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ORIGINAL ARTICLE / ÖZGÜN MAKALE

PHYTOCHEMICAL PROFILING AND HEAVY METALS COMPOSITION OF AQUEOUS AND ETHANOL EXTRACTS OF *ANOGEISSUS LEIOCARPUS*

ANOGEISSUS LEIOCARPUS'UN SULU VE ETANOLİK EKSTRELERİNİN FİTOKİMYASAL PROFİLİ VE AĞIR METAL BİLEŞİMİ

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ABSTRACT

Objective: *The present study aimed to investigate the phytochemical and heavy metals components of A. leiocarpus considering its applications in ethnomedicine.*
Material and Method: *The phytochemical components were determined qualitatively and quantitatively gravimetrically, while component identification was done using Gas spectrometer-mass spectrometer (GC-MS) technique. Heavy metals were quantified by atomic absorption spectrophotometer.*
Result and Discussion: *Saponins and flavonoids were detected in the aqueous extracts in concentrations of 10.22% ±0.48, and 38.67% ±0.17 respectively, and concentrations of 17.37% ±0.65 and 19.63% ±0.60 respectively in the ethanol extract. GC-MS analysis identified 16 and 26 compounds in the aqueous and ethanol extracts respectively. In the aqueous extract, 5-Hydroxymethylfurfural, 1,2,4-Benzenetriol, and cis-Vaccenic acid had the highest peak areas of 46.24, 17.12, and 15.13% respectively, while in the ethanol extract 5-Hydroxymethylfurfural (14.40%), 1,2,3-Benzenetriol (12.29%) and -methoxybenzene-1,4-diol (7.54%) were the highest. Chromium (0.548 ppm ±0.030) was detected only in the aqueous concentration, while Cadmium had a concentration of 0.002 ±0.001 and 0.006 ppm ±0.002 in the aqueous and ethanol extract respectively. Lead was present with aqueous and ethanol extracts concentrations of 0.096 ±0.020*

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and 0.096 ppm ±0.040 respectively. Conclusively, the present study agrees with the claims for the traditional application of the plant in folkloric medicine to manage different ailments.
Keywords: *Anogeissus leiocarpus, GC-MS, heavy metals, phytochemicals, profiling*

ÖZ

Amaç: *Bu çalışma, A. leiocarpus'un fitokimyasal ve ağır metal bileşenlerinin etnomedikaldeki uygulamalarını göz önünde bulundurmayı amaçlamıştır.*
Gereç ve Yöntem: *Fitokimyasal bileşenler kalitatif ve kantitatif olarak gravimetrik olarak belirlenirken, bileşen tanımlaması Gaz spektrometresi-kütle spektrometresi (GC-MS) tekniği kullanılarak yapılmıştır. Ağır metallerin miktarı atomik absorpsiyon spektrofotometresi ile belirlenmiştir.*
Sonuç ve Tartışma: *Saponinler ve flavonoidler sulu ekstrelerde sırasıyla %10.22 ±0.48 ve %38.67 ±0.17 konsantrasyonlarında ve etanol ekstraktında sırasıyla %17.37 ±0.65 ve %19.63 ±0.60 konsantrasyonlarında tespit edildi. Ancak alkaloidler, steroidler, glikozitler ve terpenoidler tespit edilmedi. GC-MS analizi, sulu ve etanol ekstrelerinde sırasıyla 16 ve 26 bileşik tanımladı. Sulu ekstrede 5-Hidroksimetilfurfural, 1,2,4-Benzenetriol ve cis-Vaccenic asit sırasıyla 46.24, 17.12 ve 15.13 ile en yüksek pik alanlarına sahipken, etanol ekstresinde 5-Hidroksimetilfurfural (%14.40), 1,2,3-Benzenetriol (%12,29) ve -metoksibenzen-1,4-diol (%7,54) en yüksek değerlerdi. Krom (0.548 ppm ±0,030) sadece sulu konsantrasyonda tespit edilirken Kadmiyum, sulu ve etanol özütünde sırasıyla 0,002 ±0,001 ve 0.006 ppm ±0.002 konsantrasyona sahipti. Kurşun, sırasıyla 0.096 ±0.020 ve 0.096 ppm ±0.040'lık sulu ve etanol özütlerinde mevcuttu. Sonuç olarak, bu çalışma, bitkinin farklı rahatsızlıkları tedavi etmek için folklorik tıpta geleneksel uygulamasına ilişkin iddialarla hemfikirdir.*
Anahtar Kelimeler: *Ağır metaller, Anogeissus leiocarpus, GC-MS, fitokimyasal, profil oluşturma*

INTRODUCTION

Medicinal plants sources of important bioactive compounds with pharmacological roles attributed to their phytochemical compositions. Plants are exploited as therapeutics for the management of different ailments in traditional medicine due to their phytochemical components. Formulations in form of decoctions or powder are taken orally or through topical applications in phytotherapy. The presence of phytochemicals offer a diversification of pharmacological activities which allows for the development of novel drugs for application in modern medicine [1]. Worldwide, medicinal plants are processed into different finished plant-based products of various efficacy for use at different doses as commercial products for export [2]. More than 70.000 species of plants were reported to be used in the management of various ailments worldwide [3]. Notably, in rural areas, herbalists take advantage of the diversity of medicinal plants and utilized them for various ailments [4]. Modern pharmaceutical industries rely on the supply of bioactive compounds for production, thus, providing information on these compounds through research on medicinal plants is important [5]. Different plants of varying efficacy were reported to possess different pharmacological activities in comparison to modern medicine applied in the treatment of several ailments [6].

Anogeissus leiocarpus which is called African birch in English and *Marke* in the native language (Hausa) of Northern Nigeria. Aqueous extract of *A. leiocarpus* has been reported to exert pharmacological effects against African trypanosomiasis which was credited to the phytochemicals present in the plant [7]. In another study, anti-microbial, anti-inflammatory, anti-diabetic, and wound healing were among the pharmacological activities of aqueous extract of *A. leiocarpus* reported [8]. In a similar study, the antifungal activity of ethanol extract of *A. leiocarpus* was reported which was accredited to the application of the plant in the folkloric treatment of candidiasis [9]. In another study, the butanol, hexane, and aqueous extracts of *A. leiocarpus* exerted pharmacological activities against *Klebsiella* spp., *Escherichia coli*, and *Pantoea agglomerans*, which was attributed to the phytochemical constituents of the plant, and was suggested to be a source of therapeutics against drug-resistant bacteria [10]. In a previously reported study, the methanol and aqueous extracts of *A. leiocarpus* was reported to possess antioxidant potential and suggested to be a source for the development of novel therapeutic agents [11]. A combined administration of *A. leiocarpus* and *Khaya senegalensis* indicated a better

treatment option against trypanosomosis supporting the utilization of the plants by pastoralists in the treatment of trypanosomosis [12]. Hydro alcoholic extract of *A. leiocarpus* was reported as an antioxidant and anti-diabetic agent and could be developed into an alternative for the management of diabetes [13].

Therefore, in the present study we aimed to investigate the phytochemical profile and heavy metals composition of aqueous and ethanol extracts of Anogeissus leiocarpus due to the extensive application of the plant in the management of different ailments.

MATERIAL AND METHOD

Plant Material

A sample of *A. leiocarpus* plant was collected from Girei Local Government, Adamawa state, Nigeria, and was identified by a Forest Technologist from the Forestry Technology Department of Adamawa State Polytechnic, Yola. A voucher specimen was kept in the departmental herbarium with voucher number ASP/FT/101. The drying of the stem bark was done under shade and ground to powder using a blender.

Reagents and Chemicals

All the chemicals and reagents used in this research were of AnarlaR (Xilong Scientific Co., Ltd. Guangdong, China).

Extraction

Extraction was done by maceration of 400 g of *A. leiocarpus* bark powder in 1.5 l of ethanol and distilled water for 2 days at room temperature, followed by filtration and concentration to dryness under reduced pressure at 40°C [14].

Qualitative Phytochemical Analysis

The detection of phytochemicals present aqueous (ASBE) and ethanol (ESBE) stem bark extracts of *A. leiocarpus* was carried out using a method reported previously to detect alkaloids, saponins, steroids, glycosides, terpenoids, and flavonoids [14].

Alkaloids

To 2 ml of the extract, 2 ml of 10% HCl was added, followed by the addition of 2 ml of Meyer's reagent. Formation of an orange precipitate indicate a positive result.

Saponins

To 2 ml of the extract, 2 ml distilled water was added. The mixture was agitated in a test tube for 5 min. Appearance of a layer of foam indicated a positive result.

Steroids

To 2 ml of the extract, 10 ml of chloroform was added and then, 10 ml of concentrated sulphuric acid was added by the side of the test tube. Formation of a reddish upper layer and yellow sulphuric acid layer with green fluorescence indicate a positive result.

Glycosides

To 2 ml of acetic acid, 2 ml of the extract was added. The mixture was cooled in cold water bath, and then 2 ml of concentrated H_2SO_4 was added. Colour development from blue to bluish green indicate the presence of glycosides.

Terpenoids

To 2 ml of the extract, 2 ml of chloroform and 1ml of concentrated sulphuric acid were carefully added to form a layer. A clear upper and lower layer with a reddish-brown interphase indicate a positive result.

Flavonoids

To 2 ml of the extract, 10% sodium hydroxide was added. A yellow color was formed which turned colorless upon addition of 2 ml of dilute hydrochloric acid indicating a positive result.

Quantitative Phytochemical Analysis

The quantification of phytochemicals in ASBE and ESBE of *A. leiocarpus* was carried out by the following methods:

Saponins Content

Saponins quantification was done by the method previously described [15]. Briefly, 0.5 g extract was introduced into a conical flask and 10 ml of 20% aqueous ethanol was added. The sample was heated over a water bath for 1 h with continuous stirring at about 550°C. The concentrate was transferred into a 250 ml separator funnel and 5 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered and the ether layer was discarded. About 10 ml of n-butanol was then added followed by addition of 2 ml of 5% aqueous NaCl. The remaining solution was heated over a water bath. After evaporation, the sample was dried in the oven to a constant weight and calculated as follows:

$$\% \text{ Total Saponins} = \frac{\text{weight of residue}}{\text{weight of sample}} \times 100$$

Flavonoid Content

Flavonoid quantification was carried out according to a method described previously [16]. About 0.5g of the extract was mixed with 10 ml of 80% aqueous methanol. The whole solution was filtered through Whatman filter paper. The filtrate was transferred to a pre-weighed crucible and evaporated into dryness over a water bath and weighed, and calculated as follows:

$$\% \text{ Total Flavonoids} = \frac{\text{weight of residue}}{\text{weight of sample}} \times 100$$

Gas Chromatography-mass Spectrometry (GC-MS) Analysis

Gas chromatography-mass spectrometry analysis was carried out with a combination of a Gas chromatography-mass spectrophotometer (Agilent 19091-433HP, USA). The system was fitted fused with a silica column. A column flow velocity of 1.6 ml/min was set for the carrier gas (Helium). Ion-source temperature was set to 250°C while the pressure was 8.6 psi. A split mode injection (1 µl) at 250°C was used. The initial temperature of the column was set at 100°C, and gradually increased to 180°C at 20°C/min, then 10°C/min to 280°C. The total elution time was 16 min. The National Institute of Standards and Technology (NIST) database was used for the identification and comparison of the unknown spectrum of the detected compounds with that of known standards.

Determination of Heavy Metals Composition

Atomic absorption spectrophometric method principled on the absorption of of light by different elements at different wavelenths was used to determine the concentration of heavy metals [17]. A gram of the samples was ashed at 500ºC for 1 h, which was dissolved in 25 ml of 10% HCl and made up to 100 ml. Chromium (Cr), cadmium (Cd), and lead (Pb) contents were quantified by the method previously described [17] using Atomic Absorption Spectrophotometer (AAS) (Buck Scientific AAS210).

Statistical Analysis

Data obtained in the present study were expressed as mean ± standard error of triplicate determinations' mean (± SEM) evaluated with Statistical Package for the Social Sciences (SPSS) version 22 Software.

RESULT AND DISCUSSION

The phytochemicals detected in ASBE and ESBE of *A. leiocarpus* are shown in Table 1. Saponins and flavonoids were detected in both ASBE and ESBE of *A. leiocarpus*, while alkaloids, steroids, glycosides, and terpenoids were absent in both extracts.

Table 1. Qualitative phytochemical composition of aqueous (ASBE) and ethanol (ESBE) stem bark extracts of *A. leiocarpus*

Phytochemical	**Inference**	
	Aqueous	**Ethanol**
Alkaloids	-	-
Saponins	+	+
Steroids	-	-
Glycosides	-	-
Terpenoids	-	-
Flavonoids	+	+

+ = present, - = Absent.

The phytochemicals quantified in ASBE and ESBE of *A. leiocarpus* are shown in Table 2. Although both saponins and flavonoids were detected in ASBE and ESBE of *A. leiocarpus*, saponins were in higher amounts in ESBE (38.67% ±0.17) than in the ASBE (10.22% ±0.48) of *A. leiocarpus*. Flavonoids were also detected in higher concentrations in the ESBE (19.63% ±0.60) than ASBE (17.37% ±0.65) of *A. leiocarpus*.

Table 2. Quantitative phytochemical composition of aqueous (ASBE) and ethanol (ESBE) stem bark extracts of *A. leiocarpus*

Phytochemical	**Concentration of extracts (%)**	
	Aqueous	**Ethanol**
Saponins	10.22 ±0.48	38.67 ±0.17
Flavonoids	17.37 ±0.65	19.63 ±0.60

Concentration values are in triplicates determinations (± SEM)

Although only saponins and flavonoids were present in both ASBE and ESBE of *A. leiocarpus*, their concentrations were different. Both saponins (38.67% ±0.17) and flavonoids (19.63% ±0.60) were present in higher concentrations in the ESBE than in ASBE (10.22% ±0.48 and 17.37% ±0.65 respectively). The difference in polarity of the solvents used might be attributed to the difference in the concentration of the phytochemicals quantified in the present study [18]. Saponins were reported to poses several pharmacological activities [19]. Saponins were reported to exert antitumor activities through multiple signaling pathways by blocking cellular proliferation, promoting apoptosis, and controlling the tumor microenvironment [20]. The saponin trillin was reported to exert antioxidant activity through the elevation of superoxide dismutase, catalase, and glutathione peroxidase which are antioxidant enzymes, thus protecting the heart against oxidative stress [21]. In another study, saponins were reported to raise the activity of several antioxidant enzymes, responsible for cardiac protection against reactive oxygen species [21]. Dioscin which is a saponin has been reported to be an active agent against a tumor, microbes, inflammation, and oxidative stress [22].

Flavonoids exerts antibacterial action against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* [23]. The anticancer activity of flavonoids against human hepatocarcinoma was

previously reported to be through induction of apoptosis via the mitochondria-dependent apoptotic pathway and death receptor-dependent apoptotic pathways, thereby suppressing tumor growth [24]. Flavonoids were reported to exert antibacterial activity against different species by destroying the cell membrane, blocking energy metabolism and nucleic acid synthesis, however, the antiviral activity of flavonoids against HIV was reported to be by blocking the phosphorylation of protein by cytokine II, preventing the integration of the virus [25]. In another study, flavonoids were reported to prevent cardiovascular diseases due to their antioxidant properties by modulating the functions of many inflammatory mediators and inhibiting immune cells [26]. The results reported in our study agree with the study reported previously where saponins and flavonoids were detected [27]. In a similar study, flavonoids were detected in the ESBE of *A. leiocarpus* while alkaloids were absent which agrees with the present study [28]. Previous studies on phytochemical components of stem bark extracts of *A. leiocarpus* reported results similar to our study [29, 30].

