques or combined analytical devices, analytical chemists prefer the spectroscopic methods (instead of separation

techniques) for rapid analysis with low cost and successful outcome. Particularly derivative spectrophotometry and its modified versions have been intensively utilized in fast quantitative resolution of multi-component mixtures without separation step. However, in all cases, these spectral methods may not lead good analytical results due to characteristics of strongly overlapping spectra of compounds, interference of main peaks with noise, baseline problems, decreasing signal intensity and worsening signal-to-noise ratio (S/N) for higher derivative orders [12]. In this context, to overcome the drawbacks of the above traditional methods, analytical chemists need to develop new signal analysis techniques, approaches or methods for the efficient quantitative resolution of complex mixtures. Recent developments in signal processing methods give us more opportunity for the better quantitative resolution of the complex analytical problems as well as other areas of science [13,14]. One of the newest additions is ratio-derivative spectrophotometry (RDS) method for the spectral quantification of compounds in mixtures [15]. Ratio-derivative spectrophotometry (RDS) method has gained wide acceptance as a valuable tool for signal processing tasks, due to their wide range of applications.

Several analytical methods, including derivative spectrophotometric methods (16-18) and high performance liquid chromatographic methods (19-22) were reported for the analysis of IRB and HCT in pharmaceutical and biological studies.

In this context, a rapid and powerful signal processing approach, a ratio-derivative spectrophotometry (RDS) method were developed for the simultaneous determination of irbesartan (IRB) and hydrochlorothiazide (HCT) in a tablet. IRB and HCT mixture is a combination of an angiotensin-II receptor antagonist, irbesartan, and a thiazide diuretic, hydrochlorothiazide. The binary mixture of IRB and HCT, which have closely overlapping spectra. Direct conventional absorbance measurements do not give expected results for the IRB-HCT tablet analysis mainly due to the spectral interference. However, in order to reach a reasonable spectral analysis outcome, we focused mainly on the application of the ratio-derivative spectra treatment to the quantitative resolution of IRB-HCT mixtures without having to a separation procedure. The proposed method was validated by using the recovery studies, intra-day and inter-day assays and standard addition technique.

MATERIAL AND METHOD

In the UV data collection, the absorption spectra of the compounds and their samples in the spectral region of 200-305 nm were recorded by using a Shimadzu UV-1601 double beam UV–VIS spectrophotometer having a fixed slit width (2 nm) connected with a computer loaded with Shimadzu

UVPC software and a LEXMARK E-320 printer. In the application of the approaches, the Microsoft EXCEL and Wavelet Toolbox in Matlab 7.0 software were used for data treatments, regressions and statistical analysis.

Chemicals and commercial tablet product

A commercial tablet formulation (KARVEZIDE® Tablet, Sanofi-Aventis. Ind., Istanbul, Turkey), containing 150 mg of IRB and 12.5 mg of HCT per tablet was collected from local Turkish market. IRB and HCT reference substances were kindly donated from National Pharm. Ind. Companies, Turkey).

Preparation of standard solutions

A stock standard solution of IRB and HCT was separately prepared by dissolving 25 mg of each drug in 100 mL methanol. For the spectral analysis, a calibration series for each drug between 4.0-32.0 μg mL^{-1} for IRB and 2.0-9.0 μg mL^{-1} for HCT in the above solvent was prepared from the standard stock solutions. For the standard addition technique, the sample solutions were prepared by adding the stock solution of each compound to tablets at three different concentration levels for six replicates to evaluate the interference of excipients on the analysis. In addition, the sample solutions at three different concentration levels for six times were prepared for testing intra-day and inter-day analysis.

Sample solutions preparation

For analysis of commercial tablets; twenty tablets containing IRB and HCT were weighed and crushed into fine powder. A quantity of powder equivalent to one tablet was transferred to in 100 ml volumetric flask and then volume was made up to mark with methanol. The content of the flask was mechanically shaken for 30 min. After filtration, the supernatant was diluted with methanol to obtain final concentrations.

RESULT AND DISCUSSION

This study aims to apply rapid and powerful signal processing method to the simultaneous quantification of IRB and HCT in their synthetic mixtures and tablets. The UV spectra of IRB and HCT standard series and tablet solution were recorded between 200-305 nm as shown in Figure 1. As can be seen, the UV spectra of two drugs overlapped strongly in same spectral region.

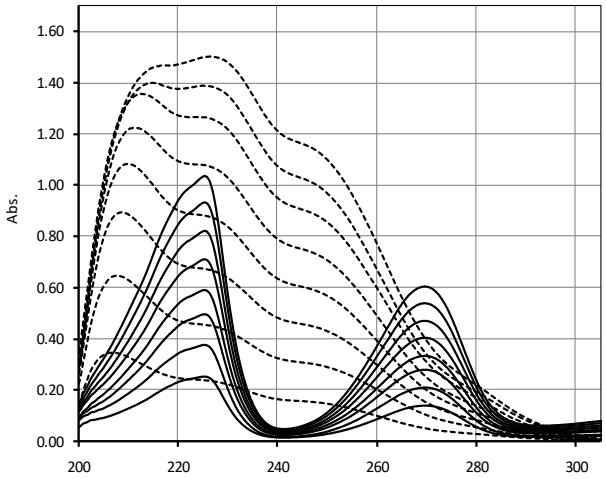

Figure 1. The UV absorption spectra of 4.0-32 µg mL^{-1} IRB (---) and 2.0-9.0 µg mL^{-1} HCT (—) in methanol

Direct conventional absorbance measurements do not give expected results for the IRB-HCT tablet analysis mainly due to the spectral interference. However, in order to reach a reasonable spectral analysis outcome, we focused mainly on the application of the derivative spectra treatment to the quantitative resolution of IRB-HCT mixtures without having to a separation procedure.

The UV spectra of the IRB and HCT, and their tablet solution were recorded in the range 200.0-305.0 nm and divided by the standard spectrum of 16 µg mL^{-1} IRB. The same procedure was repeated for the standard spectrum of 4 µg mL^{-1} HCT as a divisor, respectively. The obtained ratio spectrum was also presented in Figure 2. and Figure 3.

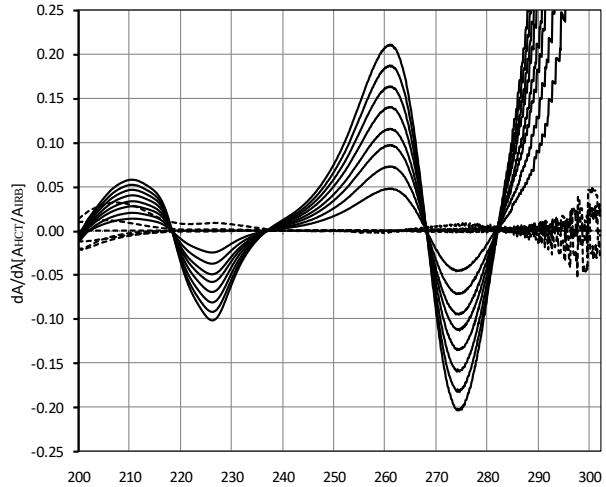

Figure 2. RDS of IRB (---) (4.0-32 µg mL^{-1}) and HCT (—) (2.0-9.0 µg mL^{-1}) in methanol (Div: 16 µg mL^{-1} IRB) ($\Delta\lambda$=10 nm))

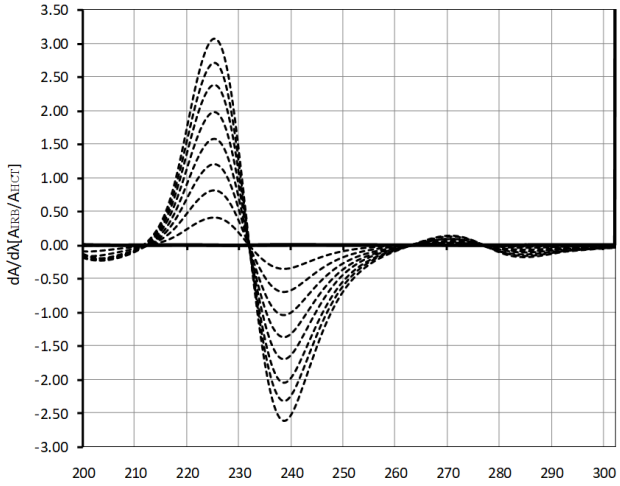

Figure 3. RDS of IRB (---) (4.0-32 µg mL^{-1}) and HCT (—) (2.0-9.0 µg mL^{-1}) in methanol (Div: 4 µg mL^{-1} HCT) (Δλ=10 nm))

Obtained regression analysis results can be seen in Table 1.

Table 1. Linear regression analysis and its statistical results for the proposed method

Method	**RDS**	
Parameter	**IRB**	**HCT**
λ (nm)	236.3	260.0
m	-7.03×10^{-2}	2.26×10^{-2}
n	-5.43×10^{-2}	3.00×10^{-3}
r	0.9993	0.9996
SE(m)	1.07×10^{-3}	2.58×10^{-4}
SE(n)	8.16×10^{-3}	1.54×10^{-3}
SE(r)	2.77×10^{-2}	1.67×10^{-3}
LOD (µg mL^{-1})	0.98	0.58
LOQ (µg mL^{-1})	3.28	1.92

m: Slope
n: Intercept
r: Correlation coefficient
SE(m): Standart error of slope
SE(n): Standart error of intercept
SE(e): Standart error of regression constant
LOD: Limit of detection
LOQ: Limit of quantification

The amount of IRB and HCT in the samples were calculated using the calibration equations calculated according to the linear regression analysis in the Table 1.

Validation of the Proposed Method

For the validation of the RDS method, a validation set consisting of 16 artificial mixture solutions at different concentrations was prepared in methanol, within the working range of 4.0-32.0

µg mL^{-1} for IRB and 2.0-9.0 µg mL^{-1} for HCT. The precision and accuracy of the RDS method was tested using this validation set. The results obtained by applying the RDS method to artificial mixtures prepared as the validation set can be seen in Table2.

Table 2. Recovery results obtained in synthetic mixtures

			RDS		
Added (µg mL^{-1})		**Found (µg mL^{-1})**		**Recovery (%)**	
IRB	**HCT**	**IRB**	**HCT**	**IRB**	**HCT**
30	2.0	28.98	1.94	96.6	96.9
30	3.0	28.73	2.93	95.8	97.6
30	4.0	28.90	3.95	96.3	98.7
30	5.0	28.73	5.01	95.8	100.3
30	6.0	28.81	5.99	96.0	99.9
30	7.0	28.72	6.96	95.7	99.4
30	8.0	28.82	7.81	96.1	97.7
30	9.0	28.65	8.85	95.5	98.3
4.0	2.5	4.09	2.52	102.3	100.6
8.0	2.5	7.86	2.46	98.2	98.5
12	2.5	12.11	2.53	100.9	101.3
16	2.5	16.28	2.47	101.7	98.7
20	2.5	20.41	2.43	102.0	97.1
24	2.5	24.25	2.44	101.1	97.7
28	2.5	27.98	2.45	99.9	97.9
32	2.5	31.42	2.46	98.2	98.5
			$\overline{X}$	100.6	98.5
			SD	1.41	1.46
			RSD	1.40	1.48

SD= Standard deviation
RSD= Relative standard deviation

To evaluate the accuracy and precision of the RDS method, intra-day and inter-day precision and accuracy studies were performed at three different concentrations (4.0, 16.0 and 30.0 µg mL^{-1} for IRB and 2.0, 5.0, and 8.0 µg mL^{-1} for HCT) within the calibration concentration range. 6 different solutions were used for concentration, and the solutions prepared on the same day and inter days were used (Table 3).

Table 3. Results obtained from the analysis of intra-day and inter-day samples by the proposed method

			Intra-day (n=6)					Inter-day (n=6)				
		Added (µg mL^{-1})	Found (µg mL^{-1})	SD	RSD	RE	Rec. (%)	Found (µg mL^{-1})	SD	RSD	RSE	Rec. (%)
RDS	**IRB**	4.0	3.92	0.08	1.92	-1.90	98.1	3.93	0.08	2.01	-1.72	98.3
		16.0	15.89	0.12	0.77	-0.66	99.3	15.82	0.13	0.81	-1.11	98.9
		30.0	29.79	0.46	1.56	-0.69	100.1	29.67	0.45	1.51	3.57	96.4
	HCT	2.0	1.99	0.01	0.63	-0.53	99.5	1.96	0.06	2.84	-1.11	98.9
		5.0	4.90	0.10	1.95	-2.08	97.9	4.83	0.07	1.35	-3.34	96.7
		8.0	7.93	0.13	1.60	-0.82	99.2	7.75	0.18	2.31	-3.34	96.8

SD= Standard deviation
RSD= Relative standard deviation
RSE= Relative standard error

Before applying the RDS signal processing method to the real commercial pharmaceutical preparation, the interference effects of tablet excipients on IRB and HCT compounds were tested. Standard addition technique was used for this purpose (Table 4).

Table 4. Results obtained from the analysis of the standard addition samples by the proposed method

		Added	Recovery					Mean	SD	RSD	RE
RDS	**IRB**	4.0	98.6	100.5	98.7	102.1	100.8	100.1	1.49	1.48	0.14
		8.0	103.7	104.8	104.8	103.5	103.3	104.0	0.73	0.70	4.02
		12.0	102.1	103.5	103.7	103.0	103.2	103.1	0.62	0.60	3.12
	HCT	2.0	103.2	105.4	104.3	104.8	104.8	104.5	0.82	0.78	4.53
		4.0	102.8	103.4	102.8	102.0	102.0	102.6	0.59	0.57	2.60
		8.0	99.6	100.2	100.3	100.2	100.2	100.1	0.26	0.26	0.09

SD= Standard deviation
RSD= Relative standard deviation
RE= Relative error

Recovery and other calculations were made for IRB and HCT in the added standards by deducting the amount of IRB and HCT coming from the preparation. These studies were carried out with five repetitions at three different concentration levels.

Tablet Results

Experimental results obtained by the application of the proposed signal processing method to the IRB-HCT tablet samples were indicated in Table 5. Successful results were obtained for the quantitative analysis of commercial tablets containing IRB and HCT compounds. In the tablet analysis, the interference of the tablet excipients on the determination of the related compounds was not observed in application of proposed method to the commercial tablets.

Table 5. Analysis results of tablets containing IRB and HCT by the proposed method

Method	**RDS**	
	IRB (mg)	**HCT (mg)**
Mean	147.12 ± 0,32	12.05 ± 0,04
SD	1.01	0.12
RSD	0.69	1.01
CL	0.63	0.08

SD= Standard deviation,
RSD= Relative standard deviation
CL= Confidence limit

In conclusion, a rapid and powerful hyphenated signal processing approach, RDS were developed and applied to the simultaneous spectral quantification of IRB and HCT in their synthetic mixtures and tablets. This signal processing method does not require any separation step for the

analysis of both compounds having the strong overlapping spectra in the same spectral region (see Figure 1). In order to demonstrate the validity and applicability of the methods, the optimal control processes of the proposed methods were performed by using the analytical validation parameters.

The proposed methods can be used for the quantitative estimation and routine quality control of the tablets containing mentioned drugs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

REFERENCES

1. Siddiqui, M.R., Al Othman, Z.A., Rahman, N. (2017). Analytical techniques in pharmaceutical analysis: A review. *Arabian Journal of Chemistry*, *10*, 1409-1421.

2. Valagaleti, R., Burns, P.K., Gill, M. (2003). Analytical support for drug manufacturing in the United States—from active pharmaceutical ingredient synthesis to drug product shelf life. *Drug Inform. J.*, *37*, 407-438.

3. Ju, H. (2013). Grand challenges in analytical chemistry: towards more bright eyes for scientific research, social events and human health. *Front. Chem.*, https://doi.org/10.3389/fchem.2013.00005.

4. El-Didamony, A. M., Saad, M. Z., Saleem, N. O. (2015). Spectrophotometric determination of some analgesic drugs in pharmaceutical formulations using N-bromosuccinimide as an oxidant. *Journal of the Association of Arab Universities for Basic and Applied Sciences*, *17*, 43-50.

5. Baghel, U. S., Singh, A., Singh, D., Sinha, M. (2017). Application of mass spectroscopy in pharmaceutical and biomedical analysis. *Spectroscopic Analyses - Developments and Applications*, DOI: 10.5772/intechopen.70655

6. Khateeb, L. A., Al-Hakami, W.E. (2020). Reliable chromatographic determination of non-steroidal anti-inflammatory drugs in real samples matrices. *International Journal of Environmental Analytical Chemistry*, https://doi.org/10.1080/03067319.2019.1689967.

7. Baciu, T., Botello, I., Borrull, F., Calull, M. Aguilar, C. (2015). Capillary electrophoresis and related techniques in the determination of drugs of abuse and their metabolites. *TrAC Trends in Analytical Chemistry*, *74*, 89-108.

8. Özdokur, K. V. (2019). Voltammetric determination of isoniazid drug in various matrix by using CuOx decorated mw-cnt modified glassy carbon electrode. *Electroanalysis*, https://doi.org/10.1002/elan.201900307.

9. Chhonker, Y. S., Edi, C., Murry, D. J. (2018). LC–MS/MS method for simultaneous determination of diethylcarbamazine, albendazole and albendazole metabolites in human plasma: Application to a clinical pharmacokinetic study. *J. Pharm. Biomed. Anal., 20-151*, 84–90.

10. Sáiz, J., García-Ruiz, C., Gómara, B. (2017). Comparison of different GC-MS configurations for the determination of prevalent drugs and related metabolites. *Analytical Methods, 9*, 2897-2908.
11. Maurer, H. H. (1994). Toxicological analysis of drugs: GC-MS screening and confirmation. *Acta Medicinæ Legalis, XLIV*, 489-492.

12. Daubechies, I. (1992). Ten Lectures on Wavelets, Society for Industrial and Applied Mathematics, Philadelphia, 1-357.

13. Dinç, E., Baleanu, D. (2007). Continuous wavelet transform applied to the overlapping absorption signals and their ratio signals for the quantitative resolution of mixture of oxfendazole and oxyclozanide in bolus. *J. Food Drug Anal., 15(2),* 109-117.

14. Dinç, E., Baleanu, D., Üstündağ, Ö. (2003). An approach to quantitative two-component analysis of a mixture containing hydrochlorothiazide and spironolactone in tablets by one dimensional continuous daubechies and biorthogonal wavelet analysis of UV-spectra. *Spectrosc. Lett., 36*, 341-355.

15. Redasani, V. K., Patel, P. R., Marathe, D.Y., Chaudhari, S. R., Shirkhedkar, A. A., Surana, S. J. (2018). A revıew on derivative uv-spectrophotometry analysis of drugs in pharmaceutical formulations and biological samples review. *Journal of the Chilean Chemical Society*, http://dx.doi.org/10.4067/s0717-97072018000304126.

16. Albero, I., Rodenas, V., Garcia, S., Sanchez, C. (2002). Determination of irbesartan in the presence of hydrochlorothiazide by derivative spectrophotometry. *J. Pharmaceut. Biomed., 29(1-2)*, 299-305.

17. El-Yazbi, A., Hammud, H., Sonji, M. (2007). Analysis of eprosartan-hydrochlorothiazide and irbesartan -hydrochlorothiazide binary mixtures by derivative spectrophotometry. *Int. J. App. Chem., 3(1),* 1-12.

18. Joseph, J., Brault, S., Boyer, C., Langlois, M., Cabrero, L., Dubost, J. (2003). Simultaneous determination of irbesartan and hydrochlorothiazide in tablets by derivative spectrophotometry. *Anal. Lett., 36(11)*, 2485-2495.

19. Zhu, J., You, S., Meng, X. (2003). Determination of irbesartan and hydrochlorothiazide in compound irbesartan tablets by HPLC. *Zhongguo Yiyao Gongye Zazhi, 34(11),* 567-569.

20. Tutunji, L. F., Tutunji, M. F., Alzoubi, M. I., Khabbas, M. H., Arida, A. I. (2009). Simultaneous determination of irbesartan and hydrochlorothiazide in human plasma using HPLC coupled with tandem mass spectrometry:Application to bioequivalence studies. *J. Pharmaceut. Biomed., 51(4),* 985-990.

21. Alanazi, A. M., Abdelhameed, A. S., Khalil, N.Y., Khan, A. A., Darwish, I. A. (2014). HPLC method with monolithic column for simultaneous determination of irbesartan and hydrochlorothiazide in tablets. *Acta Pharm., 64,* 187-198.

22. Kalyani, G., Adjuad, B. (2019). Stability indicating assay and estimation of irbesartan by first order uv derivative spectrophotometry. *International Journal of Pharmaceutical Sciences and Research, 23,* 3817-3821.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND CHARACTERIZATION OF MUCOADHESIVE-*THERMOSENSITIVE BUCCAL* GEL CONTAINING METRONIDAZOLE FOR THE TREATMENT OF ORAL MUCOSITIS

ORAL MUKOZİT TEDAVİSİ İÇİN METRONİDAZOL İÇEREN MUKOADEZİF-ISIYA DUYARLI BUKKAL JELİN GELİŞTİRİLMESİ VE KARAKTERİZASYONU

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ABSTRACT

Objective: *This study was aimed to develop and characterize, mucoadhesive-thermosensitive gel formulation containing metronidazole that can quickly gel at the buccal mucosa temperature for treatment of oral mucositis.*

Material and Method: *In this study, the combinations Poloxamer 407 (P407), Poloxamer 188 (P188) and Hydroxypropyl methylcellulose (HPMC) were used in certain concentrations to form a gel in buccal mucosa. Optimum formulations were selected by measuring the gelling time and gelling temperature of the formulations prepared. The hardness, adhesiveness, cohesiveness, resilience, sprayability, mucoadhesion and release properties of selected formulations were evaluated.*

Result and Discussion: *The results showed that F1 and F3 formulations, which quickly turn into gel form at 33 °C, could be the optimum formulations. These two formulations showed controlled release for 8 hours. Texture profile analysis (TPA), mucoadhesion studies and sprayability studies have shown that F1 formulation which the mixture of P407 (15%), P188 (20%) and HPMC (5%) is the optimum formulation for the buccal mucosa. In conclusion, mucoadhesive-thermosensitive gels containing metronidazole may be a good alternative in the treatment of oral mucositis.*

Keywords: *Metronidazole, mucoadhesive-thermosensitive gel, oral mucositis, poloxamer*

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ÖZ

Amaç: *Bu çalışmada oral mukozit tedavisi için bukkal mukoza sıcaklığında hızla jelleşebilen, metronidazol içeren mukoadhesif-ısıya duyarlı jel formülasyonu hazırlanması ve karakterize edilmesi amaçlanmıştır.*

Gereç ve Yöntem: *Bu çalışmada, belirli konsantrasyonlarda poloksamer 407 (P407), poloksamer 188 (P188) ve hidroksipropil metilselüloz (HPMC) kombinasyonları, bukkal mukozada jel oluşturmak için kullanılmıştır. Optimum formülasyonlar hazırlanan formülasyonların jelleşme süresi ve jelleşme sıcaklığı ölçülerek seçilmiştir. Seçilen formülasyonların sertlik, adhezif, kohezif, esneklik, püskürtülebilirlik, mukoadhezif ve salım özellikleri değerlendirilmiştir.*

Sonuç ve Tartışma: *Sonuçlar, 33 °C'de hızla jel formuna dönüşen F1 ve F3 formülasyonunun optimum formülasyonlar olabileceğini göstermiştir. Bu iki formülasyon 8 saat boyunca kontrollü salım göstermiştir. Doku profili analizi (TPA), mukoadhezyon ve püskürtülebilirlik çalışmaları, F1 formülasyonun, P407 (% 15), P188 (% 20) ve HPMC (% 5) kombinasyonu, bukkal mukoza için optimum formülasyon olduğunu göstermiştir. Sonuç olarak, metronidazol içeren mukoadhezif-ısıya duyarlı jeller oral mukozit tedavisinde iyi bir alternatif olabilir.*

Anahtar Kelimeler: *Metronidazol, mukoadhezif-ısıya duyarlı jel, oral mukozit, poloksamer*

INTRODUCTION

Mucositis, an inflammation of the oral mucosa, appeared in the 1980s. Oral mucositis occurs in almost all head and neck cancer patients receiving high doses of chemotherapy or radiotherapy, in about 70% of patients receiving treatment for stem cell transplantation and in about 30% of patients receiving conventional chemotherapy. Oral mucosal epithelial cells, which have the ability to divide faster than other cells, are the most affected cells during chemotherapy [1-4]. In addition, as oral mucositis makes the patient vulnerable to infections, it causes the development of opportunistic infections and increases the cost of treatment by extending the mortality and length of hospital stay. Mucositis in cancer patients can decrease the patient's quality of life. It causes inadequate oral intake and loss of body weight [5]. Since there is no effective treatment for mucositis or the pain it causes, many studies have been conducted in this area. Various strategies have been developed for treatment, such as low-level laser therapy, anti-inflammatory agents, oral decontamination, analgesics, or washing with opioids [6]. In the treatment of pain caused by oral mucositis, mixtures of oral solutions, commonly known as 'Magic Mouth Water', containing different amounts of diphenhydramine, viscous lidocaine, bismuth subsalicylate, and corticosteroids can be used [7]. There are products in the pharmaceutical market in the form of oral gels and liquids, such as Peridex® and Gelclair® [8,9].