The bioactive compounds identified in the aqueous (ASBE) of *A. leiocarpus* using GC-MS with their retention time, peak area, molecular weight, and formula are presented in Table 3. In the ASBE, 16 compounds were detected with 5-Hydroxymethylfurfural having the highest (46.24%) peak area, followed by 1,2,4-Benzenetriol (17.12%) and cis-Vaccenic acid (15.13%). Maltol and Methyl 14-methylpentadecanoate had peak areas of 5.14% and 3.96% respectively. Zidovudine, Ascorbyl dipalmitate, and Octadecanal were also identified in ASBE of *A. leiocarpus*. The structures of the identified compounds from ASBE of *A. leiocarpus* showing the different groups present are also shown in Figure 1, while the chromatogram of the GC-MS analysis showing different peaks and retention times of the identified compounds is present in Figure 2.

Table 3. Bioactive compounds identified in ASBE of *Anogeissus leiocarpus* using GC-MS

S/N	Name of compound	Retention Time	Peak Area (%)	Molecular weight	Formula
1	5-Hydroxymethylfurfural	3.613	46.24	126.11184	$C_6H_6O_3$
2	Maltol	4.535	5.14	126.11184	$C_6H_6O_3$
3	1,2,4-Benzenetriol	5.194	17.12	126.11184	$C_6H_6O_3$
4	3-Methyl-1H-pyrazole-5-carboxylic acid	6.818	1.04	126.11484	$C_5H_6N_2O_2$
5	Methyl 14-methylpentadecanoate	6.978	3.96	270.45576	$C_{17}H_{34}O_2$
6	1,3,5-Benzenetriol	7.481	2.04	126.11184	$C_6H_6O_3$
7	1,2,3-Benzenetriol	7.785	0.33	126.11184	$C_6H_6O_3$
8	9-Octadecenoic acid (Z)-, methyl ester	8.431	1.87	296.49364	$C_{19}H_{36}O_2$
9	Zidovudine	8.609	1.30	267.24432	$C_{10}H_{13}N_5O_4$
10	4-Ethylcyclohexanone	8.923	1.07	126.19856	$C_8H_{14}O$
11	1-(4-Bromobutyl)piperidin-2-one	9.124	0.83	234.13614	$C_9H_{16}BrNO$
12	Ascorbyl dipalmitate	10.749	0.30	652.95312	$C_{38}H_{68}O_8$
13	Octadecanal	11.933	0.08	268.48324	$C_{18}H_{36}O$
14	cis-Vaccenic acid	14.788	15.13	282.46676	$C_{18}H_{34}O_2$
15	cis-11-Hexadecenal	15.692	2.03	238.4136	$C_{16}H_{30}O$
16	Oxirane, tetradecyl-	15.692	2.03	240.42948	$C_{16}H_{32}O$

The compounds identified in ESBE of *A. leiocarpus* using GC-MS and their various, retention times, peak area, molecular weight, and formulas are presented in Table 4. A total of 26 compounds were detected with 5-Hydroxymethylfurfural and 1,2,3-Benzenetriol having peaks of 14.40% and 12.29% respectively. 2-methoxybenzene-1,4-diol (7.54%), 3-Methyl-1H-pyrazole-5-carboxylic acid (5.56%), and Hexadecanal (5.01%) were among the compounds detected. Methyl palmitate, cis-vaccenic acid, oleic acid, squalene, erusic acid, and hexadecanal were also identified in the ESBE of *A. leiocarpus*. The structural formula of the identified compounds is shown in Figure 3, while Figure 4

shows the chromatogram of the identified compounds with their various peaks and retention time.

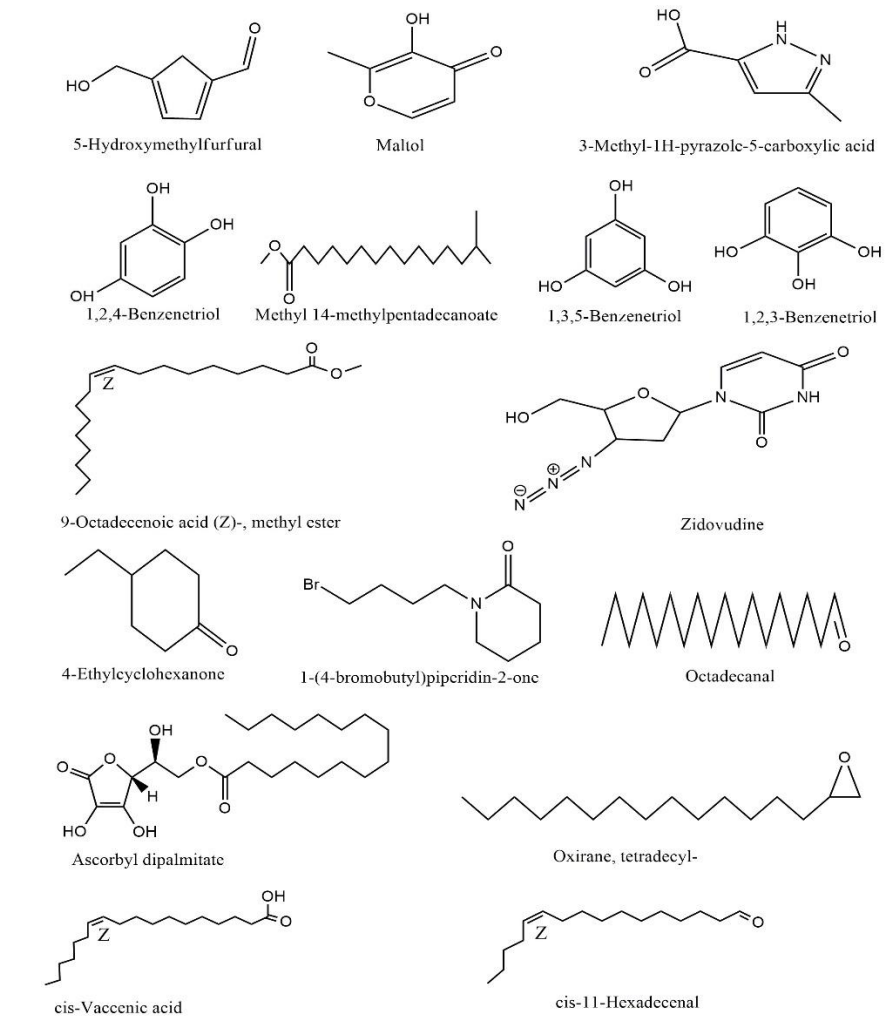

Figure 1. Structures of compounds identified in ASBE of *Anogeissus leiocarpus*

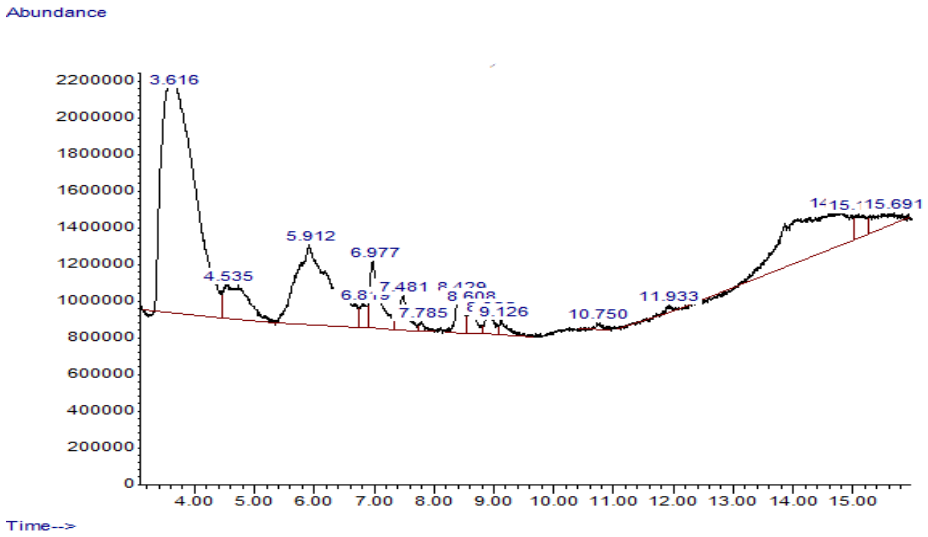

Figure 2. GS-MS Chromatogram for compounds identified in ASBE of *A. leiocarpus*

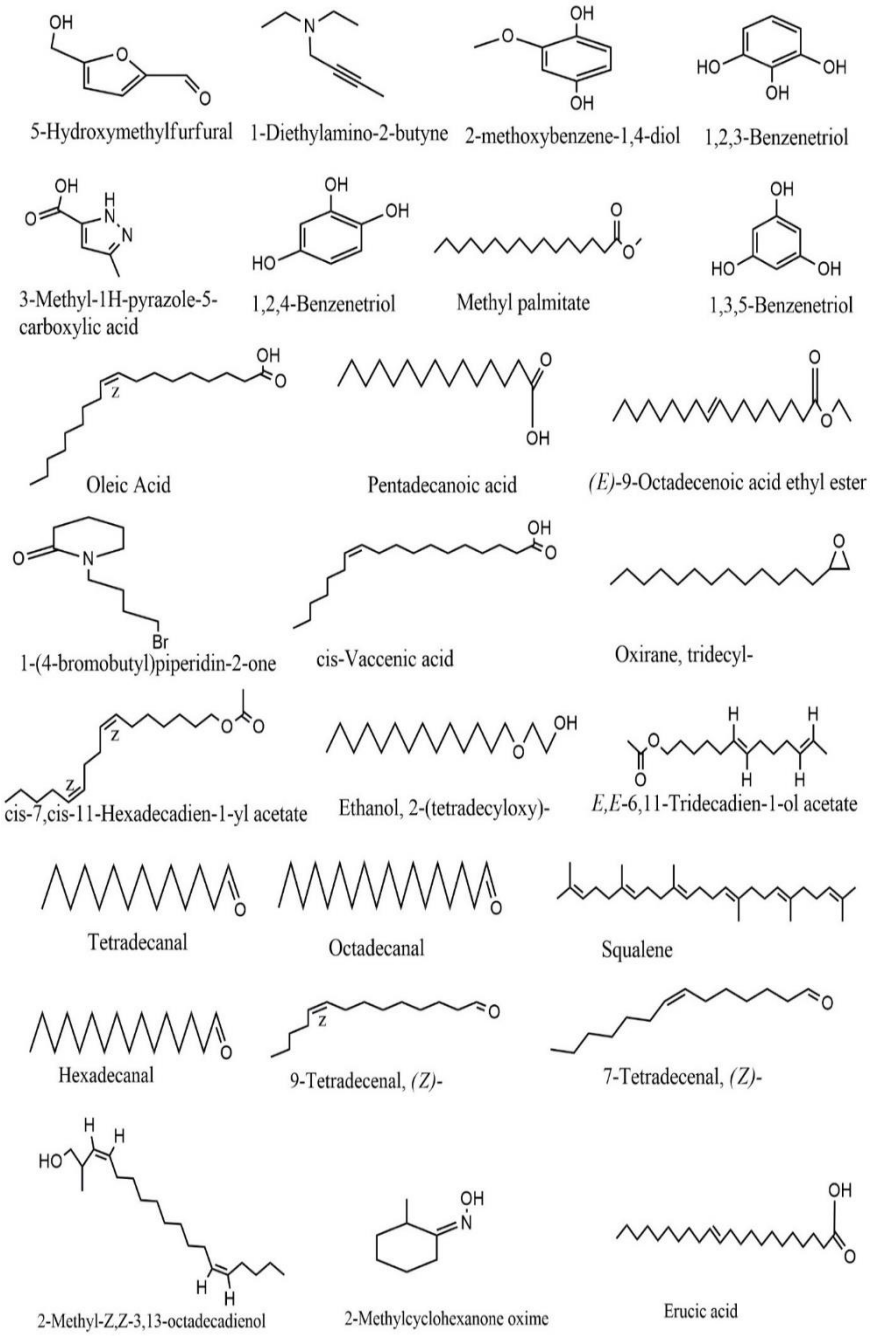

Figure 3. Structural formulas of compounds detected in ESBE of *Anogeissus leiocarpus*

GC-MS analysis of ASBE of *A. leiocarpus* identified 16 compounds (Table 3). The various compounds detected are associated with different pharmacological properties. 5-Hydroxymethylfurfural was reported to show anticancer activity against cancer cells by induction of cell apoptosis and cell circle arrest. This compound also demonstrated antioxidant potential by scavenging free radicals and increasing the activity of antioxidant enzymes [31]. The anti-inflammatory activity of 5-Hydroxymethylfurfural against lung injury was reported to be mediated through blocking of endoplasmic reticulum stress and activating of inflammasome [32]. Cis-vaccenic acid has been reported to be associated with antibacterial activity and anti-hyperglycemic effects in rats [33]. GC-MS analysis of ESBE of *D. leiocarpus* showed the presence of 26 compounds (Table 4). 1,2,3-Benzenetriol otherwise called pyrogallol demonstrated anti-malarial activity by its auto-oxidation in the presence of metallic

ions (Cu^{2+}, Fe^{3+}, and Mn^{2+}) to produce free radicals, thus inhibiting the growth of parasite, which is a characteristic of anti-malarial drugs [34]. Anti-bacterial study of pyrogallol reported inhibition of two strains of *Staphylococcus aureus* [35]. Methyl palmitate demonstrated pharmacological activity by decreasing inflammation through the reduction of the expression of cytokines promoting inflammation and increasing the expression of anti-inflammatory cytokines [36]. Methyl palmitate was reported to show antioxidant activity by reducing markers of oxidative stress and elevating the activities of the innate antioxidants [33]. Oleic acid exerts anti-inflammatory activity by acting on different pathways of immune cells that modulate inflammation [37]. Squalene detected in ethanol extract was reported to possess anti-diabetic activity against type 2 diabetes, and peroxidation [38].

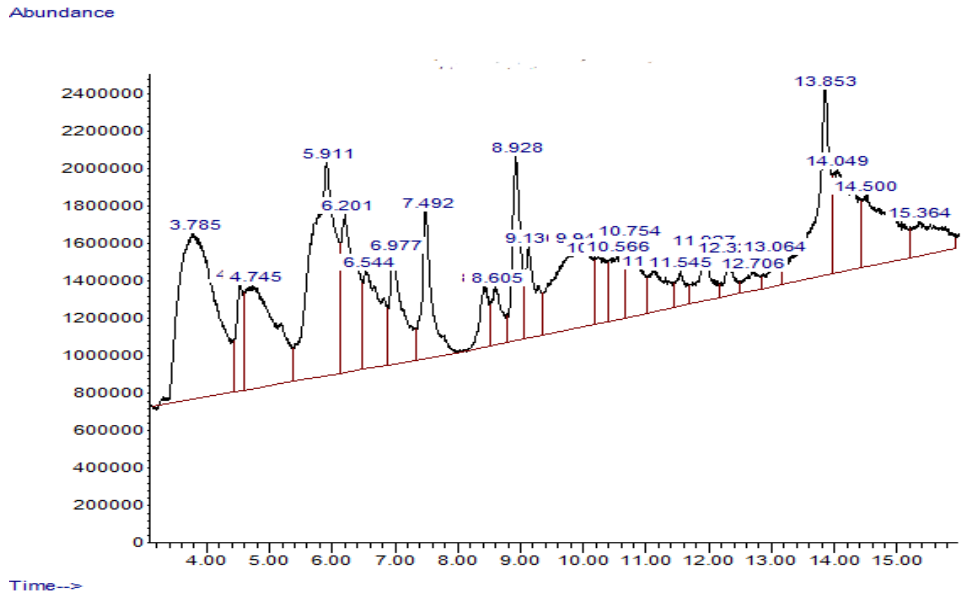

Figure 4. GS-MS Chromatogram for compounds identified in ESBE of *A. leiocarpus*

The heavy metals detected in ASBE and ESBE of *A. leiocarpus* and their respective concentrations are presented in Table 4. Chromium (Cr) was present in the highest concentration (0.548 ppm ±0.030) among all the heavy metals, though it was not detected in ESBE. Cd was detected in the least concentrations of 0.002 ppm ±0.001and 0.006 ppm ±0.002 for ABSE and ESBE respectively, while lead had concentrations of 0.096 ppm ±0.02 and 0.096 ppm ±0.04 in ASBE and ESBE respectively. Heavy metals exposure leads to acute and chronic toxicity targeting different organs of the body with effects such as cancer, gastrointestinal disturbance, and birth defects [39]. Exposure to heavy metals at lower continuous doses or high doses generate of reactive oxygen species (ROS) subsequently leading to oxidative stress and subsequently damaging the DNA, causing lipid peroxidation and modification of proteins [40]. Exposure to chromium leads to damage to the DNA by generation of ROS subsequently causing cancers of the kidney, bone, testicle, and thyroid [41]. Cd binds to the protein metallothionein, subsequently absorbed by the kidney leading to chronic toxicity of the kidney [42]. Cd exposure might also lead to carcinogenesis due to oxidative stress mediated by the generation of ROS, disturbance in gene expression and cell proliferation, and resistance to apoptosis [43]. The toxicity of lead (Pb) might be due to exposure to air and drinking water, which lead to disturbance of normal body processes [44].

The levels of heavy metals reported in our study were below the regulatory limits which are1.30, 0.02, and 2 ppm for Cr, Cd, and Pb respectively [45]. Although these heavy metals are present in the plant, traditional use of the plant occasionally might be said to be safe. In a previous study, the concentrations of lead and cadmium in the trunk bark of *A. leiocarpus* were reported to be 0.255 ppm and 0.22 ppm respectively [46]. The present study doesn't agree with this result as the concentration of lead and cadmium were lower both in the aqueous and ethanol extracts. In a similar study, the concentrations Cr, Cd, and Pb in leave of *A. leiocarpus* collected from a quarry site was reported to be

< 0.04, <0.01, and 0.060±0.01 mg/l [47], higher than values reported in the present study. In another study, the levels of Cr, Cd, and Pb in herbal drugs prepared *A. leiocarpus* were 0.061 ±0.001, 0.016 ±0.00, 0.386 ±0.001 mg/Kg [48]. The values for Cr, and Cd reported in their study were lower than the value (Table 5) reported in our study. However, the concentration of Pb was lower in our study. Weather conditions [49], location for sample collection [50], and environmental conditions [51] such as PH, temperature and dissolved oxygens were reported to influence the levels of heavy metals in soil samples. Heavy metals are absorbed by plants from the soil, the difference between concentration values reported in previous studies and our sudy might be due to the factors such as weather condition, location fro sample collection and environmental conditions.

Table 4. Bioactive compounds identified in ESBE of *Anogeissus leiocarpus*

S/N	Name of compound	Retention Time	Peak Area (%)	Molecular weight	Formular
1	5-Hydroxymethylfurfural	3.785	14.40	126.11184	$C_6H_6O_3$
2	1-Diethylamino-2-butyne	4.540	1.85	125.2138	$C_8H_{15}N$
3	2-methoxybenzene-1,4-diol	4.746	7.54	140.13872	$C_7H_8O_3$
4	1,2,3-Benzenetriol	5.913	12.29	126.11184	$C_6H_6O_3$
5	3-Methyl-1H-pyrazole-5-carboxylic acid	6.200	5.56	126.11484	$C_5H_6N_2O_2$
6	1,2,4-Benzenetriol	6.543	4.01	126.11184	$C_6H_6O_3$
7	Methyl palmitate	6.978	3.44	270.45576	$C_{17}H_{34}O_2$
8	1,3,5-Benzenetriol	7.493	3.62	126.11184	$C_6H_6O_3$
9	Oleic Acid	8.425	1.37	282.46676	$C_{18}H_{34}O_2$
10	Pentadecanoic acid	8.603	1.39	242.402	$C_{15}H_{30}O_2$
11	(E)-9-Octadecenoic acid ethyl ester	8.929	3.62	310.52052	$C_{20}H_{38}O_2$
12	1-(4-bromobutyl)piperidin-2-one	9.129	2.22	234.13614	$C_9H_{16}BrNO$
13	cis-Vaccenic acid	9.942	7.11	282.46676	$C_{18}H_{34}O_2$
14	Oxirane, tridecyl-	10.234	1.61	226.4026	$C_{15}H_{30}O$
15	cis-7,cis-11-Hexadecadien-1-yl acetate	10.565	2.17	280.45088	$C_{18}H_{32}O_2$
16	Ethanol, 2-(tetradecyloxy)-	10.754	2.52	258.44476	$C_{16}H_{34}O_2$
17	E,E-6,11-Tridecadien-1-ol acetate	11.120	1.68	238.37	$C_{15}H_{26}O_2$
18	Tetradecanal	11.544	0.84	212.37572	$C_{14}H_{28}O$
19	2-Methyl-Z,Z-3,13-octadecadienol	11.927	1.60	280.5	$C_{19}H_{36}O$
20	2-Methylcyclohexanone oxime	12.322	0.85	127.18632	$C_7H_{13}NO$
21	7-Tetradecenal, (Z)-	12.705	0.65	210.35984	$C_{14}H_{26}O$
22	Octadecanal	13.066	0.81	268.48324	$C_{18}H_{36}O$
23	Squalene	13.856	6.64	410.727	$C_{30}H_{50}$
24	Erucic acid	14.050	4.83	338.57428	$C_{22}H_{42}O_2$
25	Hexadecanal	14.502	5.01	240.42948	$C_{16}H_{32}O$
26	9-Tetradecenal, (Z)-	15.366	2.35	210.35984	$C_{14}H_{26}O$

Table 5. Heavy metals composition of *Anogeissus leiocarpus*

Heavy metal	Concentration (ppm)	
	Aqueous extract	**Ethanol extract**
Chromium (Cr)	0.548 ±0.030	-
Cadmium (Cd)	0.002 ±0.001	0.006 ±0.002
Lead (Pb)	0.096 ±0.02	0.096 ±0.04

Concentration values are in triplicates determinations (± SEM), - = Absent.

This study revealed that different bioactive compounds are present in the aqueous and ethanol extracts of *A. leiocarpus*. These are associated with different pharmacological activities, with low levels of heavy metal concentrations. Thus, this study justified the claims for the folkloric application of this plant in traditional medicine to manage different ailments. Additionally, the bioactive compounds detected might be utilized in the development of novel therapeutics with different pharmacological activities.

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AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND OPTIMIZATION OF INDOMETHACIN NANOSUSPENSIONS USING DESIGN OF EXPERIMENT APPROACHES

İNDOMETAZİN İÇEREN NANOSÜSPANSİYONLARIN GELİŞTİRİLMESİ VE FAKTÖRİYEL TASARIM YAKLAŞIMI KULLANILARAK OPTİMİZASYONU

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ABSTRACT

Objective: *In this study, it was aimed to prepare nanosuspensions that contains Indomethacin which is a BCS class II drug. To assess the cumulative impact of the chosen variables on the nanosuspension properties, a 3^4 factorial design was applied and particle size and distributions were examined.*

Material and Method: *In the study, the solvent/antisolvent method was used in the preparation of the suspensions. 3^4 factorial design. Design-Expert software was used for the evaluation of the prepared formulations in order to obtain the best formulation. PVA concentration, PVA molecular weight, solvent/antisolvent ratio, and ethanol/PEG 300 ratio were used as independent design parameters, and their effects on particle size and distribution were examined.*

Result and Discussion: *Nanosuspensions were successfully prepared by the solvent/antisolvent method. Particle size and polydispersity index of the nanosuspensions were found to be affected by both molecular weight and percentage of PVA in the antisolvent phase ($p < 0.05$). 0.2% (w/v) PVA; molecular weight of 31 000 for PVA and the solvent-antisolvent ratio as 3:50 were found to be the optimal parameters for the nanosuspension formulations. The particle size and polydispersity of optimum formulation were found 301.5 ± 31.1 nm and 0.159 ± 0.035, respectively.*

Keywords: *Factorial design, indomethacin, nanosuspension, solvent/antisolvent method*

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ÖZ

Amaç: *Bu çalışmada biyofarmasötik sınıflandırma sistemine göre (BCS) 2. sınıfta bulunan indometazinin nanosüspansiyon formülasyonlarının hazırlanması ve kritik formülasyon ve işlem basamaklarının belirlenmesi amaçlanmıştır. Formülasyonların hazırlanması sırasında 3^4 faktöriyel tasarım uygulanmış ve partikül büyüklüğü ve dağılımı incelenmiştir.*

Gereç ve Yöntem: *Çalışmada, nanosüspansiyonlar solvan/antisolvan yöntemi kullanılarak hazırlanmıştır. En iyi formülasyonu elde edebilmek adına Design Expert programı ile 3^4 faktöriyel tasarım uygulanmıştır. PVA konsantrasyonu, PVA molekül ağırlığı, solvan/antisolvan oranı ve etanol/PEG 300 oranı formülasyon parametresi olarak kullanılmıştır ve bu parametrelerin değişikliğinin partikül büyüklüğü ve dağılımı üzerine olan etkisi incelenmiştir.*

Sonuç ve Tartışma: *Nanosüspansiyonlar solvan/antisolvan yöntemi ile başarılı bir şekilde hazırlanmıştır. Nanosüspansiyonların partikül boyutu ve polidispersite indeksinin hem molekül ağırlığından hem de antisolvan fazdaki PVA yüzdesinden etkilendiği bulunmuştur ($p < 0.05$). %0.2 PVA; PVA için 3000 molekül ağırlığı ve 3:50 çözücü-antisolvan oranı, nanosüspansiyon formülasyonları için optimal parametreler olarak bulunmuştur. Optimum formülasyonun partikül boyutu 301.5 ± 31.1 nm ve polidispersite indeksi 0.159 ± 0.035 olarak tespit edilmiştir.*

Anahtar Kelimeler: *Faktöriyel dizayn, indometazin, nanosüspansiyon, solvan/antisolvan metot*

INTRODUCTION

Active substances with low water solubility have some problems with bioavailability which restrict drug development [1]. Effective gastrointestinal absorption is necessary to increase these substances' oral bioavailability. When aiming to improve the bioavailability of these kinds of compounds, efforts to improve drug solubility and dissolution rate are critical elements to take into account [2]. Therefore, it is urgent to create unique drug delivery systems that can boost the therapeutic effectiveness of these pharmacological compounds. Several unique drug delivery methods have been employed to speed up the dissolving of insoluble substances, including solid dispersion, emulsion, cyclodextrins, and nanosuspensions [3,4].

Nanosuspensions have become one of the most favourable dosage forms for the delivery of active substances that are not water soluble in recent years. They are colloidal dispersions of pure active material particles that are stabilized at the nanoscale (< 1000 nm) using the proper surfactants and/or polymers. [5,6]. In nanosuspensions, the poorly water-soluble substance is suspended in a dispersion without any matrix components. Nanosized particles and the size distribution of nanosuspensions have a considerable impact on the rate of dissolution, making them important factors in determining bioavailability. Particularly, the dissolution rate is more rapid for tiny particles with large specific surfaces [1,6]. As a result, BCS Class II and IV drugs will perform better in clinical settings due to enhanced bioavailability, quick onset of action, a decreased food effect, and other favourable pharmaceutical effects [5].

There are two ways to obtain nanosuspensions: top-down and bottom-up processes. In top-down methods, large drug particles are reduced in size by using a variety of wet-milling techniques, including media milling, microfluidization, and high-pressure homogenization. In the case of the bottom-up method, the drug is dissolved in an organic solvent and it is then precipitated by adding an antisolvent while a stabilizer is present. The solvent-antisolvent method, supercritical fluid processes, spray drying, and emulsion-solvent evaporation are a few variations of this strategy [5,6].

The solvent-antisolvent method has been generally used to form nanosuspensions recently. This method has many preparation parameters such as the selection of a suitable solvent-antisolvent ratio, optimization of sonication time and selection of polymer and surfactant [7]. These important formulation and process parameters affects the specification of nanosuspension like particle size and distribution. Optimising the formulas and proving the effects of all factors are difficult to establish a relationship between the formulation variables and their interactions, experimental design is used [9]. This strategy helps to investigate and optimize the formulation and process factors inside this design space, aiding in the creation of a predictive mathematical model. The effects can be described mathematically by a factorial design and formulations can be optimized with the fewest experiments possible. [8,9].

The safety, effectiveness, and stability of nano drug delivery systems are affected by particle size and particle size distribution. The mean particle size and the range of particle size distribution are very crucial characterization criteria as they specify the saturation solubility and dissolution rate of nanosuspensions. To create stable nanosuspension, it is crucial to maintain particle uniformity and minimize size differences to avoid differing saturation solubility and concentration gradients, which will impede Ostwald ripening. The solid-state of nanoparticles in the nanosuspension also affects how efficiently the system dissolves. As a result, particle characterisation is critical for predicting the effectiveness of nano drug delivery systems both in vitro and in vivo. Nanosuspension's in vivo pharmacokinetic and biological behaviors are highly influenced by the particle size and distribution, charge, crystallinity, and shape of the particles [4,6,10,11].

This study was aimed to evaluate the preparation process of nanosuspensions which contains Indomethacin which is BCS class II with low solubility and high permeability to determine and suggest various critical processes and formulation parameters. To assess the combined impact of the chosen factors on the properties of the nanosuspension and to optimize nanosuspension formulations, 3^4 full factorial design was performed. Design-Expert software was used, and 3^4 factorial design was selected to evaluate the effects of solvent ratio, the molecular weight the of stabilizer on particle size and polydispersity index (PDI) of Indomethacin nanosuspensions in this preparation method. With obtained nanosuspensions, it was purposed to enhance the solubility of Indomethacin in water and decrease the particle size and side effects.

MATERIAL AND METHOD

Materials

Indomethacin, Polyethylene glycol 300 (PEG 300), Polyvinyl alcohol (PVA) (MW 31 000; 30 000-70 000; 70 000-100 000), Dimethyl sulfoxide (DMSO) and Ethanol were purchased from Sigma Aldrich (Germany) All other chemicals and reagents were of analytical grade.

Preparation of Nanosuspensions Containing Indomethacin

Nanosuspensions were prepared by using the solvent/antisolvent method which is one of the bottom-up preparation approach and nanosuspensions were obtained using the bath sonicator (Branson 5200, Spectralab Scientific, Ontario, Canada). The ratios and amounts of stabilizers, solvents and antisolvents for the formulations were decided according to similar studies [9,11-14]. Briefly, to prepare the solvent phase, a solution containing Indomethacin (20 mg) was prepared by using different ratios of PEG 300 and alcohol (v/v) mixture and the antisolvent phase was prepared using different molecular weights and concentrations of PVA in water (w/v) (MW 31 000; 30 000-70 000; 70 000-100 000) which were given in Table 1. Then antisolvent phase was added to the solvent phase (v/v) the in a bath sonicator at room temperature to obtain 50 ml formulation. Samples were taken after 5 minutes [13].