Numerous bacteria that form normal oral flora can become pathogenic by immunosuppression. Examples of these bacteria are *Actinobacillus*, *Fusobacterium*, *Actinomycetemcomitans* species, which are usually found in the gram-negative anaerobic group and cause oral mucosal infection.

Metronidazole has a lethal effect on these gram-negative anaerobic bacteria, which can become pathological as a result of immunosuppression. The use of metronidazole in the treatment of oral wounds can be preferred due to its limited side effects and inexpensiveness. Many studies have been conducted on the use of metronidazole topically in the treatment of various oral infections, such as periodontitis and gingivitis [10].

Topical treatment is frequently used for the prevention or treatment of diseases locally [11,12]. Gels are commonly preferred because they are easy to prepare and can be used as carrier systems for both lipophilic and hydrophilic agents. Gels are systems that consist of two components which are cross-linked in 3D. The gel structure can consist of inorganic molecules, primarily polymers or organic macromolecules [13]. Hydrogels are often preferred because they have advantages such as high patient compliance, while the aqueous structure provides ease of application and preparation [14]. Poloxamers, which are nonionic poly (ethylene oxide) (PEO)-poly (propylene oxide) (PPO) triblock copolymers, are used very frequently in pharmaceutical formulations as surfactants, emulsifying agents, solubilizing agents, dispersing agents, and *in vivo* absorbance enhancers [15]. Poloxamers, which are approved by FDA, also called "functional excipients" because they are essential ingredients and play an important role in the formulation [16]. Due to their inverse thermosensitive properties, especially poloxamer 188 (P188) and poloxamer 407 (P407) are frequently used in pharmaceutical formulations [17]. While P407 contains 70% hydrophilic PEO and 30% hydrophobic PPO, P188 contains approximately 80% POE units and 20% PPO units [18–20]. Mucoadhesive polymers can be added to pharmaceutical formulations prepared with poloxamers and are planned to be administered mucosally. Mucoadhesive polymers are widely used in pharmaceutical formulations to increase the residence time of the prepared formulation on the mucosa. Examples of these polymers are polyacrylic acid derivatives such as carbomer and polycarbophil, cellulose derivatives, chitosan and its derivatives, hyaluronic acid, alginate, carrageenan and guar gum [21,22].

In this study, mucoadhesive-thermosensitive gels containing metronidazole were developed to the buccal mucosa for the treatment of oral mucositis. This study also, instead of using high doses of metronidazole for systemic effect was based on the idea that it would be more effective to reduce side effects and increase patient compliance by developing a topical formulation at low doses. It was thought that when metronidazole was used in low doses, a homogeneous unit dose content could be provided as it dissolved in the prepared gel. Based on these considerations, the lowest (0.5%) metronidazole dose used in commercial products is intended to be used in gel formulations developed. Poloxamers are polymers that do not have a mucoadhesive feature, and therefore retention times in mucosal tissues are short. Thus, mucoadhesive substances can be added to the gel structure

to improve their mucoadhesive properties. In our study, hydroxypropyl methylcellulose (HPMC), a cellulose derivative, was used as a mucoadhesive polymer. The goal was to develop a formulation that can be sprayed at room temperature, is able to provide controlled metronidazole release and applied once a day. Thus, patient compliance will be increased, and one of the side effects that the patient is exposed to in cancer treatment can be reduced. Primarily, gelling temperature and gelling time tests were performed on the prepared gel formulations. Afterwards, rheological studies, texture profile analysis, mucoadhesion studies, *in vitro* drug release study, and sprayability studies were performed in the selected formulations.

MATERIAL AND METHOD

Materials

Metronidazole was obtained from Abdi Ibrahim Pharmaceutical Company (Turkey). Poloxamer 188 (Lutrol® F68), poloxamer 407 (Lutrol® F127) and hydroxypropyl methylcellulose (HPMC, Methocel K100MCR Premium USP/EP) were provided from BASF (Germany) and Colorcon (UK), respectively. All other chemicals and reagents were of analytical grade.

Simulated Saliva (SS) at pH 6.8 was prepared. One liter of SS had the following composition: Na_2HPO_4 (2.38 g) KH_2PO_4 (10.19 g), NaCl (8.00 g), H_3PO_4 to adjust the final pH to 6.8 (pH meter, Schott CG 840, Germany), distilled water (1000 ml) [23].

Preparation of mucoadhesive-thermosensitive gel formulations

The mucoadhesive-thermosensitive gels were prepared according to cold method [24]. First, metronidazole was dissolved in distilled water at a concentration of 0.5% (w/w). Then, 0.5% (w/w) of HPMC was slowly added into metronidazole solution under magnetic stirring (600 rpm) at room temperature. This mixture was stirred at the room temperature for 1 h to obtain a homogeneous solution. Then P407 and P188 were slowly added to the HPMC solution containing metronidazole in an ice bath and under magnetic stirring (1000 rpm). The mixture was maintained under mild magnetic stirring (400 rpm) in ice bath overnight to allow complete dissolution.

Characterization of mucoadhesive-thermosensitive gel formulations

Appearance

Mucoadhesive-thermosensitive formulations were visually evaluated in terms of clarity, color and particle content in sol and gel states.

Determination of sol/gel temperature and time ($T_{sol-gel}$)

The formulation prepared as 20 g in a beaker at room temperature was mixed at 50 rpm by throwing a magnet on a heated magnetic stirrer. The temperature was gradually increased and controlled with the probe of the mixer. The temperature, at which the magnet in the formulation stopped due to gelation, was reported as gelation temperature (T_{gel} (°C)) [25]. The transition time (Tgel (sec)) from sol to gel was determined by holding the stopwatch. Experiments were carried out at least three times.

Drug content uniformity

0.2 g gel formulation containing metronidazole was weighed with high precision. The weighted gel formulation was mixed in a 100 mL of pH 6.8 phosphate buffer for 24 hours at room temperature and 100 rpm on a magnetic stirrer. Metronidazole concentration was measured at 320 nm via UV spectrophotometer [26].

Rheological studies of the mucoadhesive-thermosensitive gel formulations

The rheological analysis of the formulations was performed both at 25 °C and 33 °C using Brookfield DV – III + Rheometer. The formulations were kept at room temperature (25 °C) and 33 °C in a water bath, and shear stress values were measured as a function of different shear rate values. Viscosities were calculated using shear rate (ẏ) and shear stress (σs). For pseudoplastic (shear-thinning) and dilatant (shear thickening) systems, the relationship between shear stress and shear rate is expressed by a power law model (also known as The Ostwald de Waele model). K is the consistency coefficient and n is the non-Newtonian index (Eq. 1). As K increases, the viscosity of the system increases. The power law index n characterizes the rheological behavior of the samples. For the pseudoplastic systems n<1, for the dilatant systems n>1, and for the Newtonian systems n=1[27].

$$\sigma s = K (\dot{y})^n \quad \text{(Eq. 1)}$$

The curve of the logarithms of the shear stress versus the logarithms of the shear rates is expressed a slope of n and an intercept of K (Eq. 2).

$$\text{Log}(\sigma s) = \text{log}K + n \log(\dot{y}) \quad \text{(Eq. 2)}$$

Determining mechanical parameters of the mucoadhesive-thermosensitive gel formulations

Mechanical parameters such as hardness, adhesiveness, cohesiveness and resilience of chosen gel formulations were determined using a TA-XT Plus Texture Analyzer (Stable Micro Systems,

London, UK) equipped with a 5 kg load cell in TPA mode [28]. The gels were placed in a water bath to maintain at 33 °C. The 50 mm diameter back extrusion rig is immersed in chosen gel formulations at a speed of 2 mm/s by 15 mm. The hardness (N), adhesiveness (N mm), cohesiveness and elasticity of the gel formulations were determined using the force–time plot [24,29]. The adhesiveness is a necessary property to detaching the probe from the sample. Adhesiveness is a property related to mucoadhesion [28]. Hardness expresses the force required to attain a given deformation. Cohesiveness is the ratio of the area under the force-time curve produced on the second compression cycle to that produced on the first compression cycle [30]. The resilience defines the rate at which the deformed formulation returns to its undeformed condition.

Ex-vivo mucoadhesion studies of the gel formulations

The *ex-vivo* mucoadhesion test of the optimum poloxamer-HPMC gels were performed using a TA-XT Plus Texture Analyzer with a holder. Bovine cheek mucosa frozen at −20 °C was brought to 33 °C and a 2 mm thick section was taken through the surface of this mucosa and attached to the lower end of the device probe. About 1 g of gel and mucosa were contacted for 30 seconds. The instrumental parameters were specified in a previous study [25]. Mucoadhesion data were obtained by calculating the area under the curve from the force-distance plot as the work of mucoadhesion [24]. The Eq. 3 was used to calculate the work of mucoadhesion per cm^2 ($mJ \cdot cm^{-2}$). (πr^2: the area of the mucosal surface being in contact with hydrogel)

$$\text{Work of mucoadhesion (mJ/cm}^2\text{):}\ \frac{AUC}{\pi r^2} \quad \text{(Eq.3)}$$

In vitro drug release study

The *in vitro* drug release study was carried out with dialysis membrane in SS medium pH 6.8 at 33 °C by using Franz diffusion cell. At predetermined time intervals (0.25, 0.5, 1, 2, 3, 4, 6 and 8 h), 2.5 mL release medium was taken out for analysis and completed with fresh buffer (n=3). Samples were measured spectrophotometrically at 320 nm.

Drug release kinetic profiles

To study the drug release kinetics and mechanism of gel formulations containing metronidazole, the *in vitro* data was evaluated to find a suitable mathematical model to fit zero order, first order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell, Weibull release kinetic models by DDsolver software. Adjusted coefficient of determination (r^2_{adj}), Akaike information criterion (AIC) and model selection criteria (MSC) were used as fitting parameters [31].

Spraying ability assay

F1 and F3 formulations were cooled in the refrigerator, filled in 50 mL polyethylene bottles in fluid form, and closed with a spray pump. Spraying of the formulations from the spray pump was recorded with a video camera [32].

RESULT AND DISCUSSION

Preparation of mucoadhesive-thermosensitive gel formulations

Poloxamers are amphiphilic triblock copolymers that show in situ gelling at different temperatures when mixed in different proportions in the presence of different additives. It is possible that the prepared formulation can be locally effective by in situ gelling in the buccal region. In situ gelling, as well as easy of application, can provide that the formulation remains on the local lesions of the oral mucositis for a long time and can achieve antimicrobial agents such as metronidazole more successfully in the treatment of local lesions. Different poloxamer types and proportions were tried for preparing mucoadhesive-thermosensitive gel (Table 1).

While all formulations were transparent and in the fluid form at refrigerator temperature, some of the formulations started to gel at room temperature and transformed into a slightly cloudy gel form. No particle content was observed in any of the sol and gel forms. After metronidazole addition, colors of the mixtures turned to slightly yellow.

Table 1. Composition of different gel formulations

Formulation code	P407 concentration (% w/w)	P188 concentration (% w/w)	HPMC concentration (% w/w)
F1	15	20	0.5
F2	15	20	-
F3	20	15	0.5
F4	20	15	-
F5	15	-	0.5
F6	15	-	-
F7	20	-	0.5
F8	20	-	-
F9	-	20	-
F10	-	15	-
F11	-	20	0.5
F12	-	15	0.5

Characterization of gel formulations

Determination of poloxamer ratios and measurements of sol/gel transition temperature and time

Oral mucosa temperature is 35.8-37.3 °C [33]. However, the temperature changes during the 24-hour period depending on various factors (such as mouth breathing, food intake, etc.) [34]. In order to take these temperature changes into account, the temperature inside the mouth was accepted as 33 °C. Gel formulations are intended to have a gelling temperature of 28-33 ˚C for complete gelling in the mouth. The optimum formulation was determined by taking into account the gelling temperature. In a previous study, the gelling temperature in this range was achieved with a ratio of about 20% (w/w) P407 [22]. In addition to 20% (w/w) P407, addition of P188 was found to be important for keeping the gelling temperature in the range of 30-33 °C. According to results of previous studies, it was found that the increase of P188 ratio did not cause a regular increase in gelling temperature. It was aimed to increase the viscosity by increasing the total polymer concentration in order to prolong the gel formulation residence time in the oral mucosa. For this reason, P188 ratio was chosen as 15% [35,36]. The gelling time and temperatures of the formulations were compared with 20%, 15% and 0% (w/w) P407 and P188, and in the presence and absence of 0.5% (w/w) HPMC (Table 2).

Table 2. Gelation time (Tgel (sec)) and temperature (T_{gel} (°C)) of formulations with or without HPMC containing different proportions P407 and P188 (n=3).

Formulations	P407 % (w/w)	P188 % (w/w)	HPMC % (w/w)	T_{gel} (C°)	T_{gel} (sec) (Room Temperature to 33 °C)
F1	15	20	0.5	31.87±2.48	107.67±3.68
F2	15	20	-	36.5±1.15	600<
F3	20	15	0.5	28.13±0.95	68.00±6.38
F4	20	15	-	24.00±4.68	138.00±20.85
F5	15	-	0.5	20.60±0.53	13.66±4.64
F6	15	-	-	22.90±3.06	26.33±14.38
F7	20	-	0.5	19.80±1.80	3.33±4.71
F8	20	-	-	20.80±0.87	13.66±4.64
F9	-	20	-	85<	600<
F10	-	15	-	85<	600<
F11	-	20	0.5	85<	600<
F12	-	15	0.5	85<	600<

According to the results obtained, it was observed that the gelling time and temperature of F1 formulation containing 15% (w/w) P407, 20% (w/w) P188 and 0.5% (w/w) HPMC were in the desired range. In formulations containing only P407 without P188 the presence of HPMC were seen to reduce the gelling temperature. The effect of HPMC addition on the gelling temperature was different in the F1 and F3 formulations. The gelling temperature in F1 decreased with the addition of HPMC but increased in F3. In another study which P407 was used alone it was noted that the addition of HPMC reduced the gelling temperature [36]. Ban et al. have shown that addition of PEG400 to formulations containing P407 and P188 reduces the gelling temperature [35]. Koffi et al. found that propanediol-1,2 decreased the gelling temperature of the formulations prepared with P407 alone, while the addition of HPMC increased the gelling temperature of P407 and propanediol-1,2 formulations. However, the increase in the amount of HPMC caused a reduction in the gelling temperature [38]. These results support the conclusion that the presence of HPMC (as a different additive like PEG or propanediol-1,2) reduces gelling temperature in the formulations that we used P407 alone. In formulations containing only P188, T_{gel} could not be detected because the magnet did not stop at any temperature. It has been determined that the increase in P188 concentration cause an increase in the gelling temperature and gelling time (Figure 1 and Figure 2). Studies have shown that the increase of P188 concentration at constant P407 concentration does not cause a regular increase in gelling temperature [36,39]. The addition of HPMC reduced gelling time in all formulations. (Figure 2).

During this study, room temperature was not controlled as the formulations would have been under real conditions of use. Depending on the instantaneous room temperature, formulations with low gelling temperature were able to maintain their sol state. As a result, the gelling times of all formulations were measured. When the formulations were ranked according to the decreasing P407 ratio as the primary ranking criterion and the increasing P188 ratio as the secondary ranking criterion, the gelling times of the formulations showed a regular increase (Figure 2). However, the gelling temperature did not increase regularly. (Figure 1). Subsequent studies were carried out on F1 and F3 formulations due to the desired gelling temperature and acceptable gelling time. After the addition of metronidazole, a decrease was observed in the gelling temperatures and times of F1 and F3 formulations. The gelling temperature of the F1 formulation remained within the desired range (Table 3). Formulations F1 and F3 were chosen to evaluate rheological, mucoadhesive, textural and release properties.

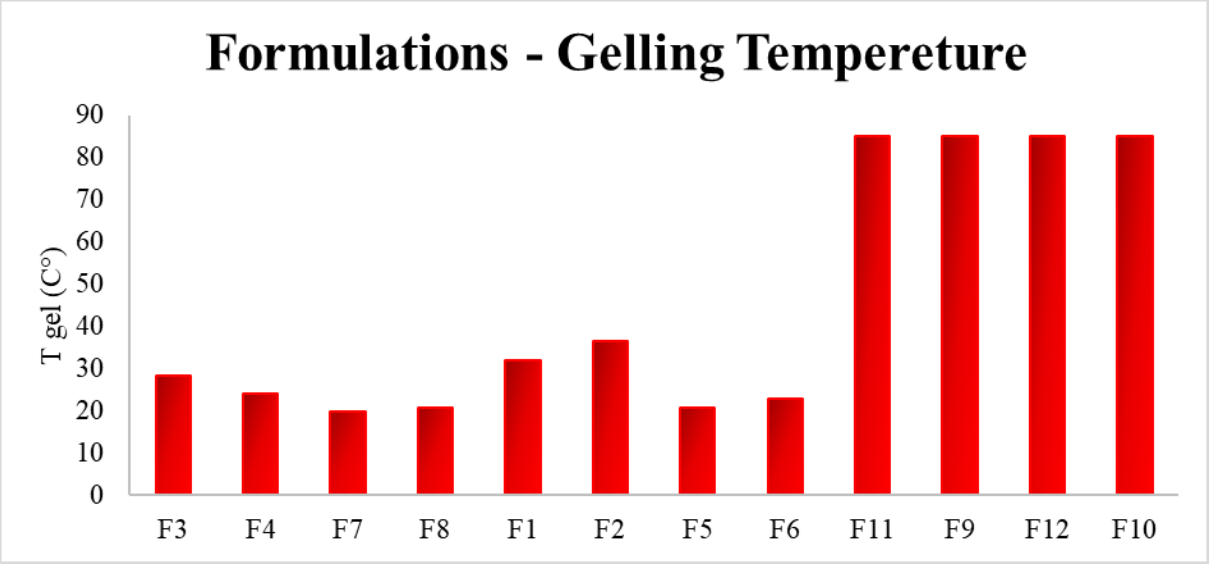

Figure 1. The formulations were sorted against gelling temperature according to the decreasing P407 ratio as the primary ranking criterion and increasing P188 ratio as the secondary ranking criterion.

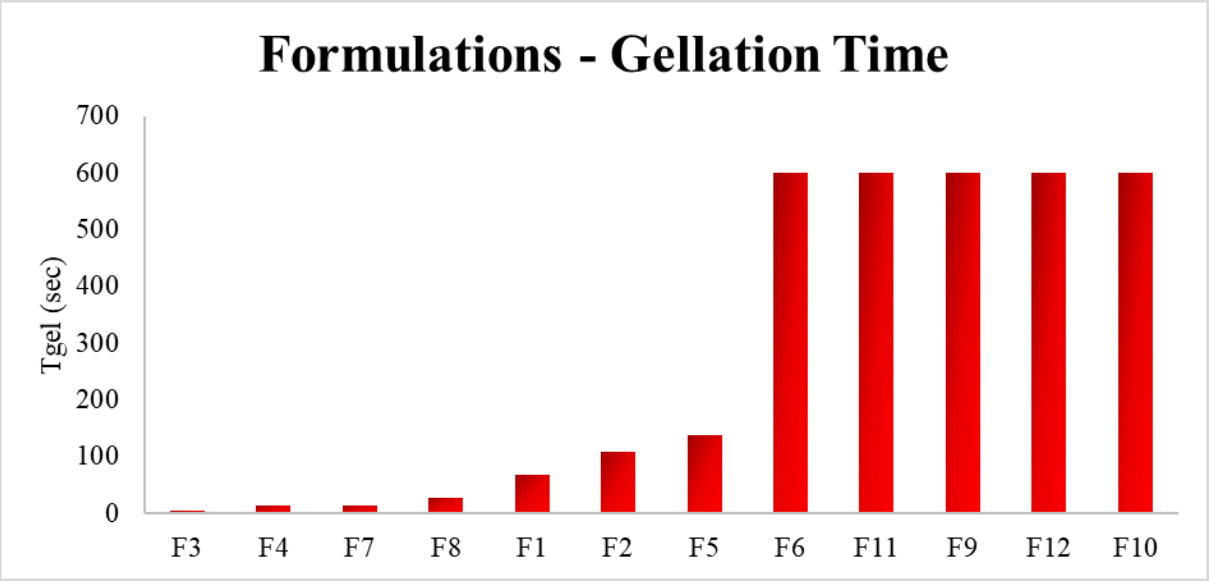

Figure 2. The formulations were sorted against gelation time according to the decreasing P407 ratio as the primary ranking criterion and increasing P188 ratio as the secondary ranking criterion.

Table 3. Gelation time and temperature of F1 and F3 formulations after metronidazole addition (n=3)

Formulations	P407 % (w/w)	P188 % (w/w)	HPMC % (w/w)	Metronidazole % (w/w)	T_{gel} (C°)	T_{gel} (sec) (Room Temperature to 33 °C)
F1	15	20	0.5	0.5	30.4±0.58	92.3±19.0
F3	20	15	0.5	0.5	26.4±0.42	16.0±5.57

After addition of metronidazole, there was no significant difference between Tgel (C°) values ($p<0.05$).

Drug content uniformity

The used spectrophotometric method for metronidazole analysis was found to be linear ($r^2 = 0.9954$). Finally, the amount of metronidazole in the F1 and F3 formulations was found as 106±2.09% and 104±1.10%, respectively.

Rheological Studies

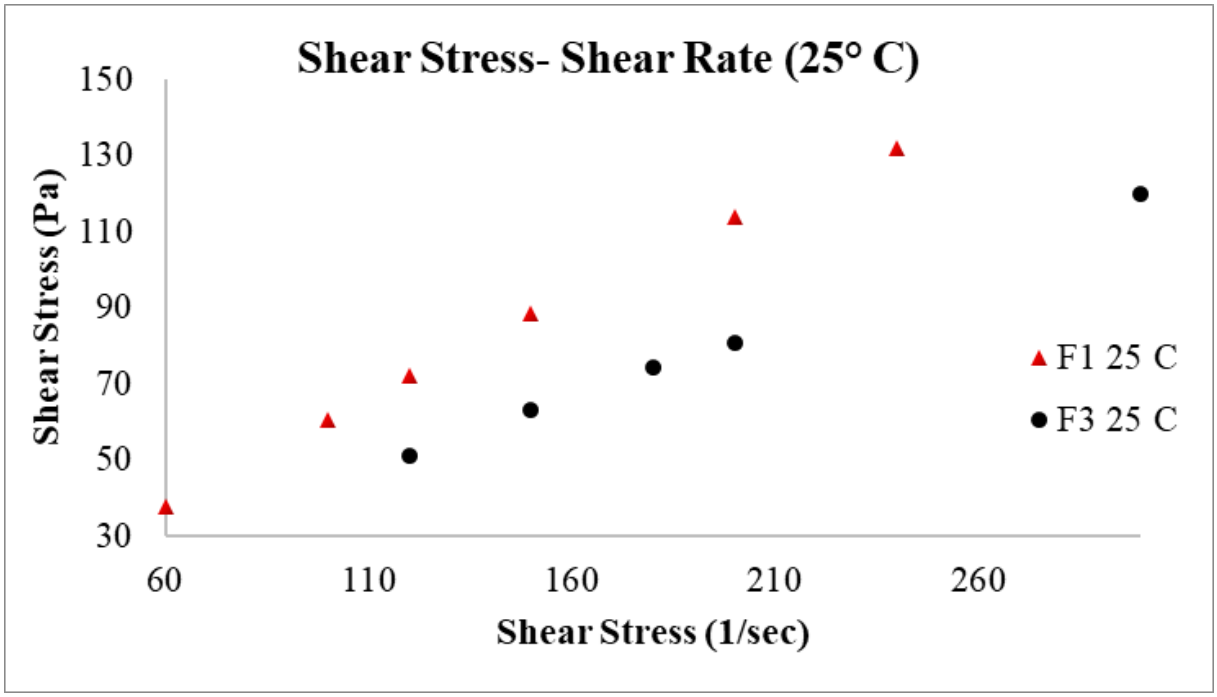

Figure 3. Viscosity (mPa.s)-Shear rate (1/sec) rheogram of F1 and F3 formulations at 25 °C (n=3).