Experimental Design

In this study, 3^4 factorial design was used for the formation of nanosuspensions containing Indomethacin and to assess the impact of four independent variables that PEG 300/ethanol ratio (A1), PVA molecular weight (B2), PVA concentration (C3) and solvent/antisolvent ratio (D4) on particle size and PDI as dependent variables. The investigational conditions were shownin Table 1. The combinations of these parameters at the four levels were created using Design Expert 7.0 (Stat-Ease, Inc., Minneapolis, USA) software, and the statistical evaluation was applied by the quadratic model. Each variable's quantitative and qualitative impact on each response was examined. The statistical design was validated using the significant response polynomial equations produced by Design Expert software [8,15].

Particle Size and Distribution

By using photon correlation spectroscopy, the average particle size and size distribution of the particles in nanosuspensions were determined (Z3000, Nicomp, Port Richey, FL, USA). Each sample was measured in triplicate [16].

Table 1. 3^4 Factorial design and the results.

Formulation Code	Ethanol: PEG 300 (v/v)	Surfactant (MW)	Surfactant Concentra-tion(% w/v)	Solvent: Antisolvent (v/v)	Particle Size (nm) ± SD	PDI ± SD
P1	4:0	PVA 31 000	0.2	3:25	233.70±25.60	1.192±16.20
P2				3:50	301.50±31.10	0.159±0.04
P3				3:100	631.30±19.60	0.402±10.10
P4			0.3	3:25	258.10±44.50	0.767±0.48
P5				3:50	278.36±18.36	0.203±0.02
P6				3:100	134.90±47.86	0.618±4.88
P7			0.4	3:25	616.40±110.40	8.468±7.80
P8				3:50	63.25±21.17	0.717±0.24
P9				3:100	9366.20±117.40	1.13±4.59
P10	0:4		0.2	3:25	568.90±102.58	3.168±1.86
P11				3:50	796.60±661.00	0.479±0.03
P12				3:100	342.90±69.80	0.238±1.58
P13			0.3	3:25	219.10±82.90	0.887±12.30
P14				3:50	322.2±20.13	0.39±0.17
P15				3:100	327.30±88.94	0.326±7.84
P16			0.4	3:25	301.80±144.30	0.334±4.52
P17				3:50	173.00±95.74	0.825±0.17
P18				3:100	90198.30±25.20	6.823±54.10
P19	2:2		0.2	3:25	712.10±58.90	21.031±78.80
P20				3:50	253.80±15.46	0.162±0.04
P21				3:100	1212.60±156.30	0.667±55.40
P22			0.3	3:25	830.75±361.54	39.476±52.92
P23				3:50	Aggregation	Aggregation
P24				3:100	285.40±113.02	0.271±4.69
P25			0.4	3:25	153.60±88.50	0.257±1.25
P26				3:50	168.47±56.71	1.570±0.92
P27				3:100	Aggregation	Aggregation
P28	4:0	PVA 30 000-70 000	0.2	3:25	552.90±15.13	9.95±0.94
P29				3:50	264.85±0.07	0.487±0.47
P30				3:100	323.75±4.17	0.093±0.09
P31			0.3	3:25	1230.50±22.16	10.12±1.65
P32				3:50	330.85±0.07	0.055±0.02
P33				3:100	1757.05±15.31	0.589±0.09
P34			0.4	3:25	451.75±14.23	3.088±2.41
P35				3:50	1347.10±24.84	10.91±0.21
P36				3:100	4019.80±10.74	0.886±0.14
P37	0:4		0.2	3:25	411.10±0.25	1.774±0.03
P38				3:50	Aggregation	Aggregation
P39				3:100	694.25±5.20	0.369±0.002

Table 1 (*continue*). 3^4 Factorial design and the results.

P40	0:4	PVA 30 000-70 000	0.3	3:25	1481.20±21.82	75.325±12.34
P41				3:50	795.80±38.01	35.42±9.36
P42				3:100	20011.80±10.26	0.712±0.25
P43			0.4	3:25	390.60±116.20	3.423±3.30
P44				3:50	597.80±121.00	24.325±4.90
P45				3:100	479.70±15.60	2.484±2.80
P46	2:2		0.2	3:25	129.40±14.07	0.247±0.043
P47				3:50	276.93±37.70	1.16±0.75
P48				3:100	539.20±52.90	1.772±56.30
P49			0.3	3:25	814.80±113.20	3.914±5.60
P50				3:50	776.50±44.70	1.16±12.90
P51				3:100	1287.20±47.90	0.828±99.10
P52			0.4	3:25	905.00±59.30	2.735±48.60
P53				3:50	779.60±91.30	0.281±69.30
P54				3:100	2603.30±123.50	0.754±63.30
P55	4:0	PVA 70 000-100 000	0.2	3:25	893.68±48.55	21.76±20.41
P56				3:50	2557.70±335.61	60.23±59.86
P57				3:100	1943.10±743.13	8.54±11.68
P58			0.3	3:25	756.14±56.75	4.67±2.82
P59				3:50	2230.40±75.68	23.79±9.58
P60				3:100	1224.35±24.62	5.57±2.35
P61			0.4	3:25	743.70±89.00	2.756±0.02
P62				3:50	1206.60±302.90	4.574±4.190
P63				3:100	344.20±49.40	2.75±0.58
P64	0:4		0.2	3:25	504.50±90.20	0.607±55.30
P65				3:50	Aggregation	Aggregation
P66				3:100	1106.20±214.10	0.734±26.30
P67			0.3	3:25	Aggregation	Aggregation
P68				3:50	548.70±101.20	1.929±16.20
P69				3:100	Aggregation	Aggregation
P70			0.4	3:25	Aggregation	Aggregation
P71				3:50	Aggregation	Aggregation
P72				3:100	Aggregation	Aggregation
P73	2:2		0.2	3:25	6250.8±302.20	2.176±26.47
P74				3:50	4135.3±250.21	27.479±58.20
P75				3:100	10278.5±312.23	28.751±29.78
P76			0.3	3:25	4613.4±105.36	56.520±32.20
P77				3:50	25372.30±191.20	37.308±10.65
P78				3:100	5379.60±271.20	1.515±56.20
P79			0.4	3:25	Aggregation	Aggregation
P80				3:50	8062.80±128.58	67.776±21.16
P81				3:100	5647.70±2214.30	0.483±19.36

Statistical Analysis

The results of *in vitro* data were analyzed by statistical software Design Expert 7.0 (New York, USA) using ANOVA to show statistical differences ($p < 0.05$). All results are expressed as mean ± standard deviation.

RESULT AND DISCUSSION

Factorial Design of Nanosuspension Formulations

Nanosuspensions that contain Indomethacin were prepared using the solvent/antisolvent method. For nanosuspension, particle size and distribution are critical parameters for nanosuspensions dissolution and this biological performance correspondingly. Many formulation and process steps can be effective on these specifications. Researchers demonstrated that suitable solvent-antisolvent ratio, sonication time and polymer and surfactant type all affect the nanoparticle formation by solvent/antisolvent method [1,17,18]. Therefore, 3^4 factorial design was employed to calculate the impact of design factors on nanosuspension preparation and optimization. In 81 formulations, just 10 formulations precipitated. Other formulations obtained successfully. It shows that Indomethacin nanosuspensions can be prepared by this method, successfully. By excluding factors from the design model that had a p-value greater than 0.05 and calculating the model for independent variables, significant variables were found [8,19].

Effect of the Independent Variables on Nanosuspensions Containing Indomethacin

Particle Size

The mean particle size of all the batches of Indomethacin nanosuspensions were shown in Table 1. The mean particle sizes of all the formulations were found in the range of 63.267±21.17 to 90198.3±25.2 nm based on the variables of solvent/antisolvent ratio, PEG 300/alcohol ratio, PVA concentration and molecular weight. Parameters of the response surfaces obtained from a 3^4 factorial design for particle size were presented in Table 2.

The mathematical equation for particle size was:

$$= 808.34 - 2892.77\,A + 463.89\,B + 2272.33\,C + 2520.64\,D + 864.08\,AB - 2423.89\,AC - 2646.54\,AD - 2434.81\,BC - 2956.75\,BD + 2841.16\,CD + 25.88\,A^2 + 3260.07\,B^2 + 592.63\,C^2 + 1127.47\,D^2$$

ANOVA analysis indicated that ethanol/PEG 300 ratio had a significant effect on particle size (Table 2).

When the effect of independent variables on dependent variables was evaluated, it was found that particle size increased with the higher PEG 300 ratios used in formulations; however smaller particles were observed when PVA-MW 30 000-70 000 was used (Figure 1a).

Particle size was increased with the higher PEG 300 ratios used in formulations; however smaller particles were observed when the PVA concentration was 0.3 %(w/v). As the percentage of the PVA concentration increases, the particle size increases. Also, as PEG 300 ratio was higher, smaller particles were observed when the solvent: antisolvent ratio was decreased. It was shown that the particle size increased as the solvent:antisolvent ratio increased (Figure 1b-1c). PEG 300 and similar agents decrease the interfacial tension, so agglomeration become harder for the particles. Because of this reason, it was expected as the PEG 300 ratio increases, particle size decreases; but in our study we showed bigger particle size with higher PEG 300 ratio conversely. The formation of micelles above the optimal critical micel concentration (CMC) may be the cause of this increase in particle size, which becomes drug particles vulnerable [9,20]. The stabilization of nanosuspensions depends mostly on the stabilizer concentration. The concentration of surfactant should be utilized under CMC. Using insufficient stabilizer will lead to prevent the drug molecules' surface from being completely covered, which is necessary to create steric repulsion between the suspended nanoparticles. Micelles, on the other hand, will form at concentrations higher than the CMC. The produced nanosuspensions' thermal instability is

significantly influenced by the micelle formation. A concentration above CMC may actually result in less surfactant adsorption, further destabilizing the nanosuspensions and contributing to the growth of the particle size [21].

Particle size was increased with the higher PVA concentrations used in formulations; however smaller particles were observed when PVA MW 30 000-70 000 was used (Figure 1d) as the solvent:antisolvent ratio decreases, the particle size decreases with higher PVA concentration (Figure 1e). When higher PVA concentrations were used, because of the high viscosity, the input energy that we used for the formation of nanosuspension may not be enough to obtain smaller particles [5]. Also the particle size can grow as a result of the existence of a thick coating on the particle surface and diffusion between the solvent and the antisolvent is prevented during precipitation if the polymer concentration is continuously raised [18,22].

As the selected solvent:antisolvent ratio decreased, the particle size decreased and when low molecular weight of PVA was selected, the decrease in the solvent:antisolvent ratio had a greater effect on the decrease in the particle size (Figure-1f). In a similar study, researchers found similar results for solvent: antisolvent ratio. This effect might be brought on by the stabilizer's ability to bind to the drug's surface. Because stabilizer adsorbed polymer molecules which leave the surface of the drug nanocrystals and travel toward the bulk of the liquid at high volumes of antisolvent (water), it may not be able to provide enough steric stabilization or assembly for the higher number of core [11].

Table 2. Parameters of the response surfaces obtained from a 3^4 factorial design for particle size.

ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	2.62E+09	14	1.87E+08	1.857926	0.0480	significant
A-Ethanol:PEG 300	4.52E+08	1	4.52E+08	4.494372	0.0378	significant
B-PVA (MW)	11620343	1	11620343	0.115576	0.7350	
C-PVA Concentration	2.79E+08	1	2.79E+08	2.773222	0.1006	
D-Solvent:Antisolvent	3.43E+08	1	3.43E+08	3.412428	0.0692	
AB	26878759	1	26878759	0.267335	0.6069	
AC	2.12E+08	1	2.12E+08	2.103669	0.1517	
AD	2.52E+08	1	2.52E+08	2.507882	0.1181	
BC	2.13E+08	1	2.13E+08	2.12266	0.1499	
BD	3.15E+08	1	3.15E+08	3.130251	0.0815	
CD	2.91E+08	1	2.91E+08	2.890281	0.0938	
A^2	12054.06	1	12054.06	0.00012	0.9913	
B^2	1.91E+08	1	1.91E+08	1.902719	0.1724	
C^2	6321790	1	6321790	0.062876	0.8028	
D^2	22881306	1	22881306	0.227577	0.6349	
Residual	6.64E+09	66	1.01E+08			
Cor Total	9.25E+09	80				

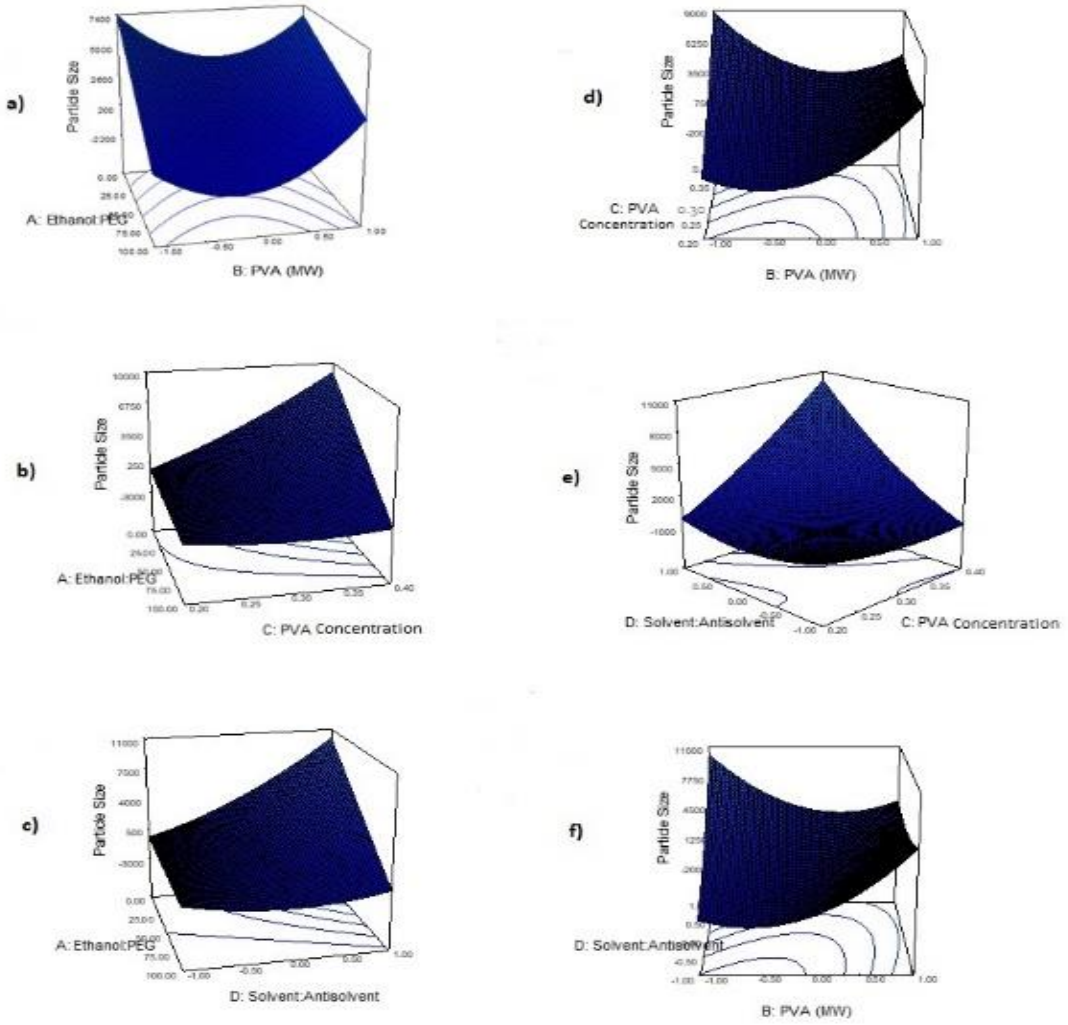

Figure 1. 3D surface response plots showing effect of factors A, B, C, and D on response on particle size.

Polydispersity Index (PDI)

The crucial characteristic known as PDI provides data on the physical stability of nanosuspensions. The PDI must be extremely low to create the perfect nanosuspension formation. When the PDI value is near to zero, the sample is said to as monodisperse. When the PDI value is less than 0.2, a limited size distribution is considered [17]. In Table 3, the PDI of each formulation is displayed. However, a polydisperse distribution is thought to exist when the PDI value is greater than 0.2. The PDI of all the batches of Indomethacin nanosuspensions were shown in Table 1. The PDI of all the formulations were found in the range of 0.159±0.035 to 75.325± 12.34 nm based on the variables. Parameters of the response surfaces obtained from a 3^4 factorial design for particle size were presented in Table 3.

The mathematical equation for particle size was:

$$= 3.76 + 0.82\ A + 3.47\ B - 0.78\ C - 1.20\ D + 1.59\ AB - 2.64\ AC - 0.93\ AD - 2.14\ BC - 0.47\ BD + 0.62\ CD + 1.36\ A^2 + 2.14\ B^2 + 1.11\ C^2 - 2.72\ D^2$$

ANOVA analysis indicated that molecular weight of PVA had a significant quadratic effect on PDI (Table 3).

The PDI was increased with the raise of the ethanol/PEG 300 ratio. Moreover smaller PDI was observed when PVA MW 31 000 was used and also when PVA concentration was 0.2% (w/v). This increase in PDI, which makes drug particles vulnerable, may be caused by the formation of micelles above the optimal CMC.

In addition that, it was observed the PDI decreased as the solvent:antisolvent ratio increased conversely high ethanol ratio. Shariare et al. found similar results in their study with furosemide nanosuspensions in 2019. It can be a result of enhanced agglomeration of drug nanoparticles at higher solvent ratios. This may be due to different mixing power between solvent and antisolvent because of the different densities of solvents used for the formulations due to different ethanol concentrations. It could result in an increased nucleation rate for crystal growth. Different mixing situation due to different density could lead to grow in crystals. [12].

The PDI was increased with the increase in the PVA concentration. As PVA concentration increases up to a certain value, PDI increases, then decreases. Similar results were found by some researchers in their studies. Researchers found higher PDI and particle size by using higher stabilizer concentration for their nanosuspension formulations [9,17]. It could be a result of formation of micelles at concentrations greater than the CMC. The nanosuspensions may become even more unstable at concentrations above CMC and may experience less surfactant adsorption, which would lead to an increase in particle size and PDI [11,21]. Another result that we foundwas smaller PDI was observed when PVA (MW 31 000) was used with higher PVA concentration.

Different molecular weights of PVA significantly affect PDI ($p<0.05$). As PVA molecular weight increases, PDI increases. In formulations prepared with large molecular weight PVA, PDI increases as the PVA concentration decreases. When using PVA (MW 70 000-100 000), the PDI increases as the percentage of PVA used decreases. When using PVA (MW 31 000), the PDI decreases as the percentage of PVA used decreases. The molecular weight and concentration of PVA have a significant impact on particle size and PDI, which may be related to the high viscosity and interfacial tension of the aqueous phase. PVA grades with significant levels of hydrolysis have reportedly been shown to be poorly soluble in water. PVA's solubility, viscosity, and surface tension are all influenced by the material's molecular weight, concentration, hydrolysis percentage, and temperature. As a result, PVA's high molecular weight and concentration formed particles with a wider size distribution [23]. It can be explained that larger molecular weight of PVAwhich formed more dense solutions are better at protecting the drug moiety than smaller molecular weight of PVA formed less viscose solution. In addition, compared to larger MW, stabilizer molecules migrate at a slower rate at the drug-polymer interface at less viscose polymer solution. The solution's viscosity increases as the number of collisions, which further slows the rate of mass transfer from the solution to the solid-liquid interface through diffusion [9,17].

Table 3. Parameters of the response surfaces obtained from a 3^4 factorial design for PDI.

ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	1627.153	14	116.2252	2.46472	0.0073	significant
A-Ethanol:PEG 300	36.09344	1	36.09344	0.765412	0.3848	
B-PVA (MW)	649.2131	1	649.2131	13.76748	0.0004	significant
C-PVA Concentration (% w/v)	32.71646	1	32.71646	0.693799	0.4079	
D-Solvent:Antisolvent	77.3286	1	77.3286	1.639862	0.2048	
AB	91.48444	1	91.48444	1.940057	0.1683	
AC	250.5203	1	250.5203	5.312637	0.0243	significant
AD	31.29843	1	31.29843	0.663727	0.4182	

Table 3 (*continue*). Parameters of the response surfaces obtained from a 3^4 factorial design for PDI.

BC	165.3089	1	165.3089	3.505608	0.0656	
BD	7.821344	1	7.821344	0.165863	0.6851	
CD	13.82724	1	13.82724	0.293226	0.5900	
A^2	33.11804	1	33.11804	0.702315	0.4050	
B^2	82.80201	1	82.80201	1.755934	0.1897	
C^2	22.1593	1	22.1593	0.469919	0.4954	
D^2	133.4615	1	133.4615	2.830239	0.0972	
Residual	3112.266	66	47.15555			
Cor Total	4739.419	80				

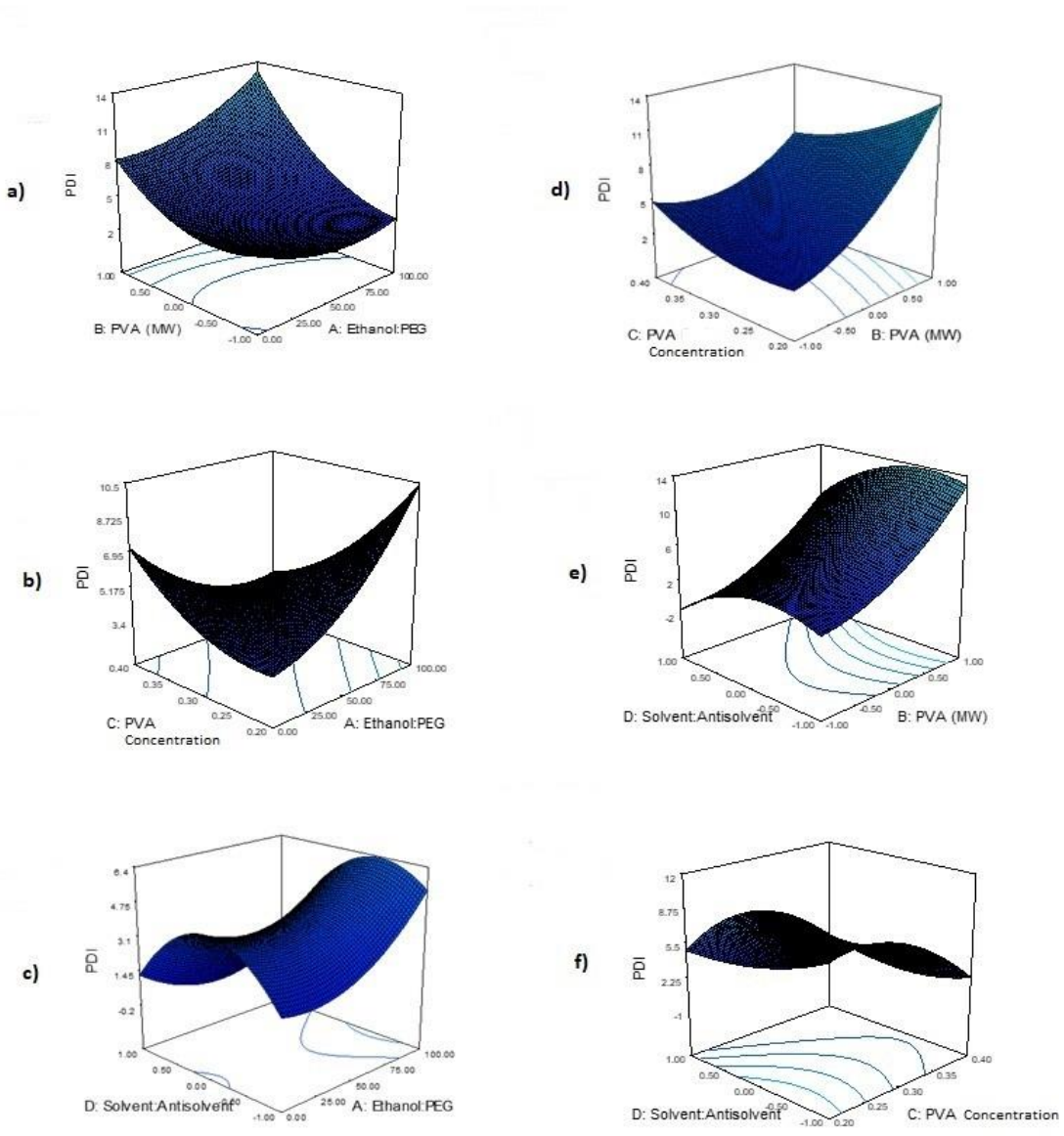

Figure 2. 3D surface response plots showing effect of factors A, B, C, and D on response on PDI

Determination of Optimal Formulation

ANOVA was used to investigate the ideal Indomethacin nanosuspension formulation, and the F test was used to evaluate each parameter. Accordingly, the Design Expert analysis of nanosuspensions was optimized based on the criteria of desired particle size and low PDI values. Table 4 shows the results according to experimental design.

Table 4. The optimum parameters according to experimental design results.

Parameters	Value
PVA concentration	0.2 % (w/v)
PVA molecular weight	31 000
Solvent/antisolvent ratio	3:50

In this study, our aim was to develop nanosuspensions including Indomethacin to overcome water-solubility problem of drug, so to increase the oral bioavalaibility. To obtain the optimum formulation Design-Expert program was used and 3^4 factorial design was planned for the preparation of formulations. Ethanol/PEG 300 ratio, PVA molecular weight, PVA concentration and solvent/antisolvent ratio were used as independent parameters for design and the effect of these parameters on particle size and distribution were evaluated.

Both molecular weight and concentration of PVA in the antisolvent phase were found to affect the particle size and polydispersity index of the nanosuspensions ($p<0.05$). The optimum parameters were found to be 0.2% (w/v) PVA (MW. 31 000) with a solvent-antisolvent ratio of 3:50 (particle size: 301.5 ± 31.1 nm, polydispersity index: 0.159 ± 0.035). As a result, nanosuspension formulations were successfully prepared using the solvent/antisolvent method. Therefore, it can be concluded that both the type and percent of stabilizer is important to obtain stable nanosuspensions.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DESIGN, SYNTHESIS AND MOLECULAR DOCKING OF SOME DERIVATIVES OF 9-METHYLPYRAZOLO[1,5-*d*][1,2,4]TRIAZOLO [3,4-*f*][1,2,4]TRIAZINE-3-THIOL

BAZI 9-METİLPİRAZOLO[1,5-d][1,2,4]TRİAZOLO[3,4-f][1,2,4]TRİAZİN-3-TİYOL TÜREVLERİNİN TASARIMI, SENTEZİ VE MOLEKÜLER YERLEŞTİRİLMESİ

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ABSTRACT

Objective: *The purpose of the work was to elaboration effective techniques for the synthesis of advanced condensed heterocyclic systems based on pyrazole and 1,2,4-triazole. In the process of realizing the set goal, a number of new pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazines have been synthesized.*

Material and Method: *Identity of synthesized compounds has been confirmed by elemental analysis, ^{1}H-NMR, LC-MS techniques. The pharmacological potential of the obtained substances has been determined by molecular docking method.*

Result and Discussion: *The optimal conditions for the preparation of pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazines has been determined. Based on the results of molecular docking, a row of substances with high potential for anti-inflammatory and antifungal activity has been identified. In silico studies were carried out using the models of cyclooxygenase-2, lanosta-8,24-dien-3β-ol 14α-demethylase, anaplastic lymphoma kinase.*

Keywords: *1,2-Diazole, 1,2,4-triazole, chemistry, molecular docking, transformation*

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ÖZ

Amaç: *Çalışmanın amacı, pirazol ve 1,2,4-triazole dayalı gelecek vaat eden kondanse heterosiklik sistemlerin sentezi için etkili yöntemler geliştirmektir. Belirlenen hedefi gerçekleştirme sürecinde, bir dizi yeni pirazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazin sentezlenmiştir.*

Gereç ve Yöntem: *Sentezlenen bileşiklerin yapısı, element analizi, ^{1}H-NMR, LC-MS teknikleri ile doğrulanmıştır. Elde edilen maddelerin farmakolojik potansiyeli moleküler modelleme yöntemi ile belirlenmiştir.*

Sonuç ve Tartışma: *Pirazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazinlerin hazırlanması için optimal koşullar belirlendi. Moleküler yerleştirme sonuçlarına dayanarak, anti-inflamatuar ve antifungal aktivite için yüksek potansiyele sahip bir dizi madde tanımlanmıştır. Siklooksijenaz-2, lanosta-8,24-dien-3β-ol 14α-demetilaz, anaplastik lenfoma kinazlarının modelleri kullanılarak in siliko çalışmalar yürütülmüştür.*

Anahtar Kelimeler: *1,2-Diazol, 1,2,4-triazol, dönüşüm, kimya, moleküler yerleştirme*

INTRODUCTION

Organic compounds of heterocyclic nature provide a wide range of opportunities for scientists in the field of medical chemistry. And here, special attention is focused on cycles with Nitrogen [1-4]. Among such compounds, we can note 1,2-diazole and 1,2,4-triazole, as well as derivatives of these structures [5]. Wide possibilities of chemical modification, availability of reagents, high pharmacological potential. It is these factors that determine the choice of research topic in favor of these groups of compounds [6-9]. The combination of these heterocycles within the same structure will contribute to the emergence of new biological properties or enhance existing properties [10,11]. In addition, the choice of heterocyclic system data makes studies of anti-inflammatory, antifungal and anti-cancer activity justified. Such a polypharmacological profile only increases the relevance and practical significance of research related to the creation of polycondensed heterocyclic systems and examine their characteristics.

The aim of the chosen direction of scientific work was to design and synthesize new derivatives of 9-methylpyrazolo[1,5-*d*][1,2,4]triazolo[3,4-*f*][1,2,4]triazine, followed by establishing the pharmacological potential of the synthesized number of compounds by computer chemistry methods.

MATERIAL AND METHOD

Chemistry

The first stage of scientific work was represented by the synthetic part. For its implementation, a pyrazole fragment was first constructed. For this purpose, freshly prepared sodium methylate and a mixture of acetone and diethyloxalate were used as starting materials (Figure 1) [12,13]. The engagement of these compounds contributed to the creation of an intermediate in the form of ethyl 2,4-dioxopentanoate (1.1). The synthesized compound 1.1 in the reaction of interaction with hydrazine hydrate made it possible to form a hydrazide of formula 1.2. Further, the resulting 5-methylpyrazole-3-carbohydrazide was converted to xanthogenate of formula 1.3 by carbon disulfide in a 9% solution of potassium hydroxide in butan-1-ol. The nature of the subsequent transformation was determined by applying a double amount of hydrazine hydrate, which made it possible to obtain 4-amino-5-(5-methylpyrazol-5-yl)-1,2,4-triazole-3-thiol (**2**). The presence of SH-group made the alkylation process, which was carried out with the participation of bromoalkanes, convenient. At the last stage of the chemical part of the work, optimal conditions for the reaction of an alkylderived thiol of formula **2** with triethoxymethane were established. All chemicals, which mentioned in this work, were obtained from "UKRORGSYNTEZ Ltd" with documental approving of its purity and quality.