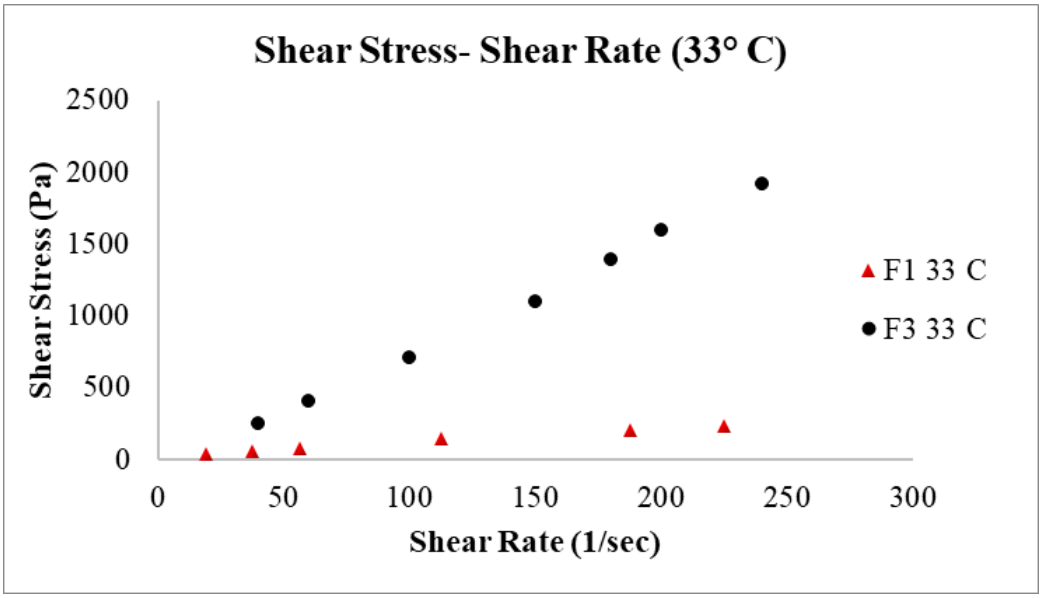

Figure 4. Viscosity (mPa.s)-Shear rate (1/sec) rheogram of F1 and F3 formulations at 33 °C (n=3).

Semi-solid systems have the advantage of ensuring that the formulation comes out of the package in the desired amount and remains in the infection area for the desired time. However, oily systems such as ointments and creams are not well tolerated by patients when applied to the mouth area due to their high viscosity values. For this reason, it will be advantageous to prepare the oral formulation as a water-based gel. Rheological properties are important factors that determine the gelling properties of thermosensitive hydrogels. The formulation should have low viscosity at room temperature and high viscosity to prevent flow and removal at 33 °C [40].

All prepared formulations were in liquid form at refrigerator temperature. F1 and F3 formulations exhibited non-newtonian rheological behavior at 25 °C (Figure 3) and 33 °C (Figure 4). According to power law equation, n and K values were calculated and were shown in Table 4. F1 and F3 formulations were determined based on the n <1 value obtained from the power law equation where it shows shear thinning properties in viscosity measurements at 25 °C. In thermosensitive hydrogels, the viscosity increases as the temperature increases to form an expected gel structure. At 33 °C, both formulations gelled above the sol/gel transition temperature. F1 formulation continued to show shear thinning properties (Figure 3), while F3 formulation has started to show shear thickening properties (Figure 4). As Todica et al. found that the K value and viscosity increased as the temperature increased at constant gel concentration. As the gel concentration increased, K value and viscosity increased as expected [41]. Similarly, as shown in a study by Rahimi S. and Natan B., it appeared that the K value increased with increasing gel concentration and temperature [42]. The

higher viscosity of the F3 formulation than the F1 can be explained by the higher P407 ratio, which has a higher molecular weight and has a different PEO/PPO ratio than the P188 [43].

In F1 and F3 formulations, containing 5% HPMC, ratio of P407 and P188 changed but the total polymer amount kept constant. While the F3 formulation was expected to exhibit a shear thinning property such as F1, it showed a shear thickening property as the rheological properties were dependent on the polymer type rather than the total polymer ratio. This may be due to the increased probability of intermolecular contact, which leads to an increase in physicochemical interactions due to the increase in flow rate. Increased physicochemical interactions may cause shear thickening behavior [44].

Table 4. K, n, R^2 and Standard Error (±SE) values obtained by applying power law equation to the results of viscosity measurements at 25 °C and 33 °C.

Power Law Equation		**F1**	**F3**
33 °C	**K**	4084	3662
	n	0.75	1.14
	R^2	0.9758	0.9737
	±SE	0.02	0.01
25 °C	**K**	1096	841
	n	0.87	0.86
	R^2	0.9115	0.9168
	±SE	0.01	0.01

Texture profile analysis of formulations

Some features of the developed formulation such as ease of removability of the product from the container, good spradability and mucoadhesion, and acceptable viscosity contribute to easy administration and increase in patient compliance. Texture profile analysis is important for the gel formulations developed to show the desired properties in the oral mucosa. Low hardness, high residence time on mucosa and easy application are among the desired properties in oral gel formulations [28]. Lower values of resilience in TPA express greater formulation elasticity [45]. Adhesiveness determines proper gel contact and retention at the mucosal surface. The cohesiveness determines the reconstruction ability of the gel after application. The high cohesiveness value increases the performance of the product. High gel adhesiveness and cohesiveness provide prolonged adhesion of the gel onto the oral mucosa and full structural recovery of the gel following application [46]. The values of hardness, adhesiveness, cohesiveness, and resilience were shown in Table 5. The

effect of the poloxamer type was evaluated in the formulations. For F1 formulation, containing P407/P188 (15%:20% w/w), mechanical properties such as hardness, cohesiveness and adhesiveness were found to be lower than for F3 formulation which contains P407/P188 (20%:15% w/w). F1 exhibited minimum hardness, adhesiveness, cohesiveness, and maximum resilience with decreasing concentration of P407. Baloglu et al. also showed that the decrease in P407 caused low hardness, cohesiveness, adhesiveness and high resilience [15].

Table 5. Hardness, adhesiveness, cohesiveness and resilience values obtained from texture profile analyses of the F1 and F3 formulations at 33 °C (n=3).

Formulations	Hardness (g)	Adhesiveness (g.sec)	Cohesiveness (g.sec)	Resilience
F1	251 ± 32.5	-78.3 ± 12.5	0.94 ± 0.05	0.14 ± 0.03
F3	845 ± 89.2	-263 ± 146	1.03 ± 0.35	0.24 ± 0.03

Evaluation of *ex-vivo* mucoadhesion studies of the gel formulations

The mucoadhesive characteristics are important to provide prolonged residence time of gel formulations on the mucosal surface. In this study, HPMC was used as a mucoadhesive polymer. The effects of poloxamer types on mucoadhesion properties were evaluated. The values of mucoadhesion of formulations were shown in Table 6. Mucoadhesive properties of F1 formulation were found to be lower than F3 formulation. According to the results obtained, the mucoadhesive force and work of mucoadhesion of the formulations increased with increasing P407 concentration. Baloglu et al. also showed that P407 increased the mucoadhesive force of the formulations [15]. Mucoadhesion is related to the elasticity of the polymer chains thus a high elasticity is required for strong mucoadhesive between the polymer and the mucosal surface [47]. Although the F1 formulation showed better elasticity, the higher P407 ratio and adhesiveness of the F3 formulation may have resulted in a better mucoadhesive property.

Table 6. Adhesiveness and work of mucoadhesion values obtained from *ex-vivo* mucoadhesion studies of the F1 and F3 formulations on bovine cheek at 33 °C (n=3).

Formulations	Peak Force (Adhesiveness) (N)	Work of Mucoadhesion (N.sec)
F1	0.01 ± 0.00	0.01 ± 0.00
F3	0.18 ± 0.01	0.218- ± 0.02

In vitro drug release study

F1 and F3 formulations showed similar profiles in an *in vitro* release study conducted with the method of Franz diffusion cell in SS pH 6.8 saliva medium at 33 °C. In the 8-hour period, the F1 formulation released 43.02% of the Metronidazole it contains, while the F3 formulation released 41.37% (Figure 5). In a recent study, metronidazole release at pH 6.8 medium from the formulation containing 15% HPMC was resulted with 23.37% after 3 hours. [48]. In our study we obtained similar release data at the end of 3 hours with the same release medium. Ibrahim et al. developed a vaginal gel containing metronidazole using P407 and P188 in the same proportions as F1 formulation, and metronidazole release was observed to be above 80% within 1.5 hours. They also found that metronidazole release in gels containing P407 and P188 in the same proportions as F3 formulation was around 40% within 1.5 hours. The difference in release rates may have been caused by different conditions, such as higher temperature (37 °C) or higher acidity (pH 4.5) [39]. In addition, HPMC used in gel formulations that we developed may decrease metronidazole release rate due to the tightening of the polymeric matrix structure of the gel and extending the diffusion distance of metronidazole [37].

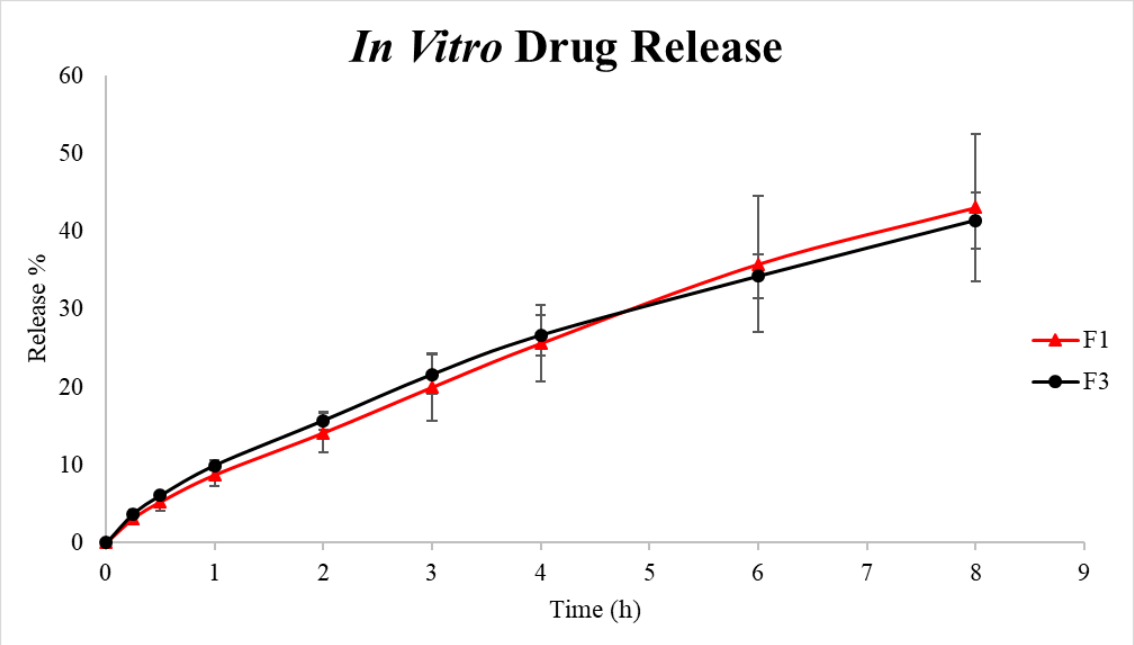

Figure 5. *In vitro* drug release studies from F1 and F3 formulations containing Metronidazole (0.5 % w/w) from dialysis membrane at 6.8 phosphate buffer medium and 33 °C (n=3). The square represents the F1 formulation and the circle represents the F3 formulation.

Drug release kinetic profiles

r^2_{adj}, AIC and MSC were chosen as fitting factors to determine the appropriate model. The AIC value is desired to be low while the MSC value is desired to be high, and the r^2_{adj} value close to 1 indicates the suitability of the model [31]. Considering these criteria, it was determined that F1 formulation fit Korsmeyer-Peppas and F3 formulation fit to both Korsmeyer-Peppas and Weibull models (Table 7). Although F1 formulation shows the compatibility of r^2_{adj} and MSC values to Weibull model, it is not accepted to be suitable for this model due to its high AIC value.

In the Korsmeyer-Peppas model, n value is used to explain the mechanism of release. The n value of 0.5 indicates that the release mechanism is Fickian diffusion. If it is in the range of 0.5-1, this indicates that the release occurs with non-Fickian diffusion. n=1 indicates that it fits to the Case II (Zero Order) model [49]. The n values of the F1 and F3 formulations were determined as 0.7590 and 0.6966, respectively. It was observed that the release from both formulations occurred by the non-Fickian diffusion mechanism.

ß value (ß <0.75 Fickian release, 0.75 <ß <1 combined release mechanism and ß>1 complex release mechanism) is the constant that describes the release mechanisms of the active substance in the Weibull model. The ß constant in the Weibull model of the F3 formulation was found to be 0.7613. This value indicates that the release occurs with the combined release mechanism [50]. Both the ß and the n values of the F3 formulation indicate that the release mechanism is non-fickian.

Inal et al. suggested that the poloxamer formulations fitting the Korsmeyer-Peppas model conform to this model due to the release by gel erosion mechanism [37]. There are various poloxamer gel formulations in the literature that fit different release kinetics such as zero order and Higuchi model [51,52].

The first order kinetics used to describe the form of release was found to be the most suitable model for release profiles of both formulations. The non-fickian release mechanism obtained from the Korsmeyer-Peppas and Weibull models supports that the release can be achieved by first order.

In the equation of the curve obtained by applying Korsmeyer-Peppas model to *in vitro* experimental drug release data of F1 formulation, when t=24 h, the percentage of the drug release was found to be 98.44% (not-shown). This result indicates that the continuous presence of metronidazole in the oral mucosa can be achieved by once a day administration.

Table 7. Adjusted coefficient of determination (r^2adj), Akaike information criterion (AIC) and model selection criteria (MSC) for different release kinetic models collected from DDsolver® by applying experimental *in vitro* drug release data.

Mathematical Models	Compliance Criteria	Formulations	
		F1	F3
Zero Order	r^2_{adj}	0.9726	0.9356
	AIC	36.8961	43.8792
	MSC	3.2057	2.3088
First Order	r^2_{adj}	0.9936	0.9751
	AIC	22.8298	35.3071
	MSC	4.7686	3.2612
Higuchi	r^2_{adj}	0.9329	0.9639
	AIC	45.0320	38.6836
	MSC	2.3017	2.8861
Korsmeyer-Peppas	r^2_{adj}	**0.9979**	**0.9987**
	AIC	**13.7807**	**8.0370**
	MSC	**5.7741**	**6.2913**
	n	0.7590	0.6966
Hixson-Crowell	r^2_{adj}	0.9892	0.9648
	AIC	27.9657	38.4336
	MSC	4.1980	2.9139
Weibull	r^2_{adj}	**0.9956**	**0.9985**
	AIC	21.3049	**8.8481**
	MSC	4.9381	**6.2011**
	ß	0.9231	0.7613

Spraying ability assay

F1 formulation was observed to be easily sprayable both at room temperature and after being removed from the refrigerator (Figure 6A and C). On the other hand, the F3 formulation was not sprayed as desired after it was freshly removed from the refrigerator (Figure 6D). At room temperature, no spraying occurred and F3 formulation came out as a leak from the pump (Figure 6B). The failure of the F3 formulation to be sprayed can be explained by its higher viscosity than the F1 formulation [53].

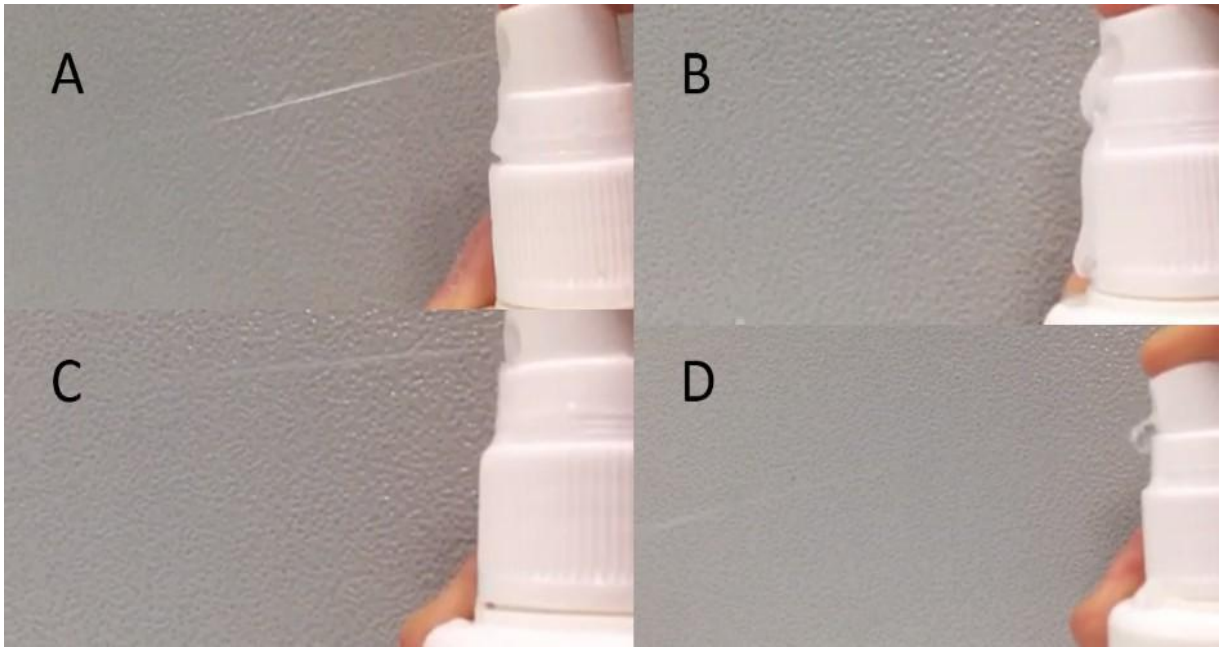

Figure 6. **A)** Spraying of the F1 formulation at room temperature, **B)** Spraying of the F3 formulation at room temperature, **C)** Spraying of the F1 formulation after removing it fresh from the refrigerator and **D)** Spraying of the F3 formulation after removing it fresh from the refrigerator.

In this study, we aim to develop thermosensitive and mucoadhesive gel formulations for oral mucositis treatment containing metronidazole, poloxamer and HPMC. We selected the optimum formulations by comparing free gel formulations with the appropriate gelling temperature and gelling time for the oral mucosa. The addition of metronidazole caused changes in the gelling temperature, and F1 formulation was within the desired range, while the F3 formulation fell below it. In addition to the gelling temperature, the lower hardness, lower resilience, better spraying ability, and pseudoplastic viscosity properties of the F1 formulation were more suitable for application to the site of action. The release of around 43% in 8 hours indicates that the desired controlled release has been achieved with optimum formulation. F1 formulation, which has the desired properties in the majority of the studies, has been determined as a candidate formulation for the future studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Lalla, R. V., Peterson, D. E. (2005). Oral mucositis. *Dental Clinics of North America*, *49* (1 SPEC.ISS.), 167–184.

2. Trotti, A., Bellm, L.A., Epstein, J. B., Frame, D., Fuchs, H. J., Gwede, C. K., Komaroff, E., Nalysnyk, L., Zilberberg, M.D. (2003). Mucositis incidence, severity and associated outcomes in patients with head and neck cancer receiving radiotherapy with or without chemotherapy: A systematic literature review. *Radiotherapy and Oncology*, 66 (3), 253-62.

3. Itoh, Y., Kubota, S., Kawamura, M., Nomoto, Y., Murao, T., Yamakawa, K., Ishihara, S., Hirasawa, N., Asano, A., Yanagawa, S., Naganawa, S. (2016). A multicenter survey of stage T1 glottic cancer treated with radiotherapy delivered in 2.25-Gy fractions in clinical practice: An initial 5-year analysis. *Nagoya Journal of Medical Science*, *78* (4), 399–406.

4. Donnelly, J. P., Bellm, L. A., Epstein, J. B., Sonis, S. T., Symonds, R. P. (2003). Antimicrobial therapy to prevent or treat oral mucositis. *Lancet Infectious Diseases*, *3* (7), 405–412.

5. Mosel, D. D., Bauer, R. L., Lynch, D. P., Hwang, S. T. (2011). Oral complications in the treatment of cancer patients. *Oral Diseases*, *17* (6), 550–559.

6. Villa, A., Sonis, S. T. (2015). Mucositis: Pathobiology and management. *Current Opinion in Oncology*, *27* (3), 159–164.

7. Chan, A., Ignoffo, R.J. (2005). Survey of topical oral solutions for the treatment of chemo-induced oral mucositis. *Journal of Oncology Pharmacy Practice*, *11* (4), 139–143.

8. Buchsel, P. C. (2008). Polyvinylpyrrolidone-sodium hyaluronate gel (Gelclair®): A bioadherent oral gel for the treatment of oral mucositis and other painful oral lesions. *Expert Opinion on Drug Metabolism and Toxicology*, *4* (11), 1449–1454.

9. Carl, W., Emrich, L. S. (1991). Management of oral mucositis during local radiation and systemic chemotherapy: A study of 98 patients. *The Journal of Prosthetic Dentistry*, *66* (3), 361–369.

10. Leiknes, T., Leknes, K. N., Böe, O. E., Skavland, R. J., Lie, T. (2007). Topical use of a metronidazole gel in the treatment of sites with symptoms of recurring chronic inflammation. *Journal of Periodontology*, *78* (8), 1538–1544.

11. Jones, D. S., Woolfson, A. D., Brown, A. F., Coulter, W. A., McClelland, C., Irwin, C. R. (2000). Design, characterisation and preliminary clinical evaluation of a novel mucoadhesive topical formulation containing tetracycline for the treatment of periodontal disease. *Journal of Controlled Release*, *67* (2–3), 357–368.

12. Kaur, J., Kaur, J., Jaiswal, S., Gupta, G. (2016). Recent advances in topical drug delivery system. *Pharmaceutical Research*, *6* (07), 6353-6369.

13. Patil, P. B., Datir, S. K., Saudagar, R. B. (2019). A review on topical gels as drug delivery system P.B. *Journal of Drug Delivery and Therapeutics*, *3* (3-s), 989–994.

14. Agrawal, A. K., Das, M., and Jain, S. (2012). In situ gel systems as "smart" carriers for sustained ocular drug delivery. *Expert Opinion on Drug Delivery*, *9* (4), 383–402.

15. Baloglu, E., Karavana, S. Y., Senyigit, Z. A., Guneri, T. (2011). Rheological and mechanical properties of poloxamer mixtures as a mucoadhesive gel base. *Pharmaceutical Development and Technology*, *16* (6), 627–636.

16. Kabanov, A. V., Batrakova, E. V., Miller, D. W. (2003). Pluronic® block copolymers as modulators of drug efflux transporter activity in the blood-brain barrier. *Advanced Drug Delivery Reviews*, *55* (1), 151–164.

17. Maynard, C., Swenson, R., Paris, J. A., Martin, J. S., Hallstrom, A. P., Cerqueira, M. D., Weaver, W. D. (1998). Randomized, controlled trial of RheothRx (poloxamer 188) in patients with suspected acute myocardial infarction. *American Heart Journal*, 135(5 I), 797–804.

18. Singh, S., Parhi, R., Garg, A. (2011). Formulation of topical bioadhesive gel of aceclofenac using 3-level factorial design. *Iranian Journal of Pharmaceutical Research*, *10* (3), 435-45.

19. Shin, S. C., Kim, J. Y., Oh, I. J. (2000). Mucoadhesive and physicochemical characterization of Carbopol-poloxamer gels containing triamcinolone acetonide. *Drug Development and Industrial Pharmacy*, *26* (3), 307–312.

20. BASF Web site. (2017). Retrieved May 22, 2020, from https://pharmaceutical.basf.com/

21. Valenta, C. (2005). The use of mucoadhesive polymers in vaginal delivery. *Advanced Drug Delivery Reviews*, 57 (11), 1692-712.