Proof of the chemical structure, purity and individuality of chemical conversion products is implemented employing common physical-and-chemical methods of analysis. The melting temperature range was determined by capillary method using SRS Inc MPA 100 equipment. The qualitative composition of the elements and their quantitative ratio were determined using the CHNS analyzer "VarioELcube". Proton-NMR spectroscopy spectra have been received using a Varian Mercury 400

spectrometer (internal standard - tetramethylsilane in dimethylsulfoxide-d_6 solution). Chromato-mass spectrometry was performed on the basis of analytical HPLC systems "1260 Infinity" (Agilent), which is equipped with a spectrometer "6120" (Agilent); ionization was performed by spraying in an electric field [15].

Molecular Docking

The second part of the scientific research was related to the preliminary determination of the potential of biological activity using computer simulations. This area of research was implemented using the molecular docking method.

Model enzyme complexes (cyclooxygenase-2, lanosterol 14α-dimethylase, receptor tyrosine kinase) with standard ligands were loaded from a Protein Data Bank. The choice of these enzymes was dictated by the properties of pharmacophore moiety in a number of target products of the chemical transformation [16,17].

The selection of biotargets were determined by the presence of similar pharmacophore snippets in the structure of substances received along with some known drugs.

Molecular docking was performed in stages. First of all, ligands were prepared, which included: 1) using the software product MarvinSketch-6.3.0 (forming the structure of the studied compounds and saving them in mol format); 2) using the Chem 3D program and a molecular mechanical algorithm, followed by saving the results in pdf files (optimizing the structures of the studied molecules); 3) working in AutoDockTools-1.5.6 and reproducing the results as pdbqt format (adding polar Hydrogen atoms) [18-20].

Then the enzyme was prepared, which included: 1) using the Discovery Studio 4.0 software and then saving the results in pdb format (removing the original ligands and water molecules); 2) using AutoDockTools-1.5.6 (converting the pdb file format to pdbqt). As a result, molecular docking was performed, which included: 1) the use of Vina software (obtaining intermolecular interaction energy values); 2) the use of the AutoDockTools-1.5.6 program (visualization of the results of the study).

RESULT AND DISCUSSION

Chemistry

The path of obtaining target chemical transformation products is shown in Figure 1. Methods for obtaining compounds 1, 1.1, 1.2**,** and 2 and their basic physico-chemical constants are described in our previous works [12,13]. Substance 1.3 was resynthesized according to the described technique [14]. The main physical and chemical constants of substance 1.3 correspond to known values [14]. The obtaining and characteristics of *S*-alkyl substituted of 4-amino-5-(5-methylpyrazol-3-yl)-1,2,4-triazole-3-thiol (2.1-2.10) were also described in previous work [13]. The final products of chemical conversion (2.12-2.20) were obtained by heating *S*-alkyl derivatives 2.1-2.10 with triethyl orthoformate to a boil for 12 hours.

9-Methyl-3-(alkylthio)pyrazolo[1,5-*d*][1,2,4]triazolo[3,4-*f*][1,2,4]triazine (2.11-2.20)

Compounds 2.1-2.10 (0.01 mol) were placed in a round-bottomed flask, 40 ml of triethyl orthoformate was added and boiled for 12 hours. Excess triethyl orthoformate was removed by low-pressure distillation. The reaction products were recrystallized from propan-2-ol.

9-Methyl-3-(methylthio)pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.11)

White crystalline solid in 64% yield, m. p. (°C): 212 – 214. ^{1}H-NMR (400 MHz), δ (ppm): 8.95 (s, 1H, Triazine CH), 7.73 (s, 1H, Pyrazole CH), 2.76 (s, 3H, S-CH_3), 2.45 (s, 3H, CH_3-pyrazole). ESI MS (m/z): $[M+H]^+$ at 221. Elemental analysis (EA) ($C_8H_8N_6S$), calculated, %: C - 43.63, H - 3.66, N - 38.16, S - 14.56; obtained, %: C - 43.74, H - 3.65, N - 38.06, S - 14.60.

3-(Ethylthio)-9-methylpyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.12)

White crystalline solid in 67% yield, m. p. (°C): 186 – 184. ^{1}H-NMR (400 MHz), δ (ppm): 8.92 (s, 1H, Triazine CH), 7.76 (s, 1H, Pyrazole CH), 3.24 (q, J=6.2 Hz, 2H, S-C$\underline{H}_2$-CH_3), 2.44 (s, 3H,

Pyrazole-CH_3), 1.32 (t, *J*=6.0 Hz, 3H, S-CH_2-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 234. EA ($C_9H_{10}N_6S$), calculated, %: C - 46.14, H - 4.30, N - 35.87, S - 13.68; obtained, %: C - 46.02, H - 4.31, N - 35.78, S - 13.71.

9-Methyl-3-(propylthio)pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.13)

White crystalline solid in 81% yield, m. p. (°C): 170 – 172. ^{1}H-NMR (400 MHz), δ (ppm): 8.94 (s, 1H, Triazine CH), 7.74 (s, 1H, Pyrazole CH), 3.12 (t, *J*=5.1 Hz, 2H, S-C$\underline{H}_2$-C_2H_5), 2.45 (s, 3H, Pyrazole-CH_3), 1.78 (q, *J*=5.3 Hz, 2H, S-CH_2-C$\underline{H}_2$-CH_3), 1.07 (t, *J*=7.0 Hz, 3H, S-$(CH_2)_2$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 249. EA ($C_{10}H_{12}N_6S$), calculated, %: C - 48.37, H - 4.87, N - 33.85, S - 12.91; obtained, %: C - 48.49, H - 4.88, N - 33.76, S - 12.87.

3-(Butylthio)-9-methylpyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.14)

White crystalline solid in 74% yield, m. p. (°C): 179 – 181. ^{1}H-NMR (400 MHz), δ (ppm): 8.96 (s, 1H, Triazine CH), 7.73 (s, 1H, Pyrazole CH), 3.27 (t, *J*=6.7 Hz, 2H, S-C$\underline{H}_2$-C_3H_7, 2.42 (s, 3H, Pyrazole-CH_3), 1.72–1.63 (m, 2H, S-CH_2-C$\underline{H}_2$-C_2H_5), 1.41–1.36 (m, 2H, S-$(CH_2)_2$-C$\underline{H}_2$-CH_3), 0.92 (t, *J*=7.1 Hz, 3H, S-$(CH_2)_3$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 263. EA ($C_{11}H_{14}N_6S$), calculated, %: C - 50.36, H - 5.38, N - 32.04, S - 12.22; obtained, %: C - 50.49, H - 5.39, N - 31.95, S - 12.19.

9-Methyl-3-(pentylthio)pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.15)

White crystalline solid in 77% yield, m. p. (°C): 173 – 175. ^{1}H-NMR (400 MHz), δ (ppm): 8.93 (s, 1H, Triazine CH), 7.76 (s, 1H, Pyrazole CH), 3.28 (t, *J*=6.0 Hz, 2H, S-C$\underline{H}_2$-C_4H_9, 2.44 (s, 3H, Pyrazole-CH_3), 1.78–1.74 (m, 2H, S-CH_2-C$\underline{H}_2$-C_3H_7, 1.45–1.33 (m, 4H, S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-CH_3), 0.93–0.84 (m, 3H, S-$(CH_2)_4$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 277. EA ($C_{12}H_{16}N_6S$), calculated, %: C - 52.15, H - 5.84, N - 30.41, S - 11.60; obtained, %: C - 52.01, H - 5.85, N - 30.48, S - 11.56.

3-(Hexylthio)-9-methylpyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.16)

White crystalline solid in 65% yield, m. p. (°C): 188 – 190. ^{1}H-NMR (400 MHz), δ (ppm): 8.95 (s, 1H, Triazine CH), 7.74 (s, 1H, Pyrazole CH), 3.27 (t, *J*=6.4 Hz, 2H, S-C$\underline{H}_2$-C_5H_{11}, 2.42 (s, 3H, Pyrazole-CH_3), 1.69–1.64 (m, 2H, S-CH_2-C$\underline{H}_2$-C_4H_9, 1.42–1.30 (m, 2H, S-$(CH_2)_2$-C$\underline{H}_2$-C_3H_7, 1.39–1.26 (m, 4H, S-$(CH_2)_3$-(C$\underline{H}_2$)$_2$-CH_3), 0.94–0.89 (m, 3H, S-$(CH_2)_5$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 291. EA ($C_{13}H_{18}N_6S$), calculated, %: C - 53.77, H - 6.25, N - 28.94, S - 11.04; obtained, %: C - 53.62, H - 6.24, N - 29.01, S - 11.07.

3-(Heptylthio)-9-methylpyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.17)

White crystalline solid in 71% yield, m. p. (°C): 194 – 196. ^{1}H-NMR (400 MHz), δ (ppm): 8.94 (s, 1H, Triazine CH), 7.75 (s, 1H, Pyrazole CH), 3.28 (t, *J*=6.2 Hz, 2H, S-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 2.45 (s, 3H, Pyrazole-CH_3), 1.67–1.63 (m, 2H, S-CH_2-C$\underline{H}_2$-C_5H_{11}, 1.44–1.35 (m, 2H, S-$(CH_2)_2$-C$\underline{H}_2$-C_4H_9, 1.34–1.26 (m, 6H, S-$(CH_2)_3$-(C$\underline{H}_2$)$_3$-CH_3), 0.93–0.88 (m, 3H, S-$(CH_2)_6$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 305. EA ($C_{14}H_{20}N_6S$), calculated, %: for: C - 55.24, H - 6.62, N - 27.61, S - 10.53; obtained, %: C - 55.11, H - 6.61, N - 27.68, S - 10.56.

9-Methyl-3-(octylthio)pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.18)

White crystalline solid in 63% yield, m. p. (°C): 187 – 189. ^{1}H-NMR (400 MHz), δ (ppm): 8.96 (s, 1H, Triazine CH), 7.74 (s, 1H, Pyrazole CH), 3.26 (t, *J*=6.3 Hz, 2H, S-C$\underline{H}_2$-C_7H_{15}, 2.44 (s, 3H, Pyrazole-CH_3), 1.69–1.64 (m, 2H, S-CH_2-C$\underline{H}_2$-C_6H_{13}, 1.40–1.23 (m, 10H, m, 2H, S-$(CH_2)_2$-(C$\underline{H}_2$)$_5$-CH_3), 0.94–0.89 (m, 3H, S-$(CH_2)_7$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 319. EA ($C_{15}H_{22}N_6S$), calculated, %: C - 56.58, H - 6.96, N - 26.39, S - 10.07; obtained, %: C - 56.43, H - 6.98, N - 26.46, S - 10.04.

9-Methyl-3-(nonylthio)pyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.19)

White crystalline solid in 68% yield, m. p. (°C): 186 – 184. ^{1}H-NMR (400 MHz), δ (ppm): 8.95 (s, 1H, Triazine CH), 7.76 (s, 1H, Pyrazole-CH_3), 3.27 (t, *J* = 6.5 Hz, 2H, S-C$\underline{H}_2$-$(CH_2)_7$-CH_3), 2.44 (s, 3H, CH_3-pyrazole), 1.68–1.64 (m, 2H, S-CH_2-C$\underline{H}_2$-C_7H_{15}, 1.41–1.30 (m, 2H, S-$(CH_2)_2$-C$\underline{H}_2$-C_6H_{13}, 1.33–1.21 (m, 10H, S-$(CH_2)_3$-(C$\underline{H}_2$)$_5$-CH_3), 0.91–0.86 (m, 3H, S-$(CH_2)_8$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$

at 333. EA ($C_{16}H_{24}N_6S$), calculated, %: C - 57.80, H - 7.28, N - 25.28, S - 9.64; obtained, %: C - 57.93, H - 7.26, N - 25.22, S - 9.67.

3-(Decylthio)-9-methylpyrazolo[1,5-d][1,2,4]triazolo[3,4-f][1,2,4]triazine (2.20)

White crystalline solid in 74% yield, m. p. (°C): 191 – 193. ^{1}H-NMR (400 MHz), δ (ppm): 8.95 (s, 1H, Triazine CH), 7.76 (s, 1H, Pyrazole CH), 3.25 (t, J=6.5 Hz, 2H, S-C$\underline{H}_2$-C_9H_{19}, 2.45 (s, 3H, Pyrazole-CH_3), 1.73–1.64 (m, 2H, S-CH_2-C$\underline{H}_2$-C_8H_{17}), 1.40–1.20 (m, 12H, S-$(CH_2)_3$-(C$\underline{H}_2$)$_6$-CH_3), 0.89–0.84 (m, 3H, S-$(CH_2)_9$-C$\underline{H}_3$). ESI MS (m/z): $[M+H]^+$ at 347. EA ($C_{17}H_{26}N_6S$), calculated, %: C - 58.93, H - 7.56, N - 24.25, S - 9.25; obtained, %: C - 58.84, H - 7.57, N - 24.21, S - 9.23.

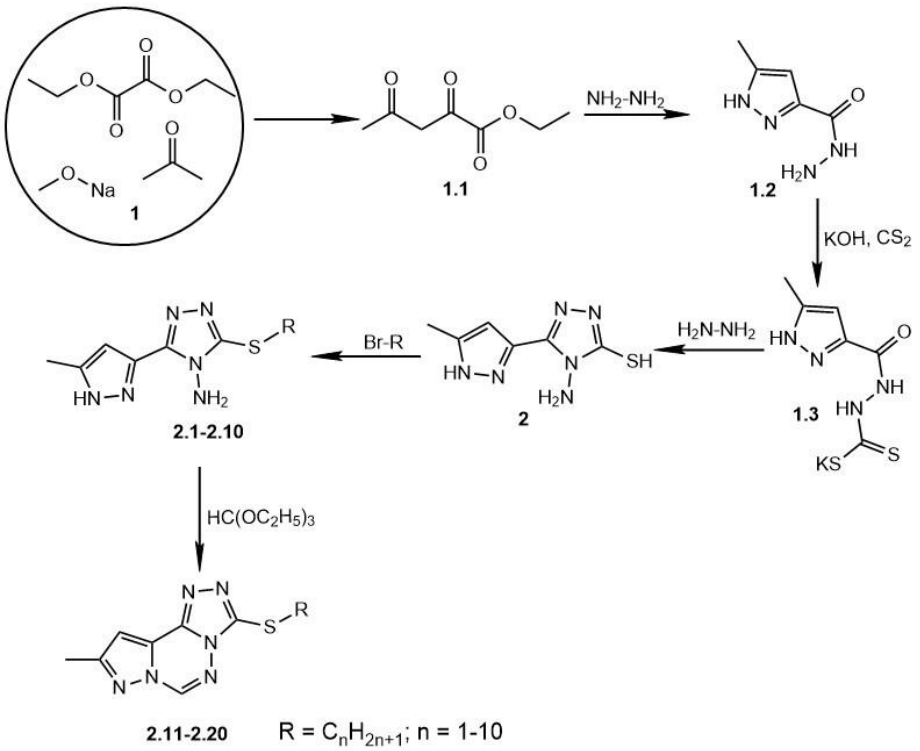

Figure 1. The scheme for the synthesis of target products of chemical transformation

The structure of all *S*-alkyl derivatives (2.11-2.20) was confirmed by physico-chemical methods, in particular by ^{1}H-NMR spectroscopy.