22. Tuğcu-Demiröz, F. (2017). Development of in situ poloxamer-chitosan hydrogels for vaginal drug delivery of benzydamine hydrochloride: Textural, mucoadhesive and *in vitro* release properties. *Marmara Pharmaceutical Journal*, *21* (4), 762–770.

23. Marques, M. R. C., Loebenberg, R., Almukainzi, M. (2011). Simulated biological fluids with possible application in dissolution testing. *Dissolution Technologies*, *18* (3), 15-27.

24. Tuğcu-Demiröz, F., Acartürk, F., Özkul, A. (2015). Preparation and characterization of bioadhesive controlled-release gels of cidofovir for vaginal delivery. *Journal of Biomaterials Science, Polymer Edition*, *26* (17), 1237-1255.

25. Tuğcu-Demiröz, F., Acartürk, F., Erdoğan, D. (2013). Development of long-acting bioadhesive vaginal gels of oxybutynin: Formulation, *in vitro* and *in vivo* evaluations. *International Journal of Pharmaceutics*, *457* (1), 25-39.

26. Patel, P., Varshney, P., Rohit, M. (2014). Analytical method development and validation for simultaneous estimation of metronidazole and amoxicillin in synthetic mixture by UV-

visible spectroscopy. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6* (SUPPL. 2), 317–319.

27. de Francisco, L. M. B., Rosseto, H. C., de Alcântara Sica de Toledo, L., dos Santos, R.S., de Souza Ferreira, S. B., Bruschi, M. L. (2019). Organogel composed of poloxamer 188 and passion fruit oil: Sol-gel transition, rheology, and mechanical properties. *Journal of Molecular Liquids*, *289,* 111170.

28. Jones, D.S., Woolfson, A. D., Brown, A. F. (1997). Textural, viscoelastic and mucoadhesive properties of pharmaceutical gels composed of cellulose polymers. *International Journal of Pharmaceutics*, *151* (2), 223–233.

29. Amasya, G., Karavana, S. Y., Şen, T., Baloğlu, E., Tarimci, N. (2012). Bioadhesive and mechanical properties of triamcinolone acetonide buccal gels. *Turkish Journal of Pharmaceutical Sciences*, *9* (1), 1–11.

30. Cevher, E., Taha, M. A. M., Orlu, M., Araman, A. (2008). Evaluation of mechanical and mucoadhesive properties of clomiphene citrate gel formulations containing carbomers and their thiolated derivatives. *Drug Delivery*, *15* (1), 57–67.

31. Sorasitthiyanukarn, F. N., Rojsitthisak, P., Rojsitthisak, P. (2017). Kinetic study of chitosan-alginate biopolymeric nanoparticles for the controlled release of curcumin diethyl disuccinate. *Journal of Metals, Materials and Minerals*, *27* (2), 17–22.

32. Mehravaran, N., Moghimi, H., Mortazavi, S. A. (2009). The influence of various mucoadhesive polymers on *in vitro* performance of the resulting artificial saliva pump spray formulations. *Iranian Journal of Pharmaceutical Research*, *8* (1), 3–13.

33. Lapum, J., Verkuyl, M., Garcia, W., St-Amant, O., Tan, A. (2018). Vital Sign Measurement Across the Lifespan, 1st Canadian edition, Springer, Toronto, p. 26.

34. Moore, R. J., Watts, J. T. F., Hood, J. A. A., Burritt, D. J. (1999). Intra-oral temperature variation over 24 hours. *European Journal of Orthodontics*, *21*(3), 249–261.

35. Ban, E., Park, M., Jeong, S., Kwon, T., Kim, E. H., Jung, K., Kim, A. (2017). Poloxamer-based thermoreversible gel for topical delivery of emodin: Influence of P407 and P188 on solubility of emodin and its application in cellular activity screening. *Molecules*, *22* (2), 246.

36. Zhang, K., Shi, X., Lin, X., Yao, C., Shen, L., Feng, Y. (2015). Poloxamer-based in situ hydrogels for controlled delivery of hydrophilic macromolecules after intramuscular injection in rats. *Drug Delivery*, *22* (3), 375–382.

37. Inal, O., Yapar, E. A. (2013). Effect of mechanical properties on the release of meloxicam from poloxamer gel bases. *Indian Journal of Pharmaceutical Sciences*, *75* (6), 700–706.

38. Koffi, A. A., Agnely, F., Ponchel, G., Grossiord, J. L. (2006). Modulation of the rheological and mucoadhesive properties of thermosensitive poloxamer-based hydrogels intended for the rectal administration of quinine. *European Journal of Pharmaceutical Sciences*, *27* (4),

328–335.

39. Ibrahim, E. S. A., Ismail, S., Fetih, G., Shaaban, O., Hassanein, K., Abdellah, N. H. (2012). Development and characterization of thermosensitive pluronic-based metronidazole in situ gelling formulations for vaginal application. *Acta Pharmaceutica, 62* (1), 59–70.

40. Tirnaksiz, F., Robinson, J. R. (2005). Rheological, mucoadhesive and release properties of Pluronic F-127 gel and Pluronic F-127/polycarbophil mixed gel systems. *Pharmazie*, *60* (7), 518-23.

41. Todica, M., Pop, C. V., Udrescu, L., Pop, M. (2010). Rheological behavior of some aqueous gels of carbopol with pharmaceutical applications. *Chinese Physics Letters*, *27* (1), 018301.

42. Rahimi, S., Natan, B. (2000). Numerical solution of the flow of power-law gel propellants in converging injectors. *Propellants, Explosives, Pyrotechnics*, 25 (4), 203-212.

43. Franzén, H. M., Draget, K. I., Langebäck, J., Nilsen-Nygaard, J. (2015). Characterization and properties of hydrogels made from neutral soluble chitosans. *Polymers, 7*, 373-389.

44. Liu, W.H., Yu, T.L., Lin, H.L. (2007). Shear thickening behavior of dilute poly(diallyl dimethyl ammonium chloride) aqueous solutions. *Polymer*, *48* (14), 4152–4165.

45. Jones, D. S., Woolfson, A. D., Djokic, J. (1996). Texture profile analysis of bioadhesive polymeric semisolids: Mechanical characterization and investigation of interactions between formulation components. *Journal of Applied Polymer Science*, *61* (12), 2229–2234.

46. Tan, Y. T. F., Peh, K. K., Al-Hanbali, O. (2000). Effect of carbopol and polyvinylpyrrolidone on the mechanical, rheological, and release properties of bioadhesive polyethylene glycol gels. *AAPS PharmSciTech*, *1* (3), E24.

47. Cevher, E., Sensoy, D., Taha, M. A. M., Araman, A. (2008). Effect of thiolated polymers to textural and mucoadhesive properties of vaginal gel formulations prepared with polycarbophil and chitosan. *AAPS PharmSciTech, 9* (3), 953-65.

48. Sarkar, U., Raha, A., Mukherjee, P., Paul, M., Bagchi, A. (2018). Development and evaluation of Metronidazole containing topical gel using different gelling agents. *Asian Journal of Pharmacy and Pharmacology*, *4* (6), 785–789.

49. Zhang, Y., Huo, M., Zhou, J., Zou, A., Li, W., Yao, C., Xie, S. (2010). DDsolver: An add-in program for modeling and comparison of drug dissolution profiles. *The AAPS Journal, 12* (3), 263–271.

50. Soares, P. I. P., Sousa, A. I., Silva, J. C., Ferreira, I. M. M., Novo, C. M. M., Borges, J. P. (2016). Chitosan-based nanoparticles as drug delivery systems for doxorubicin: Optimization and modelling. *Carbohydrate Polymers*, *147* 304–312.

51. Wang, Y., Jiang, S., Wang, H., Bie, H. (2017). A mucoadhesive, thermoreversible in situ nasal gel of geniposide for neurodegenerative diseases. *PLoS ONE, 12* (12), e0189478.

52. Parhi, R. Suresh, P. (2015). Alginate-poloxamer beads for controlled release of metoprolol succinate. *Turkish Journal of Pharmaceutical Sciences*, *12* (1), 59–66.

53. Mori, N. M., Patel, P., Sheth, N. R., Rathod, L. V., Ashara, K.C. (2017). Fabrication and characterization of film-forming voriconazole transdermal spray for the treatment of fungal infection. *Bulletin of Faculty of Pharmacy, Cairo University*, *55* (1), 41–51.

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DERLEME MAKALE / REVIEW ARTICLE

HARPAGOPHYTUM PROCUMBENS VE FİTOTERAPİDE KULLANIMI

HARPAGOPHYTUM PROCUMBENS AND USE IN PHYTOTHERAPY

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ÖZ

Amaç: *Bu derlemede, Harpagophytum procumbens ile ilgili genel bilgi verilip analjezik ve antienflamatuvar aktiviteleri üzerinde yapılmış in vitro, in vivo ve klinik çalışma bulguları sunulacaktır.*

Sonuç ve Tartışma: *H. procumbens kökleri Güney Afrika'da geleneksel halk tıbbında, romatizma ve ağrı tedavisinde uzun yıllardan beridir kullanılmaktadır. Günümüzde, standardize edilmiş kök ekstrelerinin (%1 harpagozit içerir) antienflamatuvar etkilere sahip olmasından dolayı osteoartrit hastalarında akılcı fitoterapi uygulamaları kapsamında kullanılmaktadır. Harpagozitin, nükleer faktör-kappa B'yi inhibe ederek lipopolisakkarit kaynaklı indüklenebilir nitrik oksit sentaz ve siklooksijenaz-2 ekspresyonu üzerinde inhibisyon sağlayarak antienflamatuvar etki gösterdiği tespit edilmiştir. Ayrıca standardize ekstreler, kıkırdak degredasyonunda anahtar rol oynayan matriks metalloproteinaz ve elastaz enzimlerini inhibe ederek kondroprotektif etkiler göstermektedir. Osteoartrit hastalarında minimum 2-3 ay kullanılması gerektiği önerilen ekstrenin, herhangi bir ciddi ilaç etkileşmesi oluşturmadığı da belirtilmektedir. Kanıta dayalı fitoterapi uygulamaları açısından; standardize H. procumbens kök ekstrelerinin osteoartritli hastalarda kullanımı ile ilgili yapılmış klinik çalışmalar mevcuttur ve çalışmalar devam etmektedir.*

Anahtar Kelimeler: *Fitoterapi, Harpagophytum procumbens, osteoartrit, şeytan pençesi*

ABSTRACT

Objective: *In this review, general information about Harpagophytum procumbens will be given and findings of in vitro, in vivo and clinical studies on analgesic and anti-inflammatory activities will be presented.*

Result and Discussion: *For many years, H. procumbens roots have been used to treat rheumatism and pain in traditional folk medicine in South Africa. Today, it is used within the scope of rational phytotherapy applications in osteoarthritis patients, since its standardized root extracts (containing 1% harpagozite) have anti-inflammatory effects. It has been found that harpagozite has an anti-inflammatory effect by inhibiting nuclear factor-kappa B, providing inhibition on the expression of lipopolysaccharide-inducible nitric oxide*

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synthase and cyclooxygenase-2. In addition, standardized extracts show chondroprotective effects by inhibiting matrix metalloproteinase and elastase enzymes, which play a key role in cartilage degredation. It is also stated that the extract, which should be used for a minimum of 2-3 months in patients with osteoarthritis, does not cause any serious drug interaction. In terms of evidence-based phytotherapy applications; there are clinical studies on the use of standardized H. procumbens root extracts in patients with osteoarthritis, and studies are ongoing.

Keywords: *Phytotherapy, Harpagophytum procumbens, osteoarthritis, devil's claw*

GİRİŞ

Harpagopyhtum procumbens DC. ex Meisn. (Pedaliaceae), Güney Afrika'nın Kalahari Çölü bölgesinde yetişen, yaygın tıbbi kullanımı olan bir bitkidir [1]. Meyvesinin görünüşü nedeniyle şeytan pençesi olarak adlandırılan *H. procumbens* geleneksel tıpta, artrit ve romatizmal hastalıklarda antienflamatuvar özelliklerinden dolayı kullanılmaktadır [2]. Günümüzde *H. procumbens*'in en sık kullanım alanı osteoartrittir [3].

Kronik hastalıklar arasında yer alan ve dejeneratif eklem hastalığı olarak tanımlanan osteoartrit, sinovyal eklem kıkırdaklarında bozulma, eklem yüzeyleri ve kenarlarında yeni kemik oluşumu ile karakterize kronik, non-enflamatuvar bir eklem hastalığıdır [4]. Radyolojik tanı ile erişkinlerin %33'üne, yaşlıların ise %90'ına osteoartrit teşhisi konulmaktadır [5]. Ağrı bu rahatsızlığın en yaygın semptomudur. Genel olarak; analjezikler, merkezi sinir sistemine veya periferik ağrı mekanizmalarına etki ederek, bilinci önemli ölçüde değiştirmeden ağrıyı hafifletir. Ağrının tedavisi için terapötik ilaçların etkinliği ve güvenliği sınırlıdır. Non-steroidal antienflamatuvar ilaçların (NSAİİ) sıklıkla kullanılması; gastrointestinal lezyonlar, böbrek ve karaciğer yetmezliği gibi olumsuz etkilere neden olabilmektedir [1]. NSAİİ'lerin advers etkilerinin sıklıkla görülmesi nedeniyle alternatif arayışlara ilgi büyüktür [6]. *H. procumbens* ekstreleri, hem antienflamatuvar hem de analjezik etkileri sebebiyle bu arayışta ilgi odağı olmuştur. Bu derlemede, *H. procumbens* kök ekstrelerinin osteoartrit tedavisini desteklemek üzere analjezik ve antienflamatuvar aktiviteleri ile ilgili yapılmış *in vitro, in vivo* ve klinik çalışmalar rapor edilecektir.

Botanik özellikleri

Bitki Türkiye'de yetişmemektedir. En çok Güney yarımkürede Namibya, Botswana, Güney Afrika, Angola'da 15° ile 30° enlemler arasında ve daha az oranda Zambiya, Zimbabwe ve Mozambik'te yetişmektedir. Genellikle, Kalahari Çölü'nün kırmızı kumlu toprakları gibi yıllık yağış miktarı düşük olan (150-500 mm/yıl) bölgelerde yetişmektedir [7].

Harpagophytum procumbens çok yıllık, yabani bir otsu bir bitkidir. Birçok içi boş dallara sahiptir ve sümüksü, yapışkan bir özsu yayan salgı tüyleri ile kaplıdır. Etli bir anaç kökten yayılan

sürünen bir gövdeye sahiptir. Yanal köklerinin üzerinden oluşan yumruları olan etli bir kazık köke sahiptir. Yumrular, 2 m derinliğe kadar inebilen kalıcı primer kökten filizlenir. Sekonder yumrular, 6 cm çapında ve 25 cm uzunluğunda olabilir. Yapraklar büyük ve kalp şeklindedir. Alternan dizilişli yapraklar mavi-grimsi yeşildir ve düzensiz olarak 3-5 loba ayrılmıştır. Çiçekler tüp şeklinde, sarı ve beyaz boğazı olan soluk pembe, kırmızı, mor renklidir. Korolla 25-40 mm çapındadır. Meyve etli bir tabaka ile çevrili odunsu bir iç kısma sahip olan sert bir kapsüldür, 7-20 cm. uzunluğunda, 6 cm. çapındadır. Meyveler, karakteristik olarak uzun, keskin, kıskaç benzeri kancaları olan çıkıntılara ve ayrıca üst yüzeyde iki düz dikene sahiptir. Yaklaşık 50 adet siyah tohum içerir [8-11].

H. procumbens, Devil's Claw (şeytan pençesi) ismi ile bilinmektedir. Ayrıca bitkiye "Grapple plant, Harpago" isimleri de verilmiştir [12]. Bitkinin drog olarak kullanılan kısımları yumrulu sekonder kökleridir ve bu kısmı Avrupa Farmakopesi'nde "Harpagophyti radix" olarak bilinir [13].

Kayıtlı olduğu Farmakope ve Monograflar [13-16]

- African Herbal Pharmacopoeia
- British Herbal Pharmacopoeia
- European Medicines Agency (EMA) Monografları
- European Pharmacopoeia
- European Scientific Cooperative on Phytotherapy (ESCOP) Monografları
- German Comission E
- State Pharmacopoeia of Ukraine (SPhU) 2.0
- World Health Organization (WHO)

Geleneksel Kullanımı

Güney Afrika'daki yerli San ve Khoi halkı, *Harpagophytum procumbens* yumrularını yüzyıllardır ilaç olarak kullanmıştır. Ayrıca Bantu halkının geleneksel bilgi sistemlerinde de kullanımları kayıtlıdır. Watt ve Breyer-Brandwijk (1962) ve Van den Eynden ve arkadaşları (1992), San, Khoi, Bantu Halkı gibi, Afrika'da karışık kökenli topluluklarda bitkinin sekonder yumrularının kaynatılarak veya tentür, toz, özüt şeklinde çok çeşitli sağlık problemleri için kullanıldığını rapor etmiştir. *H. procumbens* Avrupalı bilim insanları tarafından, 1820'de toplanmış ve tanımlanmıştır. Ancak, daha sonra 1900'lerin başlarında G.H. Mehnert, Namibya'daki San ve Nama halklarından bitkinin geleneksel tıbbi kullanımını öğrenmiştir [8,17]. Daha sonra yumrular Almanya'ya götürülerek incelenmiştir. Yumrular 1962 yılından itibaren ihraç edilmeye başlanmış ve Almanya bu yumruları ithal etmiştir [17]. Artrit ve ağrı tedavisinde oral ve topikal kullanımlarına ek olarak, diğer

geleneksel kullanımları arasında; dispepsi, ateş, kan hastalıkları, idrar yolu enfeksiyonu, doğum sonrası ağrı, yara, ülser, çıban sayılabilir. Kuru, toz haline getirilmiş sekonder yumru, doğrudan bir yara sargısı olarak kullanılır; yara ve yanık iyileştirici merhem yapmak için hayvansal yağ veya vazelinlerle de karıştırılabilir [7].

Kimyasal Bileşimi

Köklerde bulunan ana kimyasal bileşikler; iridoid glikozitleri, şekerler (özellikle tetrasakkarit, stakioz), triterpenoitler (oleanolik ve ursolik asit), fitosteroller (beta-sitosterol), aromatik asitler (kafeik, sinnamik ve klorojenik asitler) ve flavonoitlerdir (luteolin ve kemferol) [14].

Başlıca aktif bileşenleri iridoit glikozitleridir. Bunlar; harpagozit ve daha az miktarlarda harpagit ve prokumbittir. *Harpagophytum procumbens* %0.5-3.3 oranında total iridoit glikoziti içerir. Avrupa'daki standart ürünlerde minimum %1.2 harpagozit bulunması istenir [18]. 2002 yılında Boje ve 2003 yılında Munkombwe tarafından yapılan çalışmalarla yeni koaktif bileşenler tanımlanmıştır [19]. Sekonder yumru kökler, primer yumru köklerden yaklaşık iki misli oranda harpagozit içermektedir [20].

H. procumbens kök ekstresinin antienflamatuvar etkisinden harpagozit sorumlu tutulsa da yapılan çalışmalar etkiyi açıklamak için harpagozitin tek başına yeterli olmadığını, etkinin diğer bileşiklerle harpagozitin sinerjist etkileşiminden kaynaklandığını düşündürmektedir [18].

Standardizasyon

ESCOP, köklerin yumruların en az %1.2 oranında harpagozit içermesi gerektiğini bildirir. Standardize ekstreler en az %2.2 total iridoit glikoziti veya %1 harpagozit içermelidir. Sulu ekstre veya çay genellikle %1.1 ila %3.6 arasında harpagozit içerir [20-22].

Antienflamatuvar ve Analjezik Aktivite Çalışmaları

In vitro çalışmalar

İlk *in vitro* çalışma, *H. procumbens*'in belirgin antienflamatuvar/analjezik etkilerinin mekanizmasının aydınlatılmasına yönelik bir araştırmadır. Whitehouse ve arkadaşları (1983) bu çalışmada, *H. procumbens*'in proenflamatuvar enzim olan siklooksijenazı (COX) inhibe etme özelliğini değerlendirmiştir. Bitkinin prostaglandin sentetaz aktivitesi üzerine etkisinin olmadığı bulunmuştur [23].

H. procumbens %80'lik etanolle hazırlanmış kök ekstresinin kalsiyum iyon kanallarını (A23187) uyardığında sisteinil lökotrien (Cys-LT), lökotrien (LTC4, LTD4, LTE4) ve tromboksan B2 (TXB2) sentezlerini baskıladığı kanıtlanmıştır. Çalışmalarda eikozanoid inhibisyonunun,

%80'lik etanollü ekstrenin harpagozit konsantrasyonuna bağlı olduğu ancak ekstre halinde kullanımının tek başına harpagozit kullanımından daha iyi sonuç verdiği görülmüştür. Artritte; bağ dokunun nötrofil elastaz tarafından bozulduğu görülmüştür [24]. 2003 yılında Boje ve arkadaşları tarafından yapılan *in vitro* çalışmada, *H. procumbens*'in doz bağımlı olarak insan lökosit elastazını inhibe ettiği gösterilmiştir [25].

Inaba ve arkadaşlarının 2010 yılında farelerin makrofaj RAW 264.7 hücreleri üzerinde yaptıkları *in vitro* çalışmada bitkinin yumru ekstresi, lipopolisakkarit (LPS) nedenli interlökin (IL-1β, IL-6) ve tümör nekroz faktörü-α (TNF-α) üretimi üzerine etkilerini değerlendirmişlerdir. *H. procumbens*'in enflamatuvar sitokinlerin üretimini inhibe ettiği sonucuna varılmıştır [26].

Mevcut *in vitro* çalışma bulguları göz önüne alındığında, *H. procumbens*'in birçok proenflamatuvar bileşik üzerinde önemli etkileri olduğu açıktır. Bununla birlikte, *H. procumbens* için gözlenen antienflamatuvar/analjezik etkilerden sorumlu mekanizmalarının tam olarak açıklanmasına yardımcı olmak için daha fazla araştırma yapılması gerekmektedir [27].

In vivo ve ex vivo çalışmalar

1992 yılında Lanhers ve arkadaşları, %1.8 harpagozit ihtiva eden standardize kuru *H. procumbens* ekstresinin (50, 100, 200 ve 400 mg/kg) ve harpagozitin (5 ve 10 mg/kg) analjezik aktivitesini fareler üzerinde yaptıkları bir çalışma ile test etmişlerdir. Asetil salisilik asit (68 mg/kg) ve morfin sülfat (1, 15 mg/kg) referans bileşik olarak kullanılmıştır. Hayvanlarda görülen kıvranma ve gerilme reflekslerine karşı en yüksek korumayı; ekstre 400 mg/kg (%78 koruma) dozda; harpagozit ise ağrı uyarımına karşı koruyucu etkiyi 10 mg/kg dozda (400 mg ekstredeki harpagozit miktarının iki misline eşdeğerdir) göstermiştir. Ayrıca intraperitoneal (i.p) yoldan uygulanan %1.5 harpagozit içeren %60'lık *H. procumbens* kuru etanol ekstresinin antinosiseptif etkileri, sıcak plaka testi kullanılarak sıçanlarda incelenmiştir. Akut ve kronik tedavi sonrasında pençe çekme zamanındaki gecikmede artışlar görülmüştür. Yine farelerde zimosan nedenli artirit modelinde, 10 gün boyunca periton içine verilen 20 mg/kg dozda harpagozit; kıkırdak kaybı, kemik rezorpsiyonu ve sinovyal kavite içindeki hücre infiltrasyonunda histolojik skorlarda azalma oluşturmuştur [28].

Üçüncü ve arkadaşları (2015) deneysel romatoid artrit hayvan modelinde, hayvanlara oral olarak 30 gün boyunca *H. procumbens* ekstresi ile kombine bromelain ekstresi, glukozamin hidroklorür, kondroitin sülfat ve metilsülfonilmetan birlikte vermişlerdir. %3 harpagozit içeren ekstre, bu kombinasyonla kıkırdak yıkımını önlemiştir. Bu ajanların farmakolojik etki ilişkisi incelendiğinde, malondialdehit (MDA), nitrik oksit (NO), 8-hidroksiguanin seviyelerini ve proenflamatuvar sitokin gen seviyelerini düşürerek ortaya çıkan oksidatif stres ve enflamasyonu baskıladıkları tespit edilmiştir [29].

Sağlıklı gönüllüler üzerinde 21 gün süren bir *ex vivo* deneme yapılmıştır. %3 iridoglukozit içeren *H. procumbens* ekstre kapsüllerinin (2000 mg/gün) kullanılmasından önce ve sonra eikosanoid üretimi ölçülmüştür. Ölçülen parametrelerin hiçbiri (Prostaglandin E2 (PGE2), TXB2, 6-keto-prostoglandin F1α ve lökotrien B4), *H. procumbens* tedavisinden sonra önemli ölçüde değişmemiştir. Bu bulgular, sağlıklı bireylerde tedavinin, COX veya 5-lipoksijenaz (5-LOX) yoluyla eikosanoid üretimini değiştiremediğini göstermiştir. %9 harpagozit içeren *H. procumbens* ekstresinin eikozanoidlerin sentezi üzerine olan etkisini incelemek amacıyla yapılan *ex vivo* başka bir çalışmada, 14 gün boyunca 600, 1200 ve 1800 mg/kg dozlarda *H. procumbens* ekstresi içeren tabletleri kullanan sağlıklı erkek hastalarda gün içerisinde çeşitli zamanlarda alınan kan örneklerinde bazal ve uyarılmış Cys-LT düzeyinde, 4. saatte %28 ve 8. saatte %58 azalma olduğu görülmüştür. Farmakokinetik araştırmalar, maksimum harpagozit seviyesinin 1.3 ile 2.5 saat sonra elde edildiğini ve serum harpagozit seviyeleri ile LT inhibisyonu arasında bir ilişki olduğunu ortaya çıkarmıştır. Sağlıklı erkeklerde bazal Cys-LT'deki bifazik azalma ile eikosanoid sentezinin azaldığı görülmüştür. Ayrıca sıçanların mezengial hücrelerinde yapılan çalışmada, harpagozit konsantrasyonuna bağlı olarak nitrit ve indüklenebilir nitrik oksit (iNOS) salınımının inhibe edilerek antienflamatuvar etki gösterdiği belirlenmiştir. Yapılan çalışmalar, *H. procumbens*'in proenflamatuvar sitokinleri ve COX-2 enzimini doğrudan inhibe ettiğini göstermiştir [30].

Bitkinin analjezik aktivitesini değerlendirmek için de çeşitli *in vivo* çalışmalar yapılmıştır. Mahomed ve arkadaşları (2004) yaptıkları çalışmada, *H. procumbens* kökünün sulu ekstresini 50-800 mg/kg dozlarda farelere intraperitoneal uygulandıktan sonra termal ve kimyasal nedenli ağrıya karşı belirgin bir analjezik etki gösterdiğini bildirmiştir [22]. *H. procumbens*'in sekonder yumrularından hazırlanmış olan sulu ekstre (50-800 mg/kg), farelerde termal olarak sıcak plaka yöntemi ve kimyasal olarak da asetik asit yöntemiyle nosiseptif ağrı uyarıcılarına karşı önemli analjezik etkiler üretmiştir. Ahmed ve arkadaşları tarafından (2005) yapılan çalışmada, farelere 400 mg/kg dozda sulu *H. procumbens* ekstresi intraperitonal olarak verilmiş ve ağrı nedenli kıvranma sayısının önemli ölçüde azaldığı tespit edilmiştir [31]. Başka bir çalışmada *H. procumbens* ekstresinin, farelerde formalin testinde önemli antinosiseptif etkiler gösterdiği bildirilmiştir. 30-300 mg/kg'lık dozlarda tatbik edilen ekstre, farelerde formalin enjeksiyonu nedenli ağrıda; ağrı sürelerini doza bağlı bir şekilde azaltmıştır. Formalin enjeksiyonunun neden olduğu fare omuriliğindeki nitrit/nitrat içeriğindeki önemli artış *H. procumbens* ekstresi tarafından belirgin ölçüde azaltılmıştır [32]. Lim ve arkadaşları (2014), siyatik sinir ağrı modelinde sıçanlarda nöropatik ağrı, plantar insizyon modeli ile postoperatif ağrı oluşturmuştur. Daha sonra 300 mg/kg dozda *H. procumbens* etanol ekstresi 21 gün boyunca oral olarak verilmiştir, referans olarak intraperitoneal uygulama ile 30 mg/kg naproksen kullanılmıştır. Uygulamadan sonra elde edilen sonuçlar değerlendirildiğinde,

H. procumbens ekstrelerinin, sıçanlarda akut postoperatif ağrı ve kronik nöropatik ağrı durumunda potansiyel analjezik etki oluşturduğu görülmüştür [1]. Parenti ve arkadaşları (2015) 800 mg/kg dozda *H. procumbens* ekstresinin intraperitoneal uygulama ile sıçanlarda karragen nedenli ağrıyı önemli ölçüde azalttığını tespit etmişlerdir [33]. Yine aynı grup 2016 yılında sıçanlarda deneysel olarak oluşturulan nöropatik ağrıda, *H. procumbens* ekstresi ve morfin kombinasyonunun oluşan şiddetli ağrıya karşı sinerjistik bir şekilde analjezi oluşturduğunu belirlemişlerdir [34].

Klinik çalışmalar

Kas-iskelet sistemi ağrılarının tedavisinde *H. procumbens* preparatlarının etkinliğini ve güvenliğini belirlemek amacıyla yapılan klinik çalışmalar giderek artmaktadır. Çalışmaların tasarımı, kullanılan tedavi dozu ve kullanılan ekstraksiyon yöntemi açısından çeşitliliği, izlenen klinik koşullara ek olarak, doğrudan karşılaştırmaların yapılmasını zorlaştırmakta ve sonuç ölçümlerinin nicel değerlendirmesini engellemektedir. Bu faktörlere rağmen, klinik denemeler, bir tedavinin etkinliğini göstermenin en ikna edici aracı olarak kabul edilmektedir. *H. procumbens* denemelerinin birçoğunun farklı olmasına rağmen, bulguları tartışılabilmektedir. Kalça ve dizlerinde osteoartrit, romatoid artikülasyon, bel ağrısı veya çeşitli kas-iskelet sistemi ağrısı olan hastalar klinik çalışmalar için bitkinin etkisinin değerlendirilmesinde kullanılmaktadır [35].

Chrubasik ve arkadaşları (2003) yapmış oldukları derlemede, 20 klinik çalışmayı değerlendirmişlerdir. Bu 20 çalışmadan yalnızca 10 tanesi çift kör, randomize ve kontrollü çalışmadır. Kontrol grubu olarak 8 tanesinde plasebo ve 2 tanesinde NSAİİ (rofecoxib ve diacerein®) kullanılmıştır. Yapılan randomize kontrollü çalışmalardan biri *H. procumbens* preparatı olarak Doloteffin® ve NSAİİ olarak rofecoxib kullanılarak gerçekleştirilmiştir. Kişi sayısının azlığından kesin sonuçlara varılamamıştır. Diğer bir çalışma ise diacerein® kullanılarak yapılmıştır. *H. procumbens* ekstresi en az NSAİİ ilaç olan diacerein® kadar etkili olduğuna dair kanıt bulunmuştur. Günlük dozda en az 50 mg harpagozit içeren ürünlerin daha az miktarda harpagozit içeren ürünlere kıyasla daha iyi olduğu sonucuna varılmıştır [36]. Gagnier ve arkadaşları (2004) 12 kişiden oluşan klinik çalışmada, diz ve kalça osteoartritinin tedavisinde günde 30 mg harpagozitten az harpagozit içeren *H. procumbens*'in etanollü kuru ekstresi için kanıtların sınırlığı olduğu sonucuna varmıştır. 60 mg harpagozit içeren *H. procumbens* tozu kullanımının omurga, kalça ve diz osteoartritinde iyileştirici etkisi oluşturduğu; günlük 100 mg harpagozit içeren *H. procumbens* sulu kuru ekstresinin kronik nonspesifik bel ağrısının akut alevlenmelerinin tedavisinde iyileştirici etkisinin olduğu; 60 mg harpagozit içeren *H. procumbens*'in sulu kuru ekstresinin kısa bir süre içinde kronik nonspesifik bel ağrısı tedavisinde 12.5 mg rofecoxib'e denk etki gösterdiği görülmüştür [37]. Brendler ve

arkadaşları (2006) yaptıkları derlemede *H. procumbens*'in dejeneratif eklem hastalığında, osteoartritte, bel ağrısında etkisinin kanıtlandığını; iştah açıcı ve sindirime yardımcı olma etkisinde ise kanıt olarak gösterilebilecek yeterli veri elde edilemediğini rapor etmiştir. Dejeneratif eklem hastalığı veya osteoartritle ilişkili kısa süreli ağrı yönetiminde kullanım ile ilgili olarak yazarlar *H. procumbens*'in ilaç tedavileri olan NSAİİ'ler kadar etkili olabileceğini beyan etmiştir. Bununla birlikte, mevcut çalışmaların birçoğu metodolojik olarak sınırlı olduğunu ve *H. procumbens*'in etkinliğini ve uzun vadeli güvenliğini belirlemek için 8-12 haftadan uzun süren ek araştırmalar yapılması gerektiğini belirtmek gerekir [38]. Gagnier ve arkadaşları (2007) yaptıkları çalışmada, günde 50 mg veya 100 mg standardize harpagozit içeren *H. procumbens* ekstresi kullanan hastalarda, ağrı tedavisinde günde 12.5 mg rofecoxib kullanan hasta grubunda görülen iyileşme parametrelerini gözlemlemişlerdir [39]. Gagnier'in 2010 yılında yaptığı bir diğer çalışmada ise günde 50 mg ve 100 mg harpagozit içeren *H. procumbens* sulu kuru ekstresinin ağrıyı plasebodan daha fazla azalttığına dair güçlü kanıtlar bulunmuştur [40].

Tablo 1 ve 2'de *H. procumbens* üzerinde yapılan klinik çalışmalar ve sonuçları gösterilmiştir.

Tablo 1. *H. procumbens*'in sırt ağrısı üzerine etkisini göstermek için yapılan çalışmalar

Çalışma özellikleri	Katılımcı Sayısı (n)	Teşhis	Doz ve Süre	Sonuç	Kaynak
Randomize Çift kör Plasebo kontollü	HP: n=54 Plasebo: n=55	Bel ağrısı (en az 6 ay süre ile)	50 mg harpagozit içeren 800 mg kurutulmuş sulu ekstre *tid*,4 hafta	İki grup arasında fark yoktur	[41]
Açık prospektif çalışma	Grup J: n=51 Grup K: n=51	Bel ağrısı (en az 6 ay süre ile)	Grup J: 30 mg harpagozit içeren 600 mg sulu kuru ekstre, *tid* Grup K: Konvansiyonel tedavi, 6 hafta	İki grup arasında fark yoktur	[42]
Randomize Çift kör Plasebo kontrollü	HP 600 mg: n=65 HP 1200 mg: n=66 Plasebo: n=66	Bel ağrısı (en az 6 ay süre ile)	50 mg ya da 100 mg harpagozit içeren 600 mg ya da 1200 mg kurutulmuş sulu ekstre, 4 hafta	Her iki kontrol grubu da plasebo grubuna göre daha iyi yanıt vermiştir	[43]
Randomize Çift kör Plasebo kontrollü çalışma	HP: n=31 Plasebo: n=32	Kas gerginliği, bel ağrısı, omuz/boyun ağrısı	480 mg etanollü kuru HP ekstresi *bid*, 4 hafta	HP'nin etkisi plasebodan daha fazla gözlenmiştir	[44]
Açık Postmarketing çalışma	614 hasta	Dejeneratif kas iskelet sistemi semptomları	480 mg etanollü kuru ekstre, *bid*, 8 hafta	Hastaların %82.7'sinde iyileşme bildirilmiştir	[45]
Randomize Çift kör Pozitif kontrollü	HP: n=44 NSAİİ: n=44	Bel ağrısı (en az 6 ay süre ile)	2400 mg kurutulmuş sulu ekstre (60 mg harpagozit) Kontrol: 12.5 mg/gün Rofecoxib, 6 hafta	İki grup arasında fark yoktur	[46]
1 yıllık takip çalışması	HP: n=38 Rofecoxib: n=35	-	60 mg harpagozit içeren 2400 mg kurutulmuş sulu ekstre, 54 hafta	İki grup arasında fark yoktur	[47]
Randomize Aktif kontrollü tek merkezli çalışma	97 hasta	Spesifik olmayan bel ağrısı	Grup 1: 240 mg etanollü HP ekstresi *bid* Grup 2: 75 mg, *tid*, Diklofenak Grup 3: Günde 1 kere 12.5 mg Rofecoxib, 6 hafta	3 tedavide eşdeğer etkinlik görülmüştür	[48]

HP: *H. procumbens,* bid: günde 2 kere, tid: günde 3 kere

Tablo 2. Dejeneratif eklem hastalığı, osteoartrit ile ilgili ağrının semptomatik tedavisi

Çalışma özellikleri	Katılımcı Sayısı (n)	Teşhis	Doz ve Süre	Sonuç	Kaynak
Açık çalışma	43 hasta	Dejeneratif romatizma	250 mg HP kök tozları içeren kapsül 2 kapsül, tid, 60 gün	Ağrı şiddetinde belirgin bir düşüş ve eklem hareketliliğinde belirgin bir artış	[49]
Açık çalışma	553 hasta	Kronik kas-iskelet sistemi hastalığı	400 mg sulu kuru HP ekstresi kapsülü, *tid*, 4 hafta	Destekleyici olduğu görülmüştür	[50]
Açık çalışma	1026 hasta	Kas-iskelet sisteminin dejeneratif hastalığı	480 mg HP etanollü kuru ekstresi (%60 h/h) *bid*,6 hafta	Destekleyici olduğu görülmüştür	[51]
Çift kör Randomize Çok merkezli çalışma	HP: n=62 Diacerein®: n=60	Osteoartrit	435 mg HP tozu içeren kapsül 2 kapsül, *tid* (57 mg harpagozit) Diacerein®: Günde 100 mg, 4 ay	HP'nin Diacerein®'den daha az etkili olduğu görülmüştür	[52]
Açık çalışma	675 hasta	Osteoartrit	480 mg HP etanollü kuru ekstre (%60 h/h), *bid*, 8 hafta	Destekleyici olduğu görülmüştür	[53]
Gözlemsel çalışma	583 hasta	Diz veya kalça artrozu	HP: 480 mg etanollü kuru ekstre (%60 h/h) *bid*, 6 hafta	Destekleyici olduğu görülmüştür	[54]
Çift kör Plasebo kontrollü çalışma	HP: n=24 Plasebo: n=22	Kalça osteoartriti	HP: 480 mg etanollü kuru ekstre (%60 h/h) içeren tablet, *bid*, 20 hafta	HP tedavisi, ibuprofen kullanım dozunu azaltmıştır	[55]
Açık çalışma, Çok merkezli gözlem çalışması	75 hasta	Kalça ve/veya diz osteoartriti	HP: Günde 50 mg harpagozite eşdeğer 2400 mg sulu kuru ekstre, 12 hafta	Tipik klinik bulgularda iyileşme gözlenmiştir	[56]
Açık çalışma	259 hasta	Artrit ve diğer romatizmal durumlar	HP: 480 mg etanollü kuru ekstre (%60 h/h) *bid*, 8 hafta	Destekleyici olduğu görülmüştür	[57]
Açık çalışma	114 hasta	Spesifik olmayan bel ağrısı veya diz/kalçada osteoartrit ağrısı	400 mg sulu kuru HP ekstresi, Günde 6 tablet (günlük doz 60 mg harpagozit), 54 hafta	Destekleyici olduğu görülmüştür	[58]

HP: *H. procumbens,* h/h: Hacim/hacim, bid: günde iki kere, tid: günde 3 kere

Antienflamatuvar ve Kondroprotektif Etki Mekanizması

Harpagozit, nükleer faktör-kappa B'yi inhibe ederek LPS kaynaklı iNOS ve COX-2 ekspresyonunun da inhibisyonunu sağlayarak antienflamatuvar etki gösterir [59,60]. Bu mekanizmanın, COX-2, lökotrienler, TNF-α ve interlökin-1 (IL-1) gibi enflamatuvar mediatörlerin inhibisyonu yoluyla *H. procumbens*'in analjezik ve kıkırdak koruyucu etkisinden kısmen sorumlu olduğuna inanılmaktadır [61]. En iyi sonuçları veren araştırmalarda, günde 50-100 mg harpagozit içeren standardize *H. procumbens* sulu-alkollü ekstrelerinin kullanıldığı tespit edilmiştir [3].

Kondroprotektif etki için; COX-2, NO, TNF-α, IL-1β gibi enflamasyon mediyatörlerini inhibe etmektedir. Ayrıca kıkırdak degredasyonunda anahtar rol oynayan matriks metalloproteinaz (MMP) ve elastaz enzimlerini inhibe eder [52, 62].

Harpagozit, IL-1β'ye cevap olarak aktivatör protein-1 aktivasyonunu inhibe eder. Böylece, IL-6 ve MMP-13 üretimleri inhibe olur. IL-1β'nin uyardığı osteoartrit kondrositlerinde diğer sitokin/kemokinlerin ekspresyonunu da inhibe eder [63].

Endikasyonları ve Günlük Kullanım Dozları

ESCOP, ağrılı osteoartrit tedavisinde her gün 2-5 g ham drog veya eşdeğer miktar ekstreyi önerir. Bel ağrısı için 4.5-9 g ham drog önerilir. 4.5-9 g ham drogdan hazırlanan ekstre günlük doz olarak kullanıldığında bel ağrısının alevlendiği zamanlarda doz bağımlı olarak etki gösterdiğine dair kanıtlar mevcuttur. Bu nedenle genel olarak osteoartrit ağrılarında ham ekstre dozunu 2-5 g ile sınırlamak için belirgin bir neden görünmemektedir [21].

EMA verilerine göre *H. procumbens,* eklem ağrılarının ve hafif sindirim bozukluklarının giderilmesi ve iştah açıcı olarak kullanılmaktadır. Osteoartrit hastalarında tavsiye edilen dozlarda 4 aya kadar kullanılır [20].

Gebelik ve Laktasyon Döneminde Kullanımı

Yeterli veri olmadığından ve uterusta kasılmaya neden olabileceğinden gebelerin kullanması tavsiye edilmez. Ayrıca yeterli veri bulunmadığından laktasyon döneminde de kullanılması tavsiye edilmemektedir [20].

Çocuklarda ve Ergenlerde Kullanımı

Yeterli veri yoktur.

Advers Etkileri

Klinik çalışmalarda hafif ve seyrek olarak gastrointestinal belirtiler rapor edilmiştir [64]. Bu advers etkilerin ne kadar sıklıkla görülebileceği bilinmemektedir. Bildirilen yan etkiler, bugüne kadar çalışılmış olan doz aralığında, doza bağımlı olmaktan ziyade alerjiktir [19].

İlaç Etkileşimleri

Bu zamana kadar *H. procumbens* için ciddi bir ilaç etkileşimi bildirilmemiştir [20]. Ancak, *H. procumbens*'in farmakolojisi ve etki mekanizması nedeniyle bazı ilaçlarla etkileşimleri olabilir [3]. Kanıtlar *H. procumbens*'in bir COX-2 inhibitörü olduğunu göstermektedir. Bu inhibisyon, NSAİİ'ler veya antikoagülanlar gibi kanama riskini artıran ilaçlarla birlikte alındığında kanama riskini daha da artırabileceği şeklinde olabilir [59]. *H. procumbens* kan şekeri seviyesini düşürebilir bu sebeple bitkinin diğer hipoglisemik ilaçlarla birlikte kullanıldığında dikkatli olunması önerilmektedir [22].

H. procumbens mide asitliğini artırabilir ve bu nedenle mide asit salgısını azaltmak için kullanılan ilaçları etkiyebilir. *H. procumbens* kalp ritmini ve kasılma gücünü etkileyebilir, antiaritmik ilaç veya digoksin kullanan hastalarda dikkatli olunması önerilmektedir [38].

SONUÇ VE TARTIŞMA

Romatizmal hastalıklar, insan hayatını zorlaştıran, hayat kalitesini düşüren rahatsızlıklardır. Bu hastalıkların en önemli bulgularından olan ağrı özellikle de kronik ağrı deneyimlerinin sık yaşanması kişilerin fiziksel, psikolojik ve sosyal olarak iyi olma halini olumsuz yönde etkilemekte, işlevselliğinin bozulmasına, işgücünün düşmesine yol açmaktadır. Ağrıyı tedavi edebilmek için sağlık harcamalarında da ciddi artışlar olmaktadır. Ağrıyı dindirme insanların integratif tıbba yönelim sebeplerinden biridir. Tıbbi bitkisel ürünlerin, koruyucu, günlük rahatsızlıkları iyileştirici, tedaviyi tamamlayıcı ve destekleyici olarak kullanılan en yaygın endikasyonları; eklem, iskelet, kas rahatsızlıklarıdır.

1800'lerden beri geleneksel kullanımı olan *H. procumbens*'in, NSAİİ'lere kıyasla azımsanamayacak derecede etkili olduğu görülmüştür. Advers etkilerinin görülme ihtimali ve şiddeti, NSAİİ'lere göre daha az olması ve uzun yıllardır kullanılıyor olması *H. procumbens*'i osteoartrit nedenli enflamasyon ve ağrı olgularında iyi bir alternatif olarak öne çıkarmaktadır.

Bu derlemede kullanılan literatürlerde sunulan bilimsel verilerden yola çıkılarak;

- ESCOP, *H. procumbens* köklerinin en az %1.2 oranında harpagozit içermesi gerektiğini bildirirken; standardize ekstreler en az %2.2 total iridoit glikoziti veya %1 harpagozit içermelidir. [20, 22]
- Antienflamatuvar etkiyi; harpagozitin nükleer faktör-kappa B'yi inhibe ederek LPS kaynaklı iNOS ve COX-2 ekspresyonu üzerinde inhibisyon oluşturarak gösterdiği tespit edilmiştir [59,60]
- Bu mekanizmanın, COX-2, lökotrienler, TNF-α ve IL-1 gibi enflamatuvar mediatörlerinde inhibe edilmesini sağlayarak kök ekstrelerinin analjezik ve kıkırdak koruyucu etki göstermesine katkı verdiğine inanılmaktadır [61].
- Yine osteoartritte kıkırdak koruyucu etkileri için kıkırdak degredasyonunda anahtar rol oynayan MMP ve elastaz enzimlerini inhibe ettiği *in vitro* çalışmalarla gösterilmiştir [52, 62].

- Bu etkileri gösterebilmesi için standardize ekstrelerin 4 ay süre ile kullanılması gerekmektedir Bu süre sonunda ara verilip 2 ay sonra tekrar kür uygulamasına devam edilmesi akılcı fitoterapi uygulamalarında önerilmektedir [20].

H. procumbens gibi geleneksel tıpta kullanılan bitkilere; osteoartrit hastalarında düşük yan etki oluşturmaları, klinik ve biyokimyasal fayda göstermeleri sebebiyle yönelim gün geçtikçe artmaktadır. Bu yönelim, histolojik değerlendirme de dahil olmak üzere daha fazla araştırmayı gerektirmektedir. Dejeneratif artropatilerin tamamlayıcı tedavisi olarak etkili bitkisel takviyelerin araştırılması karmaşık bir konudur ve çalışmanın düzeni ve protokollerin zorluğu nedeniyle günümüzde yeterli miktarda kaliteli çalışma yoktur. Mevcut araştırmalar bitki ekstrelerinin, hastalığın ilerlemesi üzerindeki etkisini veya artropatilerin ağırlaşmasını durdurup durdurmadığını tam anlamıyla değerlendirememiştir. Bu amaçla, daha uzun süreli çalışmalar yapılmalıdır. Mevcut çalışmaların çoğu, sadece bitkisel tedavi nedenli semptomatik rahatlamanın değerlendirilmesine odaklanmıştır.

Avantajlarına rağmen, bitkisel tedavilerin ilaçlarla olan etkileşimleri, formülasyon sıkıntıları sebebiyle düşük biyoyararlanımları, standardizasyon eksikliği, ulusal ve uluslararası farklı yasal düzenlemeler nedeniyle dikkatle kullanılmaları gerekmektedir. Bitkisel tedavinin; osteoartrit ve romatoid artritin tedavi rehberlerinde yer alabilmesi için tıbbi bitki etkinliği, güvenliği ve etki mekanizmalarına ilişkin daha fazla kanıt gereklidir.