In the ^{1}H-NMR spectra of the final reaction products (2.11-2.20), the Hydrogen of the 1,2,4-triazine cycle forms a clear singlet signal, which has been registered at 8.96-8.92 ppm. The methine moiety of the pyrazole fragment forms a singlet at 7.76-7.73 ppm the methyl substitute of the pyrazole ring forms a three-proton singlet signal at 2.45-2.42 ppm.

The formation of *S*-alkyl derivatives is expected to be accompanied by the appearance of alkyl fragments in the aliphatic part of the proton signal spectrum. For example, in the spectrum of compound 2.11, a signal is recorded in the form of a singlet at 2.76 ppm, which is due to a methyl group bound to Sulfur.

The methylene group of the ethyl substitute of compound 2.12 is registered on the spectrum in the form of a quadruplet at 3.24 ppm the methyl fragment of this substitute forms a triplet at 1.32 ppm.

The propyl substituent of compound 2.13 contributes to the appearance on the spectrum of two triplets at 3.12 ppm and 1.07 ppm respectively, as well as a quadruplet at 1.78 ppm. Proton signals of methylene fragments of other compounds (2.14-2.20) are also recorded in the stronger magnetic field between 3.28-1.20 ppm. The elongation of the *S*-alkyl chain results in a small displacement in the proton' signals of CH_3-group to the stronger part of the field.

For example, the proton signals of the CH_3 group of the decyle substituent (-$(CH_2)_9$-C$\underline{H}_3$) manifest as a multiplet in the range 0.89-0.84 ppm. Proton signals of most methylene fragments (CH_2-C$\underline{H}_2$-C_8H_{17}, -$(CH_2)_3$-(C$\underline{H}_2$)$_6$-CH_3) also appear as multiplets in the intervals of 1.73-1.64 ppm and 1.40-1.20 ppm, but they are difficult to differentiate between each other. At the same time, the proton signals of the methylene fragment, which is directly bound to the Sulfur (C$\underline{H}_2$-C_9H_{19}), manifest as a triplet and are fixed in a weaker field at 3.25 ppm.

The mass spectra of compounds 2.11-2.20 contain peaks of molecular ions that correspond to the molecular weight. The quantitative deviation during the elemental analysis was within ± 0.3 %.

Molecular Docking

The creation of new original drugs takes into account the use of a wide range of methods that can reveal the true level of biological potential of new molecules. Among such methods, one of the primary and main is molecular docking. This method makes it possible to reliably determine the biological target for binding to the ligand and helps to rationally approach the process of creating potential candidates for the drug substance [21,22].

Analyzing the similarity of the composition of the obtained substances to the structure of known drugs, it was decided to test the possibility of the effect of the studied substances on enzymes that are associated with the inflammatory process, antifungal activity and anti-cancer effect.

The study was based on modeling complexes with cyclooxygenase-2, lanosterol 14α-demethylase, and receptor tyrosine kinase. These actions made it possible to successfully obtain the bond energy values of the studied ligand to the receptor protein and perform a visual assessment.

The results were evaluated in comparison with celecoxib, which has a pharmacophore pyrazole fragment similar to the synthesized compounds. It is a well-known fact that the nature of the interaction of celecoxib with the active COX-2 center is very diverse. This phenomenon is due to the fact that almost all structural fragments of celecoxib are involved in the interaction. The most important structural fragment that directly forms bonds with the hydrophilic "pocket" of COX-2 is the polar sulfamide group of celecoxib. An equally important role in the interaction is played by the conjugated p-π system of pyrazole, which contacts the residues Ala A: 528 and Val A: 350 (Figure 2).

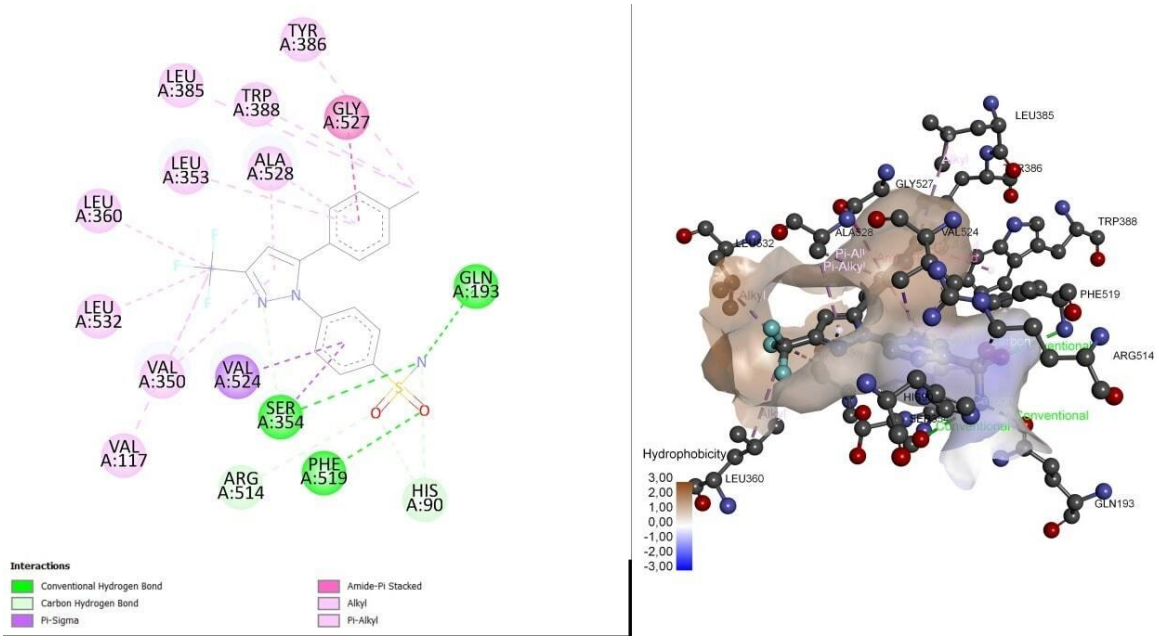

Figure 2. Visualization scheme for binding celecoxib to COX-2

Studies of the interactions of synthesized substances (2.11-2.20) with the active site of COX-2 helped to establish a certain level of similarity with celecoxib. The active participation of the thioalkyl residue in the formation of additional contacts with COX-2 was also demonstrated (Table 1).

Table 1. Main types of interactions between the studied compounds and amino acid residues of COX-2

	Nature of the amino acid residue
2.11	Ala A: 528, Leu A: 385, Phe A: 519, Met A: 523, Trp A: 388, Val A: 350, Val A: 524, Tyr A: 386, Val A: 117
2.12	Ala A: 528, Leu A: 353, Phe A: 519, Trp A: 388, Tyr A: 356, Val A: 350, Val A: 524, Leu A: 532, Val A: 117
2.13	Ala A: 528, Leu A: 353, Phe A: 519, Trp A: 388, Tyr A: 356, Leu A: 93, Val A: 89, Val A: 117
2.14	Ala A: 528, Trp A: 388, Leu A: 353, Phe A: 519, Tyr A: 386, Tyr A: 356, Val A: 350, Val A: 524, Val A: 117, Tyr A: 116
2.15	Ala A: 528, Tyr A: 386, Trp A: 388, Val A: 524, Val A: 350, Tyr A: 356, Val A: 117, Leu A: 93, Val A: 89, Tyr A: 116
2.16	Ala A: 528, Gly A: 527, Tyr A: 386, Trp A: 388, Leu A: 385, Val A: 524, Val A: 350, Tyr A: 356, Val A: 117, Leu A: 93, Tyr A: 116, Ser A: 531
2.17	Ala A: 528, Gly A: 527, Tyr A: 386, Trp A: 388, Leu A: 385, Val A: 524, Ser A: 531, Val A: 350, Tyr A: 356, Val A: 117, Leu A: 93, Val A: 89, Tyr A: 116
2.18	Ala A: 528, Leu A: 385, Val A: 350, Trp A: 349, Leu A: 532, Val A: 524, Tyr A: 386, Val A: 117, Tyr A: 116, Leu A: 93, Ile A: 113, Trp A: 100
2.19	Ala A: 528, Gly A: 527, Tyr A: 386, Leu A: 385, Trp A: 388, Phe A: 382, Val A: 524, Val A: 350, Tyr A: 356, Leu A: 93, Tyr A: 116, Val A: 117, Ile A: 113
2.20	Ala A: 528, Gly A: 527, Tyr A: 386, Phe A: 382, Leu A: 385, Trp A: 388, Val A: 524, Ser A: 531, Val A: 350, Leu A: 353, Tyr A: 356, Val A: 117, Leu A: 93, Tyr A: 116, Ile A: 113

For example, compound 2.11 performs the π-σ interaction using fragments Ala A: 528 and Val A: 350, to which 1,2,4-triazole and 1,2,4-triazine fragments are added. It helps to coordinate the ligand in the active center region and the π-alkyl interaction, which involves Leu A: 532 and Val A: 524 residues. And here the CH_3 group of pyrazole synthon makes a very significant contribution, and this is realized using Leu A: 353, Phe A: 519 and Trp A: 388. The hydrogen bond also makes a feasible contribution to the implementation of this interaction. And here the endocyclic Nitrogen atom of the triazole cycle and Tyr A: 356 actively help. The complicated complex of interactions with COX-2 π-S is enhanced by contact with the Tyr A: 356 residue and Van der Waals forces involving Gly A: 527, Ser A: 531 and Tyr A: 386.

A rise of C-atoms amount in the structure of the alkyl substituent contributed to a modest rise in the number of chemical bonds that are formed with amino acid residues. In this case, the qualitative and quantitative indicators of the interaction process were complex and complex in nature, but in most cases, it is the hydrophobic nature of the interaction (alkyl, π-σ and π-alkyl interactions) that prevails. For example, the thioethyl substitute (2.12) contributes to the formation of an alkyl type of interaction with the Val A: 117 residue. The thiopropyl fragment of compound 2.13 causes additional alkyl interactions involving Ala A: 528, Leu A: 93, and Val A: 89 residues. But at the same time, this process is compensated by an increase in the number of interactions with amino acid residues (Leu A: 353, Phe A: 519, Trp A: 388, Tyr A: 386), which occurs through the CH_3 group of the pyrazole ring. A rise in the size of the thioalkyl moiety to 5 carbon atoms (2.15) is again followed by a reduce the number of alkyl interactions with amino acid residues, which suggests a more pronounced engagement of the molecule with the enzyme binding place. However, simultaneously, the loss of intermolecular hydrogen chemical bonds is recorded, which is explained by an elongation of the hydrophobic moiety and a significant change in the orientation of the triazole fragment in the binding site. It is the Nitrogen atoms of the triazole cycle that are responsible for the formation of this type of chemical bond. It is also necessary to pay attention to the fact that S-alkyl substituted in a series of obtained substances with the number of

carbon atoms from 1 to 4 are additionally stabilized at the COX-2 π-S binding site by the interaction of the ligand with the amino acid residue Tyr A: 356 (2.11-2.14) (Figure 3).

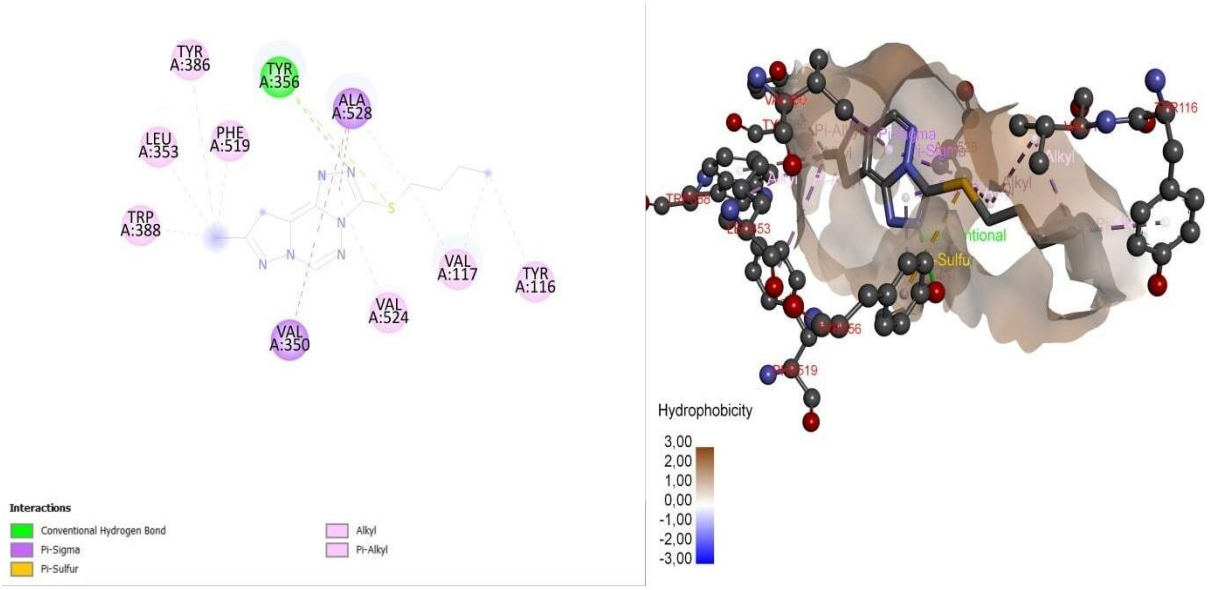

Figure 3. Visualization scheme for binding compound 2.14 to COX-2

The minimum free binding energy of the synthesized compounds to COX-2 is in the range of -4.9 ... -7.5 kcal × mol^{-1}, which, together with the results of visualization of docking with this enzyme, does not allow us to confidently speak about the high probability of anti-inflammatory activity. However, some compounds (2.14-2.16) in the future would be interesting to study for anti-inflammatory activity *in vitro* and *in vivo* (Table 2).

Table 2. Energy metrics of the molecular engagement involving COX-2

№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$
2.11	-6.4	2.15	-7.1	2.19	-4.9
2.12	-6.6	2.16	-5.9	2.20	-4.9
2.13	-7.1	2.17	-5.2	*Celecoxib*	-13.4
2.14	-7.5	2.18	-4.9		

* ε_{min} - The minimum energy of complex formation.

The vast majority of known antifungal drugs have one or more heterocyclic fragments in their structure. The most well-known such fragments include 1,2,4-triazole, imidazole, and pyridine. This type of interaction (Figure 4) contributes to the loss of lanosterol 14α-demethylase activity and disruption of the synthesis of ergosterol, which is a structural component of the fungal cell membrane.

With a view to assessing the possible influence of the obtained ligands (2.11-2.20) on the activity of this enzyme, docking studies were conducted with visualization of the results. The synthesized compounds have the ability to affect lanosterol 14α-demethylase, which is realized due to a set of certain types of interactions with the active center of this enzyme (Table 3).

For example, alkyl substituents in the structure of synthesized substances (2.11-2.20) actively affect the formation of hydrophobic interactions that occur with the participation of the corresponding fragments of amino acids of the specified demethylase (Ala A: 76, Ala A: 88, Ile A: 64, Phe A: 84, Tyr A: 92) (Figure 5).