Günümüzde oldukça sık rastlanan osteoartrit semptomlarının rahatlatılmasında, bel ve eklem ağrılarında kanıta dayalı sonuçları sunabilmek için, *H. procumbens* preparatları üzerinde daha uzun süreli, yüksek dozlarda, daha fazla katılımcı ile kontrollü, randomize ve çift körlü tasarlanmış klinik çalışmalara ihtiyaç vardır.

KAYNAKLAR

1. Lim, D.W., Kim, J.G., Han, D., Kim Y.T. (2014). Analgesic effect of *Harpagophytum procumbens* on postoperative and neuropathic pain in rats. *Molecules*, *19*(1), 1060-1068.

2. Serrano, A., Ros, G., Nieto, G. (2018). Bioactive compounds and extracts from traditional herbs and their potential anti-inflammatory health effects. *Medicines*, *5*(3), 76.

3. Sanders, M., Grundmann, O. (2011). The use of glucosamine, devil's claw (*Harpagophytum procumbens*), and acupuncture as complementary and alternative treatments for osteoarthritis. *Alternative Medicine Review*, *16*(3), 228.

4. Gümüş, K., Ünsal, A. (2014). Osteoartritli bireylerin günlük yaşam aktivitelerinin değerlendirilmesi. *Türk Osteoporoz Dergisi, 20*(3), 117-124.

5. Altindağ, Ö., Sırmatel, Ö., Tabur, H. (2006). Diz osteoartriti olan hastalarda Demografik özellikler ve klinik parametrelerle ilişkisi. *Harran Üniversitesi Tıp Fakültesi Dergisi*, *3*(2), 62-66.

6. Moura, M.D.G., Lopes, L.C., Biavatti, M.W., Busse, J.W., Wang, L., Kennedy, S.A., Bhatnaga, N., Bergamaschi, C. (2016). Brazilian oral herbal medication for osteoarthritis: A systematic review protocol. *Systematic Reviews*, *5*(1), 86.

7. Stewart, K.M., Cole, D. (2005). The commercial harvest of devil's claw (*Harpagophytum* spp.) in southern Africa: The devil's in the details. *Journal of Ethnopharmacology*, *100*(3), 225-236.

8. Mncwangi, N., Chen, W., Vermaak, I., Viljoen, A.M., Gericke, N. (2012). Devil's Claw- A review of the ethnobotany, phytochemistry and biological activity of *Harpagophytum procumbens*. *Journal of Ethnopharmacology*, *143*(3), 755-771.

9. Street, R.A., Prinsloo, G. (2012). Commercially important medicinal plants of South Africa: A review. Plant Production. Medicinal plants of South Africa. *Journal of Chemistry*, *2013*, Article ID 205048, 16 pages.

10. Kemper, K.J. (2008). Devil's claw (*Harpagophytum procumbens*). *The Longwood Herbal Task Force and The Center for Holistic Pediatric Education and Research,* 1-11.

11. Devil's claw production guideline. (2015). Directorate: plant production, Department of Agriculture, Forestry and Fisheries Republic of South Africa, November 2015, p. 2-3.

12. Rahimi, A., Razmkhah, K., Mehrnia, M., Mohamadnia, A., Sahebjamee, H., Salehi, S., Akbari Asl, E., Tahmasebi, H., Shandiz, S.A.S., Davouodbeglou, F., Ghasemi, S., Ardalan, N., Ghanbari Kordkandi, Z. (2016). Molecular docking and binding study of harpagoside and harpagide as novel anti-inflammatory and antianalgesic compound from Harpagophytum procumbens based on their interactions with COX-2 enzyme. *Asian Pacific Journal of Tropical Disease, 6*(3), 227-231.

13. Monograph. (2002). In Harpagophyti radix of the European Pharmacopoeia, 4th ed., supplement 4.3, Stuttgart, 2002.

14. Foundational Medicine Review site. Retrieved March 26, 2019, from https://www.foundationalmedicinereview.com/blog/resource/harpagophytum-procumbens-devils-claw/

15. Kriukova, A.I., Vladymyrova, I.M., Gubar, S.M., Kotov, A.G., Kotova, E.E. (2018). Question introduction to the State Pharmacopoeia of Ukraine monograph «Devil's claw root». *Upravlìnnâ, Ekonomìka ta Zabezpečennâ Akostì v Farmacìï, 1*(53), 6-12.

16. Williamson, E., Driver, S., Baxter, K. (2009). Stockley's herbal medicines interactions, Pharmaceutical Press, London, p. 165.

17. Raimondo, D., Newton, D., Fell, C., Donaldson, J.S., Dickson, B. (2005). Devil's claw, *Harpagophytum* spp. in South Africa: Conservation and livelihoods issues. *Traffic Bulletin, 20*(3), 98-112.

18. Anauate, M.C., Torres, L.M., de Mello, S.B.V. (2010). Effect of isolated fractions of *Harpagophytum procumbens* DC (Devil's claw) on COX-1, COX-2 activity and nitric oxide production on whole-blood assay. *Phytotherapy Research, 24*(9), 1365-1369.

19. Chrubasik, S. (2004). Addendum to the Escop monograph on *Harpagophytum* procumbens. *Phytomedicine, 11*(7-8), 691-695.

20. European Medicines Agency site. (1995). Retrieved March 26, 2019, from https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report harpagophytum-procumbens-dc/harpagophytum-zeyheri-decne-radix_en.pdf

21. Anonymous. (2003). Harpagophyti radix. In ESCOP monographs. Thieme Press Stuttgart, New York, pp. 233-240.

22. Mahomed, I.M., Ojewole, J.A. (2004). Analgesic, antiinflammatory and antidiabetic properties of *Harpagophytum procumbens* DC (Pedaliaceae) secondary root aqueous extract. *Phytotherapy Research, 18*(12), 982-989.

23. Whitehouse, L.W., Znamirowska, M., Paul, C.J. (1983). Devil's claw (*Harpagophytum procubens*): no evidence for anti-inflammatory activity in the treatment of arthritic disease. *Canadian Medical Association Journal, 129*(3), 249-251.

24. Loew, D., Möllerfeld, J., Schrödter, A., Puttkammer, S., Kaszkin, M. (2001). Investigations on the pharmacokinetic properties of *Harpagophytum* extracts and their effects on eicosanoid biosynthesis *in vitro* and *ex vivo. Clinical Pharmacology & Therapeutics, 69*(5), 356-364.

25. Boje, K., Lechtenberg, M., Nahrstedt, A. (2003). New and known iridoid-and phenylethanoid glycosides from *Harpagophytum procumbens* and their *in vitro* inhibition of human leukocyte elastase. *Planta Medica, 69*(9), 820-825.

26. Inaba, K., Murata, K., Naruto, S., Matsuda, H. (2010). Inhibitory effects of Devil's claw (secondary root of *Harpagophytum procumbens*) extract and harpagoside on cytokine production in mouse macrophages. *Journal of Natural Medicines, 64*(2), 219-222.

27. McGregor, G., Fiebich, B., Wartenberg, A., Brien, S., Lewith, G., Wegener, T. (2005). Devil's claw (*Harpagophytum procumbens*): An anti-inflammatory herb with therapeutic potential. *Phytochemistry Reviews, 4*(1), 47-53.

28. Lanhers, M. C., Fleurentin, J., Mortier, F., Vinche, A., Younos, C. (1992). Anti-inflammatory and analgesic effects of an aqueous extract of *Harpagophytum procumbens*. *Planta Medica, 58*(2), 117-123.

29. Ucuncu, Y., Celik, N., Ozturk, C., Turkoglu, M., Cetin, N., Kockara, N., Sener, E., Dundar, C., Arslan, A., Dogan, H., Kurt, N., Suleyman, H. (2015). Chondroprotective effects of a new glucosamine combination in rats: gene expression, biochemical and histopathological evaluation. *Life Sciences, 130*, 31-37.

30. Moussard, C., Alber, D., Toubin, M.M., Thevenon, N., Henry, J.C. (1992). A drug used in traditional medicine, *Harpagophytum procumbens*: no evidence for NSAID-like effect on whole blood eicosanoid production in human. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 46*(4), 283-286.

31. Ahmed, M.I., Afifi, M.I., Younos, I.H. (2005). *Harpagophytum procumbens* (Devil's Claw): A possible natural anti-inflammatory agent (An experimental study). *Iranian Journal of Pharmacology and Therapeutics, 4*(1), 54-63.

32. Uchida, S., Hirai, K., Hatanaka, J., Hanato, J., Umegaki, K., Yamada, S. (2008). Antinociceptive effects of St. John's Wort, *Harpagophytum procumbens* extract and grape seed proanthocyanidins extract in mice. *Biological & Pharmaceutical Bulletin, 31*(2), 240-245.

33. Parenti, C., Aricò, G., Chiechio, S., Di Benedetto, G., Parenti, R., Scoto, G. (2015). Involvement of the heme-oxygenase pathway in the antiallodynic and antihyperalgesic activity of *Harpagophytum procumbens* in rats. *Molecules, 20*(9), 16758-16769.

34. Parenti, C., Aricò, G., Pennisi, M., Venditti, A., Scoto, G.M. (2016). *Harpagophytum procumbens* extract potentiates morphine antinociception in neuropathic rats. *Natural Product Research, 30*(11), 1248-1255.

35. Grant, L., McBean, D.E., Fyfe, L., Warnock, A.M. (2007). A review of the biological and potential therapeutic actions of *Harpagophytum procumbens*. *Phytotherapy Research, 21*(3), 199-209.

36. Chrubasik, S., Conradt, C., Black, A. (2003). The quality of clinical trials with *Harpagophytum procumbens*. *Phytomedicine, 10*(6-7), 613-623.

37. Gagnier, J.J., Chrubasik, S., Manheimer, E. (2004). *Harpagophytum procumbens* for osteoarthritis and low back pain: a systematic review. *BMC Complementary and Alternative Medicine, 4*(1), 13.

38. Brendler, T., Gruenwald, J., Ulbricht, C., Basch, E. (2006). Devil's Claw (*Harpagophytum procumbens* DC) an evidence-based systematic review by the natural standard research collaboration. *Journal of Herbal Pharmacotherapy, 6*(1), 89-126.

39. Gagnier, J.J., van Tulder, M.W., Berman, B., Bombardier, C. (2007). Herbal medicine for low back pain: A cochrane review. *Spine, 32*(1), 82-92.

40. Gagnier, J.J. (2010). Evidence based review of natural health products for non-specific low back pain. *The Open Pain Journal, 3*(1), 52-59.

41. Chrubasik, S., Zimpfer, C.H., Schütt, U., Ziegler, R. (1996). Effectiveness of *Harpagophytum procumbens* in treatment of acute low back pain. *Phytomedicine, 3*(1), 1-10.

42. Chrubasik, S. (1997). Teufelskrallenwurzelextrakt, klinisch geprüfte wirksamkeit bei akuten rückenschmerzen. *Der Allgemeinarzt, 19,* 564-568.

43. Chrubasik, S., Junck, H., Breitschwerdt, H., Conradt, C., Zappe, H. (1999). Effectiveness of *Harpagophytum* extract ws 1531 in the treatment of exacerbation of low back pain: a randomized, placebo-controlled, double-blind study. *European Journal of Anaesthesiology, 16*(2), 118-129.

44. Göbel, H., Heinze, A., Ingwersen, M., Niederberger, U., Gerber, D. (2001). Harpagophytum-extrakt Li 174 (teufelskralle) bei der behandlung unspezifischer rückenschmerzen. *Schmerz, 15*(1), 10-18.

45. Kloker, B., Flammersfeld, L., (2003). Rheumatherapie mit teufelskrallenwurzel-trockenextrakt-eine multizentrische studie. *Ärztezeitsch Naturheilverfahren, 44*(2), 108-111.

46. Chrubasik, S., Model, A., Black, A., Pollak, S. (2003). A randomized double-blind pilot study comparing Doloteffin® and Vioxx® in the treatment of low back pain. *Rheumatology, 42*(1), 141-148.

47. Chrubasik, S., Kunzel, O., Thanner, J., Conradt, C., Black, A. (2005). A 1-year follow-up after a pilot study with Doloteffin® for low back pain. *Phytomedicine, 12*(1-2), 1-9.

48. Lienert, A., Ruetten, S., Kuhn, M., Wartenberg-Demand, A. (2005). A randomised, active-controlled, monocentric study of the herbal drug, Devil's claw (*Harpagophytum procumbens*) (ALLYA® tablets), Voltaren® and Vioxx® indicates equal efficacy in the treatment of patients with unspecific lumbar pain. Meeting abstract. 54. Jahrestagung der Norddeutschen Orthopâdenvereinigung e.v. Hamburg 2005, 616-618.

49. Pinget, M., Lecomte, A. (1997). Die wirkung der ‘*’Harpagophytum arkocaps*“ bei degenerativem rheuma. *Naturheilpraxis, 50*, 267-269.

50. Müller, B., Deitelhoff, P., Petrowicz, O. (2000). *Harpagophytum procumbens* ist effizient bei degenerativen erkrankungen des bewegungsapparates. *Natura Medicine, 8*, 21-29.

51. Engel S. (2000). Rivoltan (Li 174) zur behandlung von patienten mit degenarativen erkrankungen des bewegungsapparates. *Deutsche Apothekar Zeitung, 140*, 1369.

52. Chantre, P., Cappelaere, A., Leblan, D., Guedon, D., Vandermander, J., Fournie, B. (2000). Efficacy and tolerance of *Harpagophytum procumbens* versus diacerhein in treatment of osteoarthritis. *Phytomedicine, 7*(3), 177-183.

53. Ribbat, J.M., Schakau, D. (2001). Behandlung chronisch aktivierter Schmerzen am Bewegungsapparat. *Natura Medicine*, *16*, 23-30.

54. Schendel, U.M. (2001). Arthrose-Therapie: verträglich geht es auch. *Der Kassenarzt, 29/30*, 36-39.

55. Frerick, H., Biller, A., Schmidt, U. (2001). A treatment schedule for coxarthrosis: A double-blind study with Devil's claw. *Der Kassenarzt, 5*, 34-41.

56. Wegener, T., Lüpke, N.P. (2003). Treatment of patients with arthrosis of hip or knee with an aqueous extract of Devil's claw (*Harpagophytum procumbens* DC.). *Phytotherapy Research, 17*(10), 1165-1172.

57. Warnock, M., McBean, D., Suter, A., Tan, J., Whittaker, P. (2007). Effectiveness and safety of Devil's Claw tablets in patients with general rheumatic disorders. *Phytotherapy Research, 21*(12), 1228-1233.

58. Chrubasik, S., Chrubasik, C., Künzel, O., Black, A. (2007). Patient-perceived benefit during one year of treatment with Doloteffin®. *Phytomedicine, 14*(6), 371-376.

59. Huang, T.H.W., Tran, V.H., Duke, R.K., Tan, S., Chrubasik, S., Roufogalis, B.D., Duke, C.C. (2006). Harpagoside suppresses lipopolysaccharide-induced iNOS and COX-2 expression through inhibition of NF-kappa B activation. *Journal of Ethnopharmacology, 104*(1-2), 149-155.

60. Kaszkin, M., Beck, K.F., Koch, E., Erdelmeier, C., Kusch, S., Pfeilschifter, J., Loew, D. (2004). Downregulation of iNOS expression in rat mesangial cells by special extracts of *Harpagophytum procumbens* derives from harpagoside-dependent and independent effects. *Phytomedicine, 11*(7-8), 585-595.

61. Chrubasik, J.E., Lindhorst, E., Neumann, E., Gerlach, U., Faller-Marquardt, M., Torda, T., Müller-Ladner, U., Chrubasik, S. (2006). Potential molecular basis of the chondroprotective effect of *Harpagophytum procumbens*. *Phytomedicine, 13*(8), 598-600.

62. Chrubasik, S., Thanner, J., Künzel, O., Conradt, C., Black, A., Pollak, S. (2002). Comparison of outcome measures during treatment with the proprietary *Harpagophytum* extract Doloteffin® in patients with pain in the lower back, knee or hip. *Phytomedicine, 9*(3), 181-194.

63. Haseeb, A., Ansari, M.Y., Haqqi, T.M. (2017). Harpagoside suppresses IL-6 expression in primary human osteoarthritis chondrocytes. *Journal of Orthopaedic Research, 35*(2), 311-320.

64. Brien, S., Lewith, G.T., McGregor, G. (2006). Devil's Claw (*Harpagophytum procumbens*) as a treatment for osteoarthritis: a review of efficacy and safety. *Journal of Alternative and Complementary Medicine, 12*(10), 981-993.

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DERLEME MAKALE / REVIEW ARTICLE

ARONIA SP. MEYVELERİNİN KİMYASAL BİLEŞİMİ VE BİYOLOJİK AKTİVİTELERİ

CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF ARONIA SP. BERRIES

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ÖZ

Amaç: *Aronia türleri bakka tipi (berry) meyvelere sahip olup, yapılarında antosiyaninler başta olmak üzere yüksek miktarda fenolik bileşikler taşır. Zengin kimyasal içeriğinden ve güçlü antioksidan aktivitesinden dolayı fonksiyonel gıda olarak adlandırılmakta, dünya üzerinde kullanımı ve kültürü yaygınlaşmaktadır. Bu derleme ile Aronia meyvelerinin kimyasal bileşimi, biyolojik aktiviteleri ve konuyla ilgili güncel araştırmalar sunulmuştur.*

Sonuç ve Tartışma: *Aronia meyvelerinin geleneksel kullanımı yanında, kimyasal bileşimi, hastalıklardan korunma ve hastalıkların tedavisindeki rolü incelenmiştir. Biyoaktiviteden sorumlu başlıca moleküller (siyanidin türevi glikozitler, flavonoidler, prosiyanidinler ve fenolik asitler gibi fenolik yapıda bileşikler) ve oranları ile ilgili bilgiler verilmiştir. Güçlü antioksidan aktivitesi nedeniyle birçok kronik ve dejeneratif hastalık üzerinde tedavi edici etkisi olduğuna, ayrıca antiinflamatuvar, antidiyabetik, antimutajenik ve kardiyoprotektif aktivite gösterdiğine ilişkin detaylı veriler bulunmaktadır.*

Anahtar Kelimeler: *Antioksidan, antosiyanin, Aronia melanocarpa, biyolojik aktivite, fenolik bileşikler*

ABSTRACT

Objective: *Aronia species have bacca (berry) type fruits and contain high amount of phenolics especially anthocyanins. Due to their rich chemical content and strong antioxidant activity, they are called functional food, their uses and cultivation is becoming widespread in the world. In this review, the data on the chemical composition, biological activity of Aronia fruits, and current researches on the fruits are presented.*

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Result and Discussion: *Besides the traditional use of Aronia fruits, their chemical composition and their role in the treatment and protection of diseases have been presented. Information about percentages and structures of main compounds (phenolic compounds such as cyanidin glycosides, flavonoids, procyanidins, and phenolic acids) which are responsible for bioactivity of berries are mentioned. Detailed data are available on the therapeutic effects of fruits on many chronic and degenerative diseases thanks to the their strong antioxidant activity, Also there are substantial studies regarding anti-inflammatory, antidiabetic, antimutagenic, and cardioprotective activities of Aronia fruits.*

Keywords: *Anthocyanin, antioxidant, Aronia melanocarpa, biological activity, phenolic compounds*

GİRİŞ

Önleyici tedavi yaklaşımlarına olan ilginin artması nedeniyle sağlığı destekleyici doğal kaynaklar ve içerikleri üzerinde yapılan çalışmalara olan ihtiyaç artmıştır. Günlük diyette sıklıkla kullanılan üzümsü meyveler (*Fragaria vesca, Rubus fruticosus, Rubus idaeus, Ribes nigrum, Vaccinium myrtillus, Sambucus nigra* gibi) lezzetli, düşük enerjili, antioksidan etkili, lif ve polifenolik bileşikler açısından zengin meyvelerdir. Üzümsü (kırmızı ve mor meyveler, berry) tipte meyveler kimyasal içerikleri nedeniyle potansiyel antioksidanlardır ve birçok dejeneratif hastalığın korunma ve tedavi süreçlerinde rol alırlar [1].

Rosaceae familyasına ait çalımsı bir bitki olan *Aronia melanocarpa* türünün, meyve üretimi için kültürü yapılmaktadır. *Aronia* türleri, Kuzey Amerika ve Kanada'nın doğu bölgesinde doğal olarak yetişmektedir. Avrupa'ya yayılışı 1900'lerde Almanya'dan Rusya'ya doğru olmuştur. *Aronia* türleri 2-3 metreye kadar büyüyebilen çalımsı bitkiler olup Mayıs-Haziran aylarında çiçeklenirler, meyveleri olgunlaştığında parlak kırmızı veya siyah (6–13mm, 0,5–2 g) renktedir [2].

"Chokeberry" olarak bilinir ve tanınmış iki türü vardır. *Aronia melanocarpa* (black chokeberry) ve *Aronia arbutifolia* (red chokeberry) meyveleri yaygın olarak meyve şurubu, meyve suyu, yumuşak marmelatları, meyve reçelleri, ekstre ve çay üretmek için Avrupa'nın farklı bölgelerinde kullanılmaktadır [2,3].

Aronia meyveleri zengin kimyasal içeriğinden ve yüksek antioksidan aktivitesinden dolayı fonksiyonel gıda olarak adlandırılmakta, dünya üzerinde kullanımı ve kültürü yaygınlaşmaktadır. Önemli derecede antioksidan aktivite gösterdiğinden dolayı birçok kronik ve dejeneratif hastalığın önlenmesinde ve tedavisinde rol almaktadır [4-8].

Antosiyaninlerin sağlık üzerine etkilerine olan ilgi zamanla daha da artmaktadır. Yüksek antosiyanin içeriğine sahip *Aronia* meyveleri de bu etkileri açısından araştırılmış ve insan sağlığı üzerinde, antioksidan ve diğer biyolojik aktivitelerine dayanarak önemli derecede koruyucu ve faydalı etkilere sahip olduğu gösterilmiştir [9].

Aronia meyveleri antosiyanin, flavonol, flavanol, proantosiyanidin, fenolik asitler gibi fenolik bileşikler açısından çok önemli bir kaynaktır [1,4,5]. *Aronia* ağızda belirgin bir büzülme hissine (dokunsal, kuruma ve sıkılaştırma etkisi) neden olur. Bu astrenjan etki içeriğindeki kondanse tanenlerden kaynaklanır [10].

Bitkinin meyvelerinin zengin içerikli bir gıda olarak tüketilmesinin yanında, geleneksel bitkisel ilaç olarak da kullanımları bulunmaktadır. *Aronia* meyveleri Rusya ve bazı Doğu Avrupa ülkeleri başta olmak üzere antihipertansif ve anti-aterosklerotik etkileri nedeniyle kullanılmıştır [11]. *Aronia* meyvelerinin başta antioksidan aktivite olmak üzere, antiinflamatuvar, antidiyabetik, antikanser, antimutajenik, antibakteriyel aktivitesi ile ilgili ayrıca obezite, kardiyovasküler hastalıklar, otoimmün hastalıklar gibi çeşitli hastalıklar üzerine etkisi ve terapötik potansiyeli ile ilgili birçok çalışma yapılmıştır [2,12]. Bu makalede, Türkiye'de ve dünya genelinde fonksiyonel bir besin olarak kullanımı artan *Aronia* meyveleri ile ilgili şu ana kadar yapılmış fitokimyasal çalışmalar ve biyolojik aktivite çalışmaları derlenmiştir. Bu şekilde *Aronia* bitkilerinin kültürünün ve doğrudan gıda olarak veya çeşitli ürünleri şeklinde kullanımının yaygınlaşması sürecinde bir kaynak teşkil etmesi amaçlanmıştır.

Kimyasal Bileşimi

Aronia meyveleri diyet lifi, organik asitler, şeker, yağ protein, mineral (özellikle çinko, potasyum) ve vitaminler (vitamin C) ile birlikte yüksek fenolik içeriğe sahiptir [13].

Meyvelerdeki fenolik bileşiklerin içeriği ve miktarının incelenmesinde tek ve standart bir metot olmaması, araştırmalar sonucunda elde edilen verilerin karşılaştırılması güçlüğünü ortaya çıkarmaktadır. Bu araştırmalar, bazı pratik spektroskopik metodlar ile veya daha gelişmiş HPLC/DAD, HPLC/ESI-MS gibi yöntemler ile yürütülmektedir [14]. Ayrıca meyvelerin kimyasal kompozisyonu ve bu bileşiklerin miktarları; bitkinin yetiştirilme şeklinden, gübrelenmesinden, meyvelerin olgunluğundan, hasat tarihinden, habitatından, saklanma yönteminden ve diğer tüm çevresel koşullardan dolayı değişiklikler gösterebilir [15,16].

Aronia meyveleri diğer "berry" tipi meyvelerden yüksek sorbitol ve polifenol içeriği ile ayrılır [2]. Kurutulmuş *Aronia* meyvelerindeki diyetsel lif içeriği katı hal NMR tekniği ile incelenmiştir. Buna göre total kütlenin % 66,6'sı çözünemeyen lif (lignin, selüloz, hemiselüloz), %5,4'ü çözünebilir lif olmak üzere %72 oranında diyetsel lif içermektedir [17]. Taze meyvelerdeki diyetsel lif oranı ise 5,62 g/100 g şeklindedir [2].

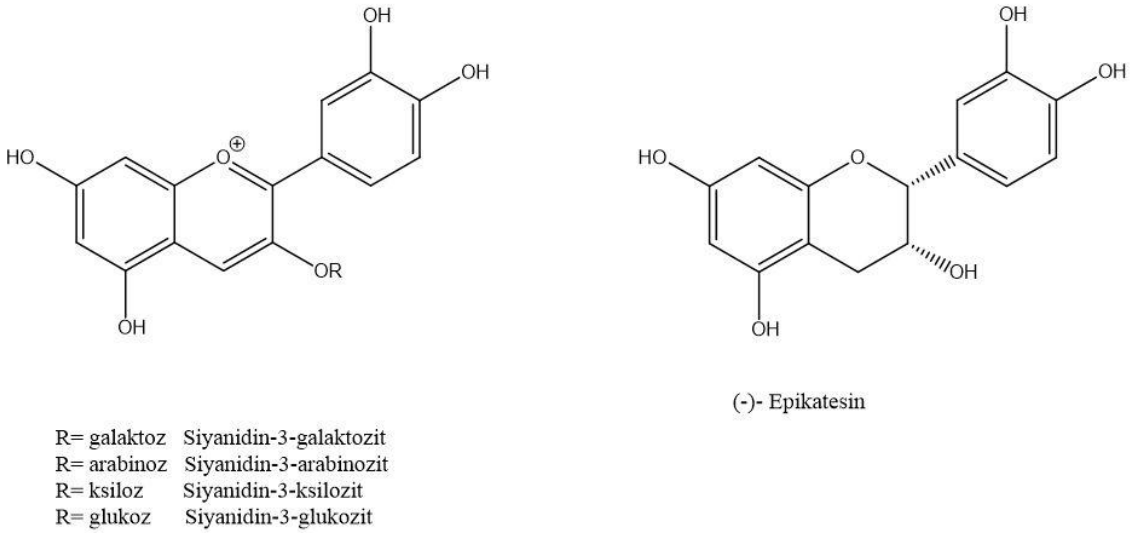
Taze meyvelerdeki indirgen şeker oranı %16–18 arasında bulunmuştur. Başka bir çalışmada glikoz ve fruktoz oranı taze ağırlıkta 13– 17,6 g/100 g arasında bulunmuştur. Taze meyvelerin yağ içeriği 0,14 g/100 g ve protein içeriği 0,7 g/100 g bulunmuştur [2,7]. Meyvelerde ortalama su miktarı

%70-80 dolaylarında olmakla birlikte bitkinin türüne, yetiştiği yere ve yıla göre değişiklikler göstermektedir [18].

Aronia Meyvelerinin Fenolik Bileşenleri

Aronia meyvelerinin içerdiği ve meyvelere rengini veren antosiyaninler başta olmak üzere fenolik bileşikler, bitkinin gösterdiği biyoaktivitelerden sorumlu majör bileşiklerdir. Meyvelerin kimyasal bileşimi incelendiğinde, başlıca (−)-epikateşin olmak üzere proantosiyanidinler *Aronia* meyvelerinin polifenol içeriğinin %66 ile en büyük kısmını oluşturur [19].

Meyvelerdeki siyanidin ve (−)-epikateşin bileşiklerinin kimyasal yapısı **Şekil 1.**'de verilmiştir [2].



Şekil 1. *Aronia melanocarpa* türünün kimyasal bileşiminde bulunan siyanidin glikozitleri ve epikateşin

Siyanidin glikozitleri, flavonoidler, prosiyanidinler, fenolik asitler gibi bitkinin fenolik bileşenleri ve meyvelerdeki oranı üzerine yapılan çalışmalar **Tablo 1.**'de gösterilmiştir.

Tablo 1. *Aronia* meyvelerindeki fenolik bileşikler ve oranları

BİLEŞİK GRUBU	MİKTAR/BİRİM	KULLANILAN METOD	KAYNAK
Total antosiyanin	4341,06±22 SGE mg.kg^{-1} (TA)	pH- diferansiyel metodu	[13]
	460,5±2,9 SGE mg.100 g^{-1} (TA)	pH- diferansiyel metodu	[20]
	4,28 SGE mg.g^{-1} (TA)	pH- diferansiyel metodu	[21]
	1480,0 SGE mg.100g^{-1} (TA)	HPLC-MS/MS metodu	[22]
	4,5 ± 0,20 SGE g·kg^{-1} (TA)	pH- diferansiyel metodu	[23]
	SGE g·kg^{-1} (TA) (kurutulmuş) 3,1 ± 0,1 / 1,4 ± 0,1		
	SGE g·kg^{-1} (TA) (meyve suyu) 0,7 ± 0,01 / 0,4 ± 0,02 / 0,6 ± 0,02		
	SGE g·kg^{-1} (TA) (konsantre) 3,6 ± 0,1		
	SGE g·kg^{-1} (TA) (posa) 10 ± 0,4		
Total polifenol	10637,20±571 GAE mg.kg^{-1} (TA)	Folin-Ciocalteau mikro metodu	[13]
	690,2 ± 8,8 GAE mg.100 g^{-1} (TA)	Folin-Ciocalteau metodu	[20]
	25,56 GAE mg.g^{-1} (TA)	Folin-Ciocalteu metodu	[21]
	GAE g.kg^{-1} (TA) 13,3 ± 0,03	Folin-Ciocalteu metodu	[23]
	GAE g.kg^{-1} (TA) (kurutulmuş) 39,9 ± 0,3 / 50,1 ± 0,4		
	GAE g.kg^{-1} (TA) (meyve suyu) 6,6 ± 0,1 / 6,5 ± 0,03 /6,3 ± 0,04		
	GAE g.kg^{-1} (TA) (konsantre) 29,6 ± 0,1		
	GAE g.kg^{-1} (TA) (posa) 63,1 ± 0,5		
	20,1 GAE mg.g^{-1} (TA)	Folin-Ciocalteu metodu	[22]
Total flavonoid	5,3 ± 0,2 KE g·kg^{-1} (TA)	Kolorimetrik metod	[23]
Siyanidin-3-galaktozit	2794,74± 4,0 mg.kg^{-1} (TA)	HPLC metodu	[13]
	1256,3 ± 11,5 µg.g^{-1} (TA)	HPLC metodu	[21]
	989,7 mg.100 g^{-1} (TA)	HPLC-MS/MS metodu	[22]
	2 917,2 ± 129,3 mg·kg^{-1}(TA)	Ters faz HPLC metodu	[23]
Siyanidin-3-glikozit	121,69±0,1 mg.kg^{-1} (TA)	HPLC metodu	[13]
	16,9 ± 2,5 µg.g^{-1} (TA)	HPLC metodu	[21]
	37,6 mg.100 g^{-1} (TA)	HPLC-MS/MS metodu	[22]
	127 ± 5,4 mg.kg^{-1} (TA)	Ters faz HPLC metodu	[23]
Siyanidin-3-arabinozit	993,77±1,1 mg.kg^{-1} (TA)	HPLC metodu	[13]
	1424,3 ± 18,2 µg.g^{-1} (TA)	HPLC metodu	[21]
	399,3 mg.100 g^{-1} (TA)	HPLC-MS/MS metodu	[22]
	1359,4 ± 0,2 mg.kg^{-1} (TA)	Ters faz HPLC metodu	[23]
Siyanidin-3-ksilozit	146,02±0,3 mg.kg^{-1} (TA)	HPLC metodu	[13]
	469,0 ± 8,6 µg.g^{-1} (TA)	HPLC metodu	[21]
	51,5 mg.100 g^{-1} (TA)	HPLC-MS/MS metodu	[22]
	165,8 ± 1,3 mg.kg^{-1} (TA)	Ters faz HPLC metodu	[23]
Kersetin	71,13±1,5 mg.kg^{-1} (TA)	HPLC metodu	[13]
Kersetin-3-galaktozit	302,4 ± 6,4 µg.g^{-1} (TA)	HPLC metodu	[21]
Kersetin-3-glikozit	273,1 ± 5,7 µg.g^{-1} (TA)	HPLC metodu	[21]
Kempferol	5,30±0,5 mg.kg^{-1} (TA)	HPLC metodu	[13]
Fenolik asit	669,03 mg.100 g^{-1} (KA)	LC-MS, UPLC-PDA-FL	[24]
Kafeik asit	1411,4 ± 14,3 µg.g^{-1}(TA)	HPLC metodu	[21]
(−) Epikateşin	15,04 mg.100 g^{-1}(KA)	HPLC metodu	[19]

SGE: Siyanidin-3-glikozit ekivalanı, GAE: gallik asit ekivalanı, KE: kateşin ekivalanı, TE: trolox ekivalanı, KA: kuru ağırlık, TA: taze ağırlık

Biyoyararlanım

Aronia meyvelerinin sindirimi sırasında, antosiyaninler hızla mikrobiyal katabolizasyona uğrar. Antosiyaninler arasından siyanidin-3-O-galaktozit hızlı bir şekilde peonidin-3-O-galaktozite

metabolize edilir. Katabolizasyon ürünleri biyoyararlanım sırasında, plazma ve idrarda antosiyaninlerin yaklaşık 10 katı kadar olur. Antosiyaninler ve diğer polifenol katabolitleri ile plazma ve idrarda 1,0 ila 6,33 saat arasında bir t_{max} ile yoğun şekilde metabolize edilir [25].

Farmakolojik Aktivite

Antioksidan Aktivite

Serbest radikaller vücudun normal enerji metabolizması sonucunda veya çeşitli çevresel etkenlere (radyasyon, sigara kullanımı, çeşitli ilaçlar, çevre kirliliği) bağlı olarak üretilebilir. Bu reaktif oksijen ve azot türleri vücudun çeşitli fonksiyonları için gereklidir. Ancak bu radikallerin aşırı üretiminde veya vücut tarafından elimine edilemediği durumlarda, vücuttaki doğal denge bozulur ve oksidatif stres meydana gelir. Oluşan oksidatif stres; kanser, otoimmün hastalıklar, yaşlanma, katarakt, kardiyovasküler ve nörodejeneratif (Alzheimer Hastalığı) hastalıklar gibi önemli kronik ve dejeneratif hastalıklara neden olabilir [26].

Bu tür hastalıklardan korunmada ve oluşan hasarın düzeltilmesinde antioksidan etkili bileşikler önemli rol oynar. Antioksidan aktivitenin belirlenmesi için radikal süpürme kapasitesinin ölçülmesine ve metal indirgeme etkisinin ölçülmesine dayanan çeşitli *in vitro* metotlar geliştirilmiştir.

Antosiyaninler, flavonoidler gibi fenolik bileşiklerce zengin olan *Aronia melanocarpa* türünün meyveleri bu açıdan değerlendirilmiş ve önemli derecede antioksidan aktiviteye sahip olduğu yapılan çalışmalarda gösterilmiştir (**Tablo 2)**.

Tablo 2: Antioksidan aktivite deneyleri

METOT	**SONUÇ**	**KAYNAK**
DPPH Serbest Radikal Süpürücü Aktivitesi	1,8 ± 0,3 (EC_{50})	[20]
	11,3 ± 0,5 $g \cdot kg^{-1}$ (TE)	[23]
CUPRAC	67,7 $g \cdot kg^{-1}$ (TE)	[23]
ABTS	11 ± 0,04 $g \cdot kg^{-1}$ (TE)	[23]
ORAC	160,2 $\mu mol.g^{-1}$ (TE)	[21]
Ferric Reducing Antioxidant Power (FRAP)	36,64 ± 0,01 mM .100 g^{-1} (TE),(KA)	[24]
Lipofilik $ORAC_{FL}$ metodu	2,42 $\mu mol.g^{-1}$ (TE)	[22]
Hidrofilik $ORAC_{FL}$ metodu	158,2 $\mu mol.g^{-1}$ (TE)	[22]

EC_{50}: Başlangıçtaki DPPH• radikalinin 50%'sini giderecek meyve miktarının miligram cinsinden değeri

DPPH: 1,1-Difenil-2-pikrilhidrazil, CUPRAC: Bakır (II) indirgeme esaslı antioksidan kapasite, ORAC : Oksijen radikal absorbans kapasitesi, FRAP: Demir (III) iyonu indirgeyici antioksidan güç

TE: trolox ekivalanı, KA: kuru ağırlık, TA: taze ağırlık

İmmünomodülatör Aktivite

İnflamasyon vücuttaki değişikliklere, oluşan hasara ve vücut homeostazisini etkileyebilecek etkenlere karşı verilen bir yanıt, doğal savunma mekanizmasıdır. Makrofaj hücreleri üzerindeki tool like reseptörler (TLR) tarafından patojen endotoksini veya lipopolisakkarit yapısının tanınması sonrasında inflamatuvar gen ilişkili nükleer faktörü kappa B (NF-κB) transkripsiyon faktörü salınır. NF-κB, inflamayon ve immün yanıt, hücresel stres reaksiyonları, karsinojenez, hücre sağkalımı ve apoptoz gibi birçok fizyolojik ve patolojik süreçte rol oynar.

İmmün sitemin uyarılması ile interlökinler (IL), tümör nekrozis faktör (TNF)-α, reaktif oksijen türleri (ROS), nitrik oksit (NO), prostaglandinler (PG) gibi bir takım proinflamatuvar medyatörler salınır ve inflamasyon süreci devam eder. Bu medyatörlerin aşırı veya kontrolsüz üretimi kanser, Alzheimer, otoimmün hastalıklar ve diğer kronik ve dejeneratif hastalıklarda rol oynar. İnflamatuvar hastalıklarda kullanılan non-steroid antiinflamatuvar ilaçların neden olduğu yan etkiler nedeniyle, daha az yan etki gösteren doğal kaynaklı, fenolik yapıdaki antiinflamatuvar bileşiklere olan ilgi artmıştır [27].

Aronia meyveleri ve polifenollerinin primer C57/BL6 fare splenosit hücreleri üzerindeki antiinflamatuvar aktivite çalışması ile *A. mitschurinii* ekstresinin 125 μg gallik asit ekivalanı/ml'de, *A. arbutifolia* ve *A. prunifolia* ekstrelerinin daha düşük konsantrasyonlarda, lipopolisakkarit (LPS) ile stimüle edilmiş IL-6'yı inhibe ettiği gösterilmiştir [28]. Ayrıca yapılan çalışmalarda, antiinflamatuvar aktivitenin bitkinin çoğunlukla flavonoid ve fenolik asit içeriğinden kaynaklandığı belirlenmiştir. Aktiviteden başlıca sorumlu bileşiklerin kersetin, siyanidin 3-arabinozit ve hidroksisinnamik asit türevi bileşikler olduğu belirtilmiştir. [28, 29].

Yapılan bir diğer araştırma, *Aronia* konsantresinin, insan periferik monositlerinde TNF-α, IL-6 ve IL-8 salınımını ve RAW264.7 makrofaj hücrelerinde NF- κB yolağını inhibe ettiğini göstermiştir. Ayrıca, *Aronia* meyvelerinin NF-κB aktivasyonunda, sitokin salınımının ve PGE2 sentezinin inhibisyonunda sodyum selenit ile sinerjize olduğu belirtilmiştir [30].

A. melanocarpa türünden izole edilmiş proantosiyanidin bileşiklerince zengin fraksiyonlar ve prosiyanidin C1, B5, B2 ve antosiyaninler, komplement modülatör aktiviteleri, LPS-indüklenmiş RAW 264.7 makrofajların nitrik oksit üretimini inhibisyon aktiviteleri ve hücre canlılık testleri açısından incelenmiştir. Buna göre, siyanidin, prosiyanidin B2, B5, C1 ve proantosiyanidin bileşiklerince zengin fraksiyonlar kompleman fiksasyon testinde oldukça aktif çıkmıştır. Ayrıca, oligomerik prosiyanidinler, hücre proliferasyonunu etkilemeden murin RAW 264.7 makrofajlarında LPS ile indüklenen NO üretimi üzerinde doza bağlı inhibitör etkiler göstermiştir [31].

Obezite ve Diyabet Üzerine Etki

Aronia meyvelerinin, içerdikleri antiinflamatuvar ve antioksidan bileşenlerden dolayı hiperglisemiye bağlı oksidatif stresin ve bunun sonucunda ortaya çıkan komplikasyonların azaltılmasında faydalı etkileri olduğu gösterilmiştir [32].

Yine *Aronia* meyve ekstresinin, insülin sinyali, adipogenez ve inflamasyon ile ilişkili çoklu yolları modüle ederek insülin direnciyle ilgili risk faktörlerini azalttığı belirlenmiştir [33].

Pankreatik α-amilaz, α-glikozidaz, lipaz gibi enzimler sindirim sırasında, kompleks moleküllerin daha küçük sindirilebilir forma dönüştürülmesinde önemli rol oynar. *Aronia* ekstreleri ve etkili bileşikleri, bu enzimlerin inhibisyonu ile, besinlerin biyoyararlanımını ve enerji değerini düşürdüğünden dolayı diyabet ve obezite tedavisinde etkili bir yaklaşım teşkil eder [34,35].

Yapılan klinik bir çalışma ile *Aronia* meyve suyunun Tip- 2 diyabetli hastaların açlık kan şekerini düşürdüğü gösterilmiştir [36].

Bir diğer çalışmada ise intraperitonal streptozotosin (50 mg/kg) uygulanarak sıçanlarda diyabet modeli oluşturulmuş, *Aronia melanocarpa* meyve suyu 10 ve 20 ml/kg dozlarda, sağlıklı ve hasta sıçanlara gavaj yolu ile uygulanarak deneklerin kan glikoz seviyeleri ve obezite parametreleri incelenmiştir. Diyabetik sıçanlarda plazma glikozu ve trigliseritinde streptozotosine bağlı anormalliklerde önemli ölçüde azalma görülmüştür. Diabetes mellitus ve diyabetle ilişkili komplikasyonların önlenmesi ve kontrolünde faydalı olabileceği belirlenmiştir [37].

Aronia meyve suyu verilen diyabet modelli KK-Ay farelerde, plazma glikozu, vücut ağırlığı, beyaz adipoz doku azalmıştır. Ayrıca DPP IV inhibisyonu sağlanmıştır. Bu inhibisyon glikoz ilişkili insülin sekresyonunun arttırılması, gastrik boşalmanın yavaşlaması ve postprandiyal glukagon ve gıda alımının azaltılması gibi yollarla tip-2 diyabet tedavisinde önemli bir yaklaşım teşkil eder [38].

Kardiyoprotektif Aktivite

Aronia meyveleri, lipit metabolizması, peroksidasyon, iltihaplanma süreci, pıhtılaşma ve oksidasyon üzerindeki çoklu aktiviteler nedeniyle kardiyoprotektif etkiler gösterir [39].

Metabolik sendromlu hastalar ile yapılan bir çalışmada *Aronia melanocarpa* ekstresinin kullanımı ile total kolesterol (TC), düşük yoğunluklu lipoprotein kolesterolün (LDL-C), ve trigliserit (TG) seviyelerinde belirgin düşüş kaydedilmiştir**.** 1 aylık ekstre kullanımı sonucunda platelet agregasyonunda önemli derecede inhibisyon görülmüş ve ayrıca pıhtı oluşumunda ve fibrinolizde azalma gözlemlenmiştir [40].

Aronia meyve suyunun arteriyel kan basıncı ve lipid parametleri üzerindeki etkilerini araştırmak üzere yapılan çalışmada, düzenli *Aronia* suyu içilmesinin, toplam kolesterol seviyesinin,

LDL kolesterolün ve trigliseritlerin azalmasına ve yüksek yoğunluklu lipoprotein 2 (HDL2) kolesterolünün artmasına neden olduğu gösterilmiştir [41].

Aronia ekstresinin Caco-2 hücrelerinde doza bağlı bir şekilde, kolesterol akışında rol alan genlerin ekspresyonunu etkilediği ve bir şekilde hücresel kolesterolün bağırsak lümenine akışını teşvik ettiği bulunmuştur. Bu çalışma ile ekstrenin hipolipidemik etkileri, en azından kısmen LDL türevi kolesterolün apikal atımının artmasına ve bağırsakta azalmış şilomikron oluşumuna bağlanabileceği ve sirtuinin spesifik izoformlarının bu süreçte önemli bir rol oynayabileceği düşünülmektedir [42].

Miyokard enfarktüsü geçirmiş en az altı ay statin tedavisi gören hastalar ile yapılan çalışmada, hastalarda 6 hafta *Aronia* ekstresi kullanımı sonrasında, kontrole karşı, statinlerden bağımsız olarak inflamasyon şiddetinde azalmaya sebep olduğu gösterilmiştir. Klinikte iskemik kalp hastalığının ikincil korumasında kullanılabileceği belirtilmiştir [43].

Bir diğer çalışmada *A. melanocarpa* meyve zengin ekstreleri, yalnızca kardiyovasküler risk faktörleri olan hastalarda süperoksit üretiminde önemli bir konsantrasyona bağlı azalmaya neden olurken, kontrol grubunda herhangi bir etki gözlenmemiştir.

A. melanocarpa meyve ekstreleri, her iki çalışma grubunda da konsantrasyona bağlı olarak önemli bir antiagregan etki gösterilmiş ve bu etkilerin süperoksit üretimini üzerindeki düzenleyici etkiden bağımsız olabileceğini gösterilmiştir [44].

Antosiyaninle zenginleştirilmiş ekstrelerin domuz koroner arterlerinde endotel bağımlı gevşemeyi sağladığı gösterilmiştir. Buna göre *Aronia* ekstresi gibi polifenolce zengin ekstrelerin, vasküler hastalıklarda önemli faydalı etkilere sahip olabileceğini düşünülmektedir [45].

Antikanser ve Antimutajenik Aktivite

Yapılan birçok in *vitro* ve hayvan deneyi çalışmaları, *Aronia* meyvelerinin ve ekstrelerinin başta kolon kanseri olmak üzere anti-proliferatif veya koruyucu etkilerini göstermektedir [2].

Aronia melonocarpa meyvelerinden yarı saflaştırılmış antosiyanin bakımından zengin ekstresinin normal kolon ve kolon kanseri hücre hatları üzerindeki etkisi araştırıldığında, 50 mg monomerik antosiyanin / ml Aronia ekstresine 24 saatlik maruz kalma sonucu, insan HT-29 kolon kanseri hücrelerinde % 60 büyüme inhibisyonu gözlenmiştir. Hücrelerin, hücre döngüsünün G1/G0 ve G2/M fazlarında bir blokaj gözlemlenmiştir. Kolon karsinogenezinde koruyucu etkileri ve çoklu etki mekanizmaları belirlenmiştir [46,47]. Sitostatik etkide antosiyaninlerin kimyasal yapısının önemli rol oynadığı belirtilmiştir [48].

Aronia melanocarpa meyvelerinden izole edilmiş antosiyaninlerin, Ames testi ile antimutajenik aktivitesi olduğu belirlenmiştir. Bu aktivitenin, esas olarak serbest radikalleri süpürücü

aktivitesi yanı sıra promutajenleri aktive eden ve mutajenleri DNA-reaktif türevlere dönüştüren enzimlerin inhibisyonu yoluyla oluştuğu düşünülmektedir [49].

Diğer Aktiviteler

Bitkinin ayrıca hepatoprotektif, gastroprotektif, antibakteriyel, antiviral aktiviteleri ile ilgili çalışmalar yapılmıştır [2,11].

SONUÇ VE TARTIŞMA

Sonuç olarak günümüzde kullanımı yaygınlaşan *Aronia melanocarpa* başta olmak üzere *Aronia* türleri üzerine yapılan çalışmalar göstermiştir ki; *Aronia* meyvelerinin yüksek fenolik içeriğinden dolayı ve potansiyel terapötik faydaları nedeniyle fonksiyonel bir gıda olarak kullanımı önem kazanmaktadır. Antosiyanin, prosiyanidin, fenolik asit bileşikleri farmakolojik aktiviteden sorumlu başlıca bileşiklerdir. Güçlü antioksidan aktivitesi nedeniyle sağlığı destekleyici etkileri yapılan birçok araştırma ile gösterilmiştir. Antidiyabetik, kardiyoprotektif, antimutajenik aktiviteleri, obezite, çeşitli inflamatuvar ve kronik hastalıklardaki olumlu etkileri belirlenmiştir. Güncel kullanımına ek olarak, klinikte koruyucu ve tedavi edici etkileri nedeni ile kullanımının yaygınlaşması için, bitkinin kimyasal bileşimi ve biyoaktivitesi üzerine yapılan çalışmaların artarak devam etmesi gerekmektedir.

KAYNAKLAR

1. Beattie, J., Crozier, A., Duthie, G. (2005). Potential health benefits of berries. *Current Nutrition & Food Science* 1: 71-86

2. Kulling, S.E., and Rawel, H.M. (2008). Chokeberry (*Aronia melanocarpa*) - A review on the characteristic components and potential health effects. *Planta Medica*, *74*(13), 1625–1634. https://doi.org/10.1055/s-0028-1088306

3. Chrubasik, C., Li, G., & Chrubasik, S. (2010). The clinical effectiveness of chokeberry: A systematic review. *Phytotherapy Research*, *24*(8), 1107–1114. https://doi.org/10.1002/ptr.3226

4. Oszmiański, J., & Lachowicz, S. (2016). Effect of the production of dried fruits and juicefrom chokeberry (*Aronia melanocarpa* L.) on the content and antioxidative activity of bioactive compounds. *Molecules*, 21, 1098.

5. Tolić, M. T., Krbavčić, I. P., Vujević, P., Milinović, B., Jurčević, I. L., & Vahčić, N. (2017). Effects of weather conditions on phenolic content and antioxidant capacity in juice of chokeberries (*Aronia melanocarpa* L.). *Polish Journal of Food and Nutrition Sciences*, 67,67–74.

6. Sidor, A., Drożdżyńska, A., & Gramza-Michałowska, A. (2019). Black chokeberry (*Aronia melanocarpa*) and its products as potential health-promoting factors - An overview. *Trends in Food Science and Technology*, *89*(March 2018), 45–60. https://doi.org/10.1016/j.tifs.2019.05.006

7. Tanaka T., & Tanaka A. (2001) Chemical components and characteristics of black chokeberry. *Journal of the Japanese Society For Food Science and Technology*; 48: 606–610

8. Chrubasik, C., Li, G., & Chrubasik, S. (2010). The clinical effectiveness of chokeberry: A systematic review. *Phytotherapy Research*, *24*(8), 1107–1114. https://doi.org/10.1002/ptr.3226
9. Konić-Ristić, A., Šavikin, K., Zdunić, G., Janković, T., Juranic, Z., Menković, N., & Stanković, I. (2011). Biological activity and chemical composition of different berry juices. *Food Chemistry*, *125*(4), 1412–1417. https://doi.org/10.1016/j.foodchem.2010.10.018

10. Bajec, M. R., & Pickering, G. J. (2008). Astringency: Mechanisms and Perception. *Critical Reviews in Food Science and Nutrition*, *48*(9), 858–875. https://doi.org/10.1080/10408390701724223

11. Kokotkiewicz, A., Jaremicz, Z., & Luczkiewicz, M. (2010). Aronia plants: a review of traditional use, biological activities, and perspectives for modern medicine. *Journal of Medicinal Food*, *13*(2), 255–269. https://doi.org/10.1089/jmf.2009.0062

12. Jurikova, T., Mlcek, J., Skrovankova, S., Sumczynski, D., Sochor, J., Hlavacova, I., Snopek, l., Orsavova, J. (2017). Fruits of black chokeberry *Aronia melanocarpa* in the prevention of chronic diseases. *Molecules*, *22*(6), 1–23. https://doi.org/10.3390/molecules22060944

13. Jakobek, L., Šeruga, M., Medvidović-Kosanović, M., Novak, I., & Strossmayer, J. J. (2007). Antioxidant activity and polyphenols of *Aronia* in comparison to other berry species. *Agriculturae Conspectus Scientificus*, *72*(4), 301–306. Retrieved from https://pdfs.semanticscholar.org/b5a2/cd69aca19e5f3efae06f5b10497b8585a47e.pdf

14. Krenn, L., Steitz, M., Schlicht, C., Kurth, H., & Gaedcke, F. (2007). Anthocyanin- and proanthocyanidin-rich extracts of berries in food supplements--analysis with problems. *Die Pharmazie*, 62(11), 803–812.

15. Jeppsson, N., & Johansson, R. (2000). Changes in fruit quality in black chokeberry (*Aronia melanocarpa*) during maturation. *Journal of Horticultural Science and Biotechnology*, *75*(3), 340–345. https://doi.org/10.1080/14620316.2000.11511247

16. Skupień, K., & Oszmiański, J. (2007). The effect of mineral fertilization on nutritive value and biological activity of chokeberry fruit. *Agricultural and Food Science*, *16*(1), 46–55. https://doi.org/10.2137/145960607781635822

17. Wawer, I., Wolniak, M., & Paradowska, K. (2006). Solid state NMR study of dietary fiber powders from aronia, bilberry, black currant and apple. *Solid State Nuclear Magnetic Resonance*, *30*(2), 106–113. https://doi.org/https://doi.org/10.1016/j.ssnmr.2006.05.001

18. Brand, M. H., Connolly, B. A., Levine, L. H., Richards, J. T., Shine, S. M., & Spencer, L. E. (2017). Anthocyanins, total phenolics, ORAC and moisture content of wild and cultivated dark-

fruited *Aronia* species. *Scientia Horticulturae*, *224*(November), 332–342. https://doi.org/10.1016/j.scienta.2017.06.021

19. Oszmiański, J., & Wojdylo, A. (2005). Aronia melanocarpa phenolics and their antioxidant activity. *European Food Research and Technology*, *221*(6), 809–813. https://doi.org/10.1007/s00217-005-0002-5

20. Benvenuti, S., Pellati, F., Melegari, M., & Bertelli, D. (2006). Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus, Ribes, and Aronia*. journal of food science, 69(3), FCT164–FCT169. https://doi.org/10.1111/j.1365-2621.2004.tb13352.x

21. Zheng, W., & Wang, S. Y. (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *Journal of Agricultural and Food Chemistry*, *51*(2), 502–509. https://doi.org/10.1021/jf020728u

22. Wu, X., Gu, L., Prior, R. L., & McKay, S. (2004). Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes, Aronia,* and *Sambucus* and their antioxidant capacity. *Journal of Agricultural and Food Chemistry*, *52*(26), 7846–7856. https://doi.org/10.1021/jf0486850

23. Kapci, B., Neradová, E., Čížková, H., Voldřich, M., Rajchl, A., & Capanoglu, E. (2013). Investigating the antioxidant potential of chokeberry (*Aronia melanocarpa*) products. *Journal of Food and Nutrition Research*, *52*(4), 219–229.

24. Teleszko, M., & Wojdyło, A. (2015). Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. *Journal of Functional Foods*, *14*, 736–746. https://doi.org/10.1016/j.jff.2015.02.041

25. Xie, L., Lee, S. G., Vance, T. M., Wang, Y., Kim, B., Lee, J.-Y., Chun, O.K., & Bolling, B. W. (2016). Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract. *Food Chemistry*, *211*, 860–868. https://doi.org/10.1016/J.FOODCHEM.2016.05.122

26. Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science*, *4*(2), 89–96.

27. Ambriz-Pérez, D. L., Leyva-López, N., Gutierrez-Grijalva, E. P., & Heredia, J. B. (2016). Phenolic compounds: Natural alternative in inflammation treatment. A Review. *Cogent Food & Agriculture*, *2*(1). https://doi.org/10.1080/23311932.2015.1131412

28. Martin, D. A., Taheri, R., Brand, M. H., Draghi, A., Sylvester, F. A., & Bolling, B. W. (2014). Anti-inflammatory activity of aronia berry extracts in murine splenocytes. *Journal of Functional Foods*, *8*, 68–75. https://doi.org/10.1016/J.JFF.2014.03.004

29. Xu, J., & Mojsoska, B. (2013). The immunomodulation effect of aronia extract lacks association with its antioxidant anthocyanins. *Journal of Medicinal Food*, *16*(4), 334–342. https://doi.org/10.1089/jmf.2012.0151

30. Appel, K., Meiser, P., Millán, E., Collado, J. A., Rose, T., Gras, C. C., … Muñoz, E. (2015). Chokeberry (*Aronia melanocarpa* (Michx.) Elliot) concentrate inhibits NF-κB and synergizes with selenium to inhibit the release of pro-inflammatory mediators in macrophages. *Fitoterapia*, *105*, 73–82. https://doi.org/10.1016/J.FITOTE.2015.06.009

31. Ho, G. T. T., Bräunlich, M., Austarheim, I., Wangensteen, H., Malterud, K. E., Slimestad, R., & Barsett, H. (2014). Immunomodulating activity of *Aronia melanocarpa* polyphenols. *International Journal of Molecular Sciences*, *15*(7), 11626–11636. https://doi.org/10.3390/ijms150711626

32. Banjari, I., Misir, A., Šavikin, K., Jokić, S., Molnar, M., De Zoysa, H. K. S., & Waisundara, V. Y. (2017). Antidiabetic Effects of *Aronia melanocarpa* and Its Other Therapeutic Properties. *Frontiers in Nutrition*, *4*(November), 1–6. https://doi.org/10.3389/fnut.2017.00053

33. Qin, B., & Anderson, R. A. (2012). An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet. *British Journal of Nutrition*, *108*(4), 581–587. https://doi.org/10.1017/S000711451100599X

34. Worsztynowicz, P., Napierała, M., Białas, W., Grajek, W., & Olkowicz, M. (2014). Pancreatic α-amylase and lipase inhibitory activity of polyphenolic compounds present in the extract of black chokeberry (Aronia melanocarpa L.). *Process Biochemistry*, *49*(9), 1457–1463. https://doi.org/10.1016/j.procbio.2014.06.002

35. Bräunlich, M., Slimestad, R., Wangensteen, H., Brede, C., Malterud, K. E., & Barsett, H. (2013). Extracts, anthocyanins and procyanidins from *Aronia melanocarpa* as radical scavengers and enzyme inhibitors. *Nutrients*, *5*(3), 663–678. https://doi.org/10.3390/nu5030663

36. Banjari, I., Misir, A., Šavikin, K., Jokić, S., Molnar, M., De Zoysa, H. K. S., & Waisundara, V. Y. (2017). Antidiabetic effects of *Aronia melanocarpa* and ıts other therapeutic properties. *Frontiers in Nutrition*, *4*(November), 1–6. https://doi.org/10.3389/fnut.2017.00053

37. Valcheva-Kuzmanova, S., Kuzmanov, K., Tancheva, S., & Belcheva, A. (2007). Hypoglycemic and hypolipidemic effects of *Aronia melanocarpa* fruit juice in streptozotocin-induced diabetic rats. *Methods and Findings in Experimental & Clinical Pharmacology*, 29(2), 101–105. https://doi.org/10.1358/mf.2007.29.2.1075349

38. Yamane, T., Kozuka, M., Konda, D., Nakano, Y., Nakagaki, T., Ohkubo, I., & Ariga, H. (2016). Improvement of blood glucose levels and obesity in mice given aronia juice by inhibition of dipeptidyl peptidase IV and α-glucosidase. *The Journal of Nutritional Biochemistry*, *31*, 106–112. https://doi.org/10.1016/J.JNUTBIO.2016.02.004

39. Jurikova, T., Mlcek, J., Skrovankova, S., Sumczynski, D., Sochor, J., Hlavacova, I., Snopek, L., & Orsavova, J. (2017). Fruits of black chokeberry Aronia melanocarpa in the prevention of chronic diseases. *Molecules*, *22*(6), 1–23. https://doi.org/10.3390/molecules22060944

40. Sikora, J., Broncel, M., Markowicz, M., Chałubiński, M., Wojdan, K., & Mikiciuk-Olasik, E. (2012). Short-term supplementation with *Aronia melanocarpa* extract improves platelet aggregation, clotting, and fibrinolysis in patients with metabolic syndrome. *European Journal of Nutrition*, *51*(5), 549–556. https://doi.org/10.1007/s00394-011-0238-8

41. Skoczyñska, A., Jêdrychowska, I., Porêba, R., Affelska-, A., Turczyn, B., Wojakowska, A., & Andrzejak, R. (2007). Influence of chokeberry juice on arterial blood pressure and lipid parameters in men with mild hypercholesterolemia. *Pharmacological Reports.* 177–182.

42. Kim, B., Park, Y., Wegner, C. J., Bolling, B. W., & Lee, J. (2013). Polyphenol-rich black chokeberry (*Aronia melanocarpa*) extract regulates the expression of genes critical for intestinal cholesterol flux in Caco-2 cells. *Journal of Nutritional Biochemistry*, *24*(9), 1564–1570. https://doi.org/10.1016/j.jnutbio.2013.01.005

43. Naruszewicz, M., Laniewska, I., Millo, B., & Dłuzniewski, M. (2007). Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (MI). *Atherosclerosis*, *194*(2), e179–e184. https://doi.org/10.1016/j.atherosclerosis.2006.12.032

44. Ryszawa, N., Kawczyńska-Drózdz, A., Pryjma, J., Czesnikiewicz-Guzik, M., Adamek-Guzik, T., Naruszewicz, M., Korbut, R., & Guzik, T. J. (2006). Effects of novel plant antioxidants on platelet superoxide production and aggregation in atherosclerosis. *Journal of Physiology and Pharmacology :an Official Journal of The Polish Physiological Society*, *57*(4), 611–626.

45. Bell, D. R., & Gochenaur, K. (2006). Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *100*(4), 1164–1170. https://doi.org/10.1152/japplphysiol.00626.2005

46. Malik, M., Zhao, C., Schoene, N., Guisti, M. M., Moyer, M. P., & Magnuson, B. A. (2003). Anthocyanin-rich extract from *Aronia meloncarpa* E induces a cell cycle block in colon cancer but not normal colonic cells. *Nutrition and cancer*, 46(2), 186–196. https://doi.org/10.1207/S15327914NC4602_12

47. Lala, G., Malik, M., Zhao, C., He, J., Kwon, Y., Giusti, M. M., & Magnuson, B. A. (2006). Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats. *Nutrition and Cancer*, *54*(1), 84–93. https://doi.org/10.1207/s15327914nc5401_10

48. Zhao, C., Giusti, M. M., Malik, M., Moyer, M. P., & Magnuson, B. A. (2004). Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *Journal of Agricultural And Food Chemistry*, *52*(20), 6122–6128. https://doi.org/10.1021/jf049517a

49. Gasiorowski, K., Szyba, K., Brokos, B., Kołaczyńska, B., Jankowiak-Włodarczyk, M., & Oszmiański, J. (1997). Antimutagenic activity of anthocyanins isolated from *Aronia melanocarpa* fruits. *Cancer Letters*, *119*(1), 37–46. https://doi.org/10.1016/s0304-3835(97)00248-6

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 hiçbir yerde yayınlanmamış, Türkçe veya İngilizce 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 yayımlanmadan 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.

Yayım Gönderme

Yazarlar makalelerini https://dergipark.org.tr/tr/pub/jfpanu adresinden online olarak yükleyeceklerdir.

Yazım Kuralları

1. Metinler, A4 normunda (21 x 29,7 cm) yazılmış olmalıdır.
2. Bütün tablo ve şekiller metin içindeki yerlerine yazım alanından taşmadan yerleştirilmiş olmalıdır.
3. Metinler A4 normundaki sayfanın sağ ve sol tarafından 2,5 cm., üst ve alt kenarlarından 3 er cm boşluk bırakılarak (ilk sayfada yukarıdan 5 satır aralığı) 1,5 satır aralıkla yazılmalıdır. Yayımı kabul edilen makaleler doğrudan "Microsoft Word" dosyası halinde online olarak sisteme yüklenecektir (online submission). Ana metin yazı karakteri "Times New Roman" ve 11 punto olmalıdır.
4. Sayfa numaraları makalede belirtilmemelidir.
5. Yazar adı (küçük harf) ve soyadı (büyük harf) koyu olarak başlığın altına üç 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 açıkça yazılmalıdır.
6. Başlık sayfasında yayın adı, yazar/yazarların adları ve yazışma yapılacak yazarın açık adresi, telefon ve faks numaraları, varsa e-mail adresi belirtilmelidir. Sorumlu yazarın soyadının üstüne (*) işareti konularak belirtilmelidir. Bu kişinin açık adresi, faks numarası, telefon numarası ve e-mail adresi başlık sayfasının en altında belirtilmelidir.
7. Tablolar üstlerine, şekiller (formül, grafik, şema, spektrum, kromatogram, fotoğraf v.b.) de altlarına arabik rakamlarla (**Şekil 1**., **Tablo 2**.,) numaralandırılmalıdır. "Tablo", "Şekil" sözcükleri ile bunlara ait numaralar koyu yazılmalı ve 11 punto olmalıdır. Şekil/Resim (JPG formatında) makale içinde yerleşmiş olmalıdır.
8. Tablo adları Tabloların üstüne ve şekil adları da Şekillerin altına birer satır aralıkla ve bunların genişliğini aşmayacak şekilde 11 punto yazılmalıdır. Tabloya ait açıklama varsa tablonun altına 1 boşluk bırakılarak 9 punto ile yazılmalıdır. Tablo ve Şekiller metin içine yerleştirilirken metin ile aralarında net ayırımı sağlayacak kadar boşluk bırakılmalıdır.
9. Paragraf başları 1 cm içeriden başlamalıdır.
10. Uluslararası kısaltmalar kullanılabilir. Metin içinde mililitre için ml; dakika için dak. olarak belirtilen şekliyle yazılmalıdır.
11. Makalelerin bölümleri Başlık, Öz, Anahtar kelimeler, Giriş, Gereç ve Yöntem, Sonuç ve Tartışma, Teşekkür ve Kaynaklar sırasına uygun olarak hazırlanmalıdır. Derleme makalelerinde Gereç ve Yöntem bölümü bulunmayabilir. Bu bölümler birbirlerinden 2 satır aralık ile ayrılmalıdır. Bu bölümleri ifade eden başlıklar 12 punto ile koyu olarak büyük harflerle ve sayfanın solundan başlanarak yazılmalıdır. Bölüm başlıkları ile metin arasında ayrıca aralık bırakılmamalıdır.

- **Başlık:** Türkçe ve İngilizce olarak büyük harf ve ilk başlık 14 punto, koyu ve ikinci başlık 12 punto, italik olarak yazılmalıdır. Başlık metine uygun, kısa, çalışmayı tanıtıcı ve açık ifadeli olmalıdır.
- **Öz:** Türkçe ve İngilizce (Abstract) olarak makalelerin başında 200'er kelimeyi geçmeyecek şekilde 10 punto ile, *italik* olarak ve çerçeve içinde yazılmalıdır. Yabancı dilde yazılmış makalelerde mutlaka Türkçe özet bulunmalıdır. Ayrıca öz, kendi içinde amaç, gereç ve yöntem, sonuç ve tartışma olarak alt başlıklar halinde yazılmalıdır.
- **Anahtar kelimeler:** En fazla 5 sözcükten oluşmalı ve özetlerin hemen altına ilgili dilde alfabetik ve italik olarak yazılmalıdır.
- **Giriş:** Araştırmanın amacı ve konuyla ilgili çalışmaların yer aldığı bölüm olmalıdır.
- **Gereç ve Yöntem**: Kullanılan gereç belirtilerek, uygulanan yöntem hakkında gerekli bilgiler açıkça ifade edilmelidir. Deneylerde hayvan kullanılması durumunda lokal etik komiteden veya ilgili düzenleyici makamlardan onay alınmalıdır ve bilgilendirilmiş onam belgelendirilmelidir.
- **Sonuç ve Tartışma:** Bulguların verilerek değerlendirildiği bölümdür.

- **Teşekkür:** Varsa araştırmayı destekleyen kuruluşa ve katkısı olan kişilere kaynaklardan önce yer alan bu bölümde kısaca teşekkür edilebilir.
- **Kaynaklar:** Kaynak yazım stili Amerikan Psikoloji Derneği'ne (APA) göredir. Metinde, geçiş sırasına göre köşeli parantez içinde, örneğin: [1,2,…] gibi numaralandırılmalı ve metin sonunda bu numaralara göre sıralanmalıdır. Kaynaklar aşağıdaki örneklere uygun olarak yazılmalıdır.

  i. **Makale için:** Yazarın soyadı, adının baş harfleri, makalenin tam başlığı derginin adı, cilt no, varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa no, yıl yazar isimlerinden sonra (parantez içinde) olarak 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.

  Moncada, S., Palmer, R.M.J., Higgs, E.A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. Biochemistry and Pharmacology, 38, 1709 – 1715.

  ii. **Elektronik Makale için:**

  Perneger, T. V. and 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/

  iii. **Web sitesi için:**

  Clinical Pharmacology Web site. (2001). Retrieved June 16, 2004, from http://cpip.gsm.com/

  iv. **Kitap için:** Yazarın soyadı, adının baş harfleri, kitabın adı, cilt no (varsa), kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

  Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

  v. **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.

  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.

12. Bileşiklerin karakterizasyonu ayrı bir paragraf ile gösterilmeli ve yeni bileşiklerin saflıkları ve yapı aydınlatılmaları sağlanmalıdır.

Instruction 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 English 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.
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    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.

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1. Manuscripts should be typed on A4 size papers marked in 21 x 29,7 cm area.
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3. Manuscripts should be typed with 1.5 line spacing with a margin of 2,5 cm on left-hand and right-hand sides, 3 cm on the top (5 line spacing on the first page) and bottom. Since articles will be loading online, authors are requested to submit their manuscripts as "Microsoft Word" file. The font of main text should be "Times New Roman" with 11 pt font size.
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7. All tables and figures/images must be cited in the text consecutively. Every table must have a descriptive title at the top and should be numbered with Arabic numerals (**Table 1.**, **Table 2.**) Please submit tables as editable text and not as images. Figures (chemical formulas, graphics, photographs, chromatographs, spectra etc) should also be numbered with Arabic numerals (**Figure 1.**, **Figure 2.**,) Captions should be typed with 11 pt font size. Figures/Images (JPG) should be embedded in the Manuscript file.
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9. In each paragraph, indentation must be done (1 cm space).
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- **Title**: It should be written in Turkish and English. Font size must be 14 pt as a bold for first title. In the second title, font size must be 12 pt as an italic. The title must be appropriate to the text.
- **Abstract:** It should be written in Turkish and English no longer than 200 words, 10 pt, *Italic*. Abstract should be written in a border. If manuscript is written in a foreign language, must include Turkish abstract. In addition, the abstract should be written in subheadings as objective, material and method, result and discussion within itself.
- **Keywords:** Up to 5 key words should be provided in alfabetic and *italic* at the end of the abstract.
- **Introduction:** It should contain a clear statement of the aim and novelty of the study.
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- **Result and Discussion**: The results must be clearly and concisely described with the help of appropriate illustrative material. The discussion should deal with the interpretation of the results.
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- **References:**The style of references is that of the American Psychological Association (APA). They should be numbered with Arabic numerals consecutevily in the order in which they first appear in the paper, for example: [1, 2,…]. Cited publications should be listed in numerical order at the end of the paper. If there is more than one author, all the names of the authors should be written. Examples are given below;

  i. **Article:** Reference to a journal publication (journal names in full, not abbreviated)

  Moncada, S., Palmer, R.M.J., Higgs, E.A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication, Biochemistry and Pharmacology, 38, 1709 – 1715.

  ii. **Electronic Article:**

  Perneger, T. V. and 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/

  iii. **Web page:**

  Clinical Pharmacology Web site. (2001). Retrieved June 16, 2004, from http://cpip.gsm.com/

  iv. **Book:**

  Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

  v. **Chapter in a book:**

  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.

12. The characterization of compounds should be presented in a seperate paragraph and for all new compounds, evidence to confirm both identity and purity have to be provided.

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