Table 3. Main types of interactions between the studied compounds and amino acid residues of lanosterol 14α-demethylase

	Nature of the amino acid residue
2.11	Ala A: 88, Ile A: 64, Phe A: 84, Lys A: 79, Lys B: 79
2.12	Ala A: 76, Ala A: 88, Lys A: 79, Lys B: 79, Ser A: 80, Phe A: 84, Tyr A: 92, Ile A: 64
2.13	Ala A: 76, Ala B: 76, Ala A: 88, Ser A: 80, Ser B: 80, Phe A: 84
2.14	Ala A: 76, Ala B: 76, Ala A: 88, Ser A: 80, Ser B: 80, Phe A: 84, Phe B: 84, Ile A: 64, Tyr A: 92, Lys B: 79
2.15	Ala A: 76, Ala B: 76, Ala A: 88, Glu A: 83, Ile A: 64, Ser A: 80, Ser B: 80, Phe A: 84, Tyr A: 92
2.16	Ala A: 76, Ala B: 76, Ala A: 88, Glu A:83, Phe B: 84, Phe A: 84, Tyr A: 92, Ile A: 64
2.17	Ala A: 76, Ala A: 88, Glu A: 83, Phe B: 84, Phe A: 84, Ser A: 80, Tyr A: 92, Ile A: 64
2.18	Ala A: 76, Ala A: 88, Glu A: 83, Lys A: 79, Ser A: 80, Phe B: 84, Phe A: 84, Tyr A: 92, Ile A: 64
2.19	Ala A: 76, Ala A: 88, Asn A: 87, His A: 73, Ile A: 64, Phe A: 84, Ile A: 64
2.20	Ala A: 76, Ala A: 88, Ile A: 64, Phe A: 84, Phe B: 84, Ile A: 64, Tyr A: 92

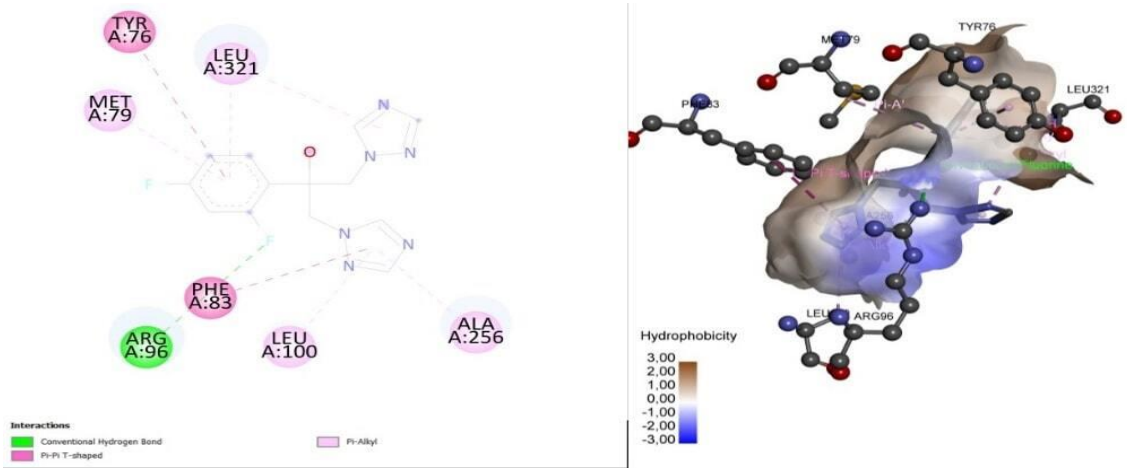

Figure 4. Imaging scheme for binding fluconazole to lanosterol 14α-demethylase

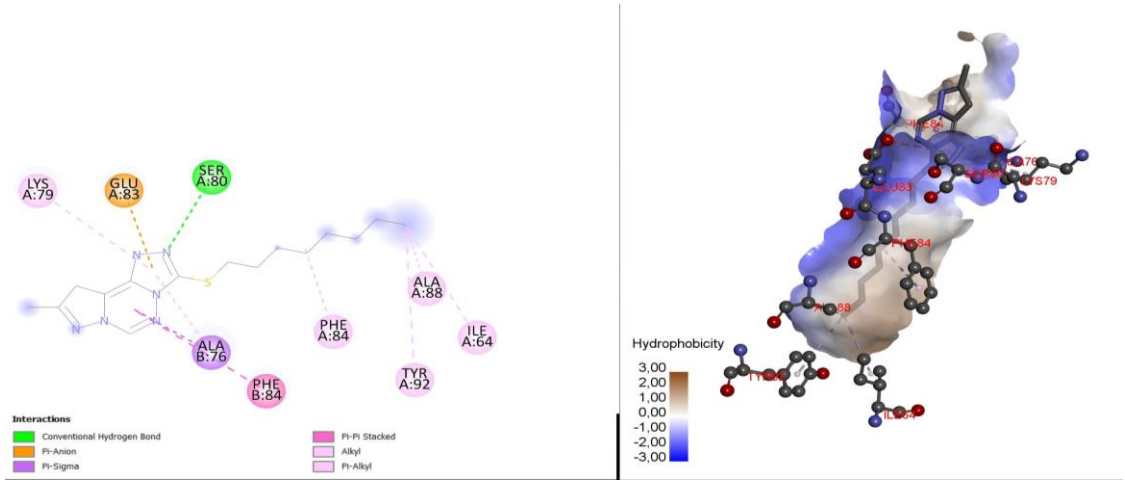

Figure 5. Imaging scheme for binding compound 2.18 to lanosterol 14α-demethylase

Attention is also drawn to the π-anionic interactions that are formed with the participation of triazole and triazine cycles upon contact with Glu A: 83. Additionally, ligand-protein complexes are stabilized by the formation of intermolecular hydrogen chemical bonds. This type of chemical bond is

formed with the direct participation of triazole synthon nitrogen in interaction with the Ser A: 80 residue, as well as with the participation of triazine nitrogen in the process of contact with the Asn B: 87 fragment. The triazine fragment in the structure of synthesized compounds plays one of the key roles in the formation of interactions with lanosterol 14α-demethylase due to the stacking interaction with the amino acid residue Phe B: 84. The largest number of interactions is recorded for compound 2.18. The occurrence of such a large number of interactions allows us to predict a positive effect on the possibility of antifungal activity. Probably, the structure of this compound makes it possible to occupy the most favorable position in the active site of lanosterol 14α-demethylase. But despite this fact, compounds 2.19 and 2.20 have the best indicator of the binding energy of the ligand-receptor complex (Table 4).

The value of the minimum free binding energy of the synthesized compounds to lanosterol 14α-demethylase is in the range of -6.1 ... -9.5 kcal × mol^{-1} (Table 4). Taking into account these values and the results of docking imaging, we can conclude that an extended study of certain compounds (2.19, 2.20) for antifungal activity is promising.

Table 4. Energy metrics of the molecular engagement involving lanosterol 14α-demethylase

№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$
2.11	-6.1	2.15	-8.0	2.19	-9.0
2.12	-6.9	2.16	-8.2	2.20	-9.5
2.13	-7.4	2.17	-8.1	*Fluconazole*	-10.9
2.14	-8.1	2.18	-8.4		

* ε_{min} - The minimum energy of complex formation.

The structure of crizotinib, a well-known anti-cancer drug, includes, among other things, a fragment of pyrazole. Therefore, among other things, it was interesting to test the possibility of the effect of synthesized substances on the crizotinib-dependent enzyme.

Receptor tyrosine kinase was chosen as the model enzyme, an increase in the activity of which is associated with the development of certain types of cancer. In line with the outcomes of molecular docking, the specified drug is captured in the receptor tyrosine kinase binding site using H-bonds that are formed between the N-atom of heterocyclic fragments of the crizotinib molecule and the residues of the Met A: 1199, Glu A: 1197 enzyme.

Additional stabilization of crizotinib at the binding site occurs with the active help of alkyl and π-alkyl interactions involving Ala A: 1148 and Leu A: 1122 (Figure 6). A crucial part is also carries out by the π-σ interaction, that are being implemented through to Leu A: 1256. Attention should also be paid to the spatial interaction of Chlorine and Fluorine with Asn A: 1254 and Gly A: 1269.

Trends in the amount for C-atoms within the structure of the thioalkyl substitute of synthesized compounds actively determine the nature and number of amino-acid snippets in the tyrosine kinase binding site, which will be involved in interaction with the ligand (Figure 7).

The synthesized compounds (2.11-2.20) can form the following types of interactions with the active site of tyrosine kinase: alkyl-alkyl hydrophobic interaction of the thioalkyl substitute with residues Leu A: 1196, Leu A: 1256 and Lys A: 1150; π-alkyl – with residues Ala A: 1148 and Leu A: 1256, π-σ - with residue Gly A: 1202 (Table 5).

Interactions that enhance contact with the enzyme can be noted C-H interaction. A lengthening of the alkyl chain leads in some cases to an increase in the number of additional hydrophobic interactions, for example, with the amino acid residue Ile A: 1171 (with the methyl group of the octyl and nonyl substituent). The transition to decyl substitute is accompanied by a decrease in the number of amino-acid pieces of the active zone of tyrosine kinase, which interact with the ligand and, in turn, is characterized exclusively by an alkyl interaction.

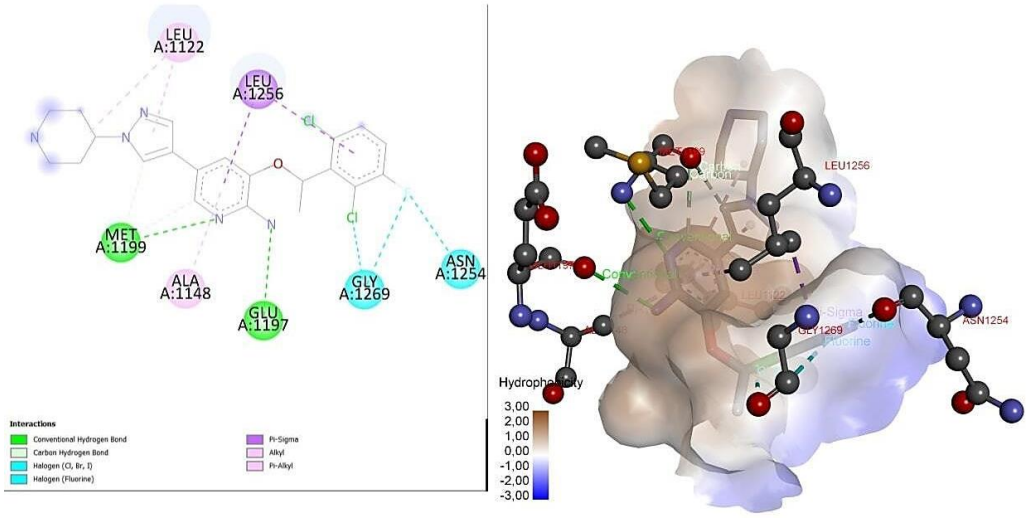

Figure 6. Imaging scheme for the binding of crizotinib to tyrosine kinase

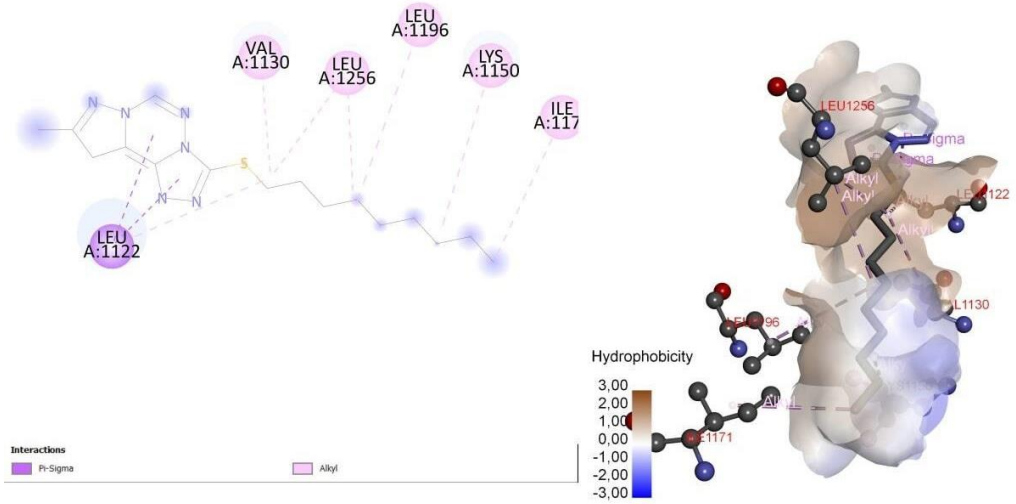

Figure 7. Imaging scheme for the binding of compound 2.19 to tyrosine kinase

Table 5. Main types of interactions between the studied compounds and amino acid residues of receptor tyrosine kinase

	Nature of the amino acid residue
2.11	Ala A: 1148, Leu A: 1122, Leu A: 1196, Leu A: 1256, Val A: 1130
2.12	Ala A: 1148, Leu A: 1122, Leu A: 1196, Leu A: 1256, Val A: 1130
2.13	Ala A: 1148, Leu A: 1122, Leu A: 1196, Leu A: 1256, Val A: 1130
2.14	Ala A: 1148, Leu A: 1122, Leu A: 1256, Lys A: 1150, Val A: 1130
2.15	Ala A: 1148, Ala A: 1200, Leu A: 1122, Leu A: 1196, Leu A: 1256, Val A: 1130
2.16	Ala A: 1148, Gly A: 1202, Leu A: 1122, Leu A: 1256, Leu A: 1196, Lys A: 1150
2.17	Ala A: 1148, Leu A: 1122, Leu A: 1256, Leu A: 1196, Lys A: 1150, Ile A: 1171
2.18	Leu A: 1122, Leu A: 1196, Leu A: 1256, Met A: 1199, Val A: 1130, Lys A: 1150
2.19	Leu A: 1122, Leu A: 1196, Leu A: 1256, Lys A: 1150, Ile A: 1171, Val A: 1130
2.20	Leu A: 1122, Leu A: 1196, Leu A: 1256, Lys A: 1150, Val A: 1130

The test substances are in contact with the receptor tyrosine kinase with a minimum free binding energy of -6.0 to -7.6 kcal × mol^{-1} (Table 6).

Table 6. Energy metrics of the molecular engagement with the receptor tyrosine kinase

№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$	№	ε_{min}, $\frac{kcal}{mol}$
2.11	-6.0	2.15	-6.3	2.19	-7.5
2.12	-6.2	2.16	-7.5	2.20	-7.6
2.13	-6.4	2.17	-7.5	*Crizotinib*	-10.8
2.14	-6.6	2.18	-7.4		

*ε_{min} - The minimum energy of complex formation.

Thus, docking studies of the synthesized thioalkyl-substituted of 9-methylpyrazolo[1,5-*d*][1,2,4]-triazolo[3,4-*f*][1,2,4]triazine (2.11-2.20) indicate a low the probability of impact on receptor tyrosine kinase of these ligands.

A series of mercaptoalkyl-9-methylpyrazolo[1,5-*d*][1,2,4]triazolo[3,4-*f*][1,2,4]triazines has been successfully synthesized. The structure of the synthesized substances is proven by modern methods of analysis. The conducted molecular docking made it possible to identify a number of promising compounds for subsequent research of anti-inflammatory and fungicidal activity.

The results of studies have shown that the synthesized compounds show the greatest affinity for lanosterol 14α-demethylase. The obtained values of the predicted free binding energy with the active center of the specified enzyme allowed us to determine the most promising compound for further research, which turned out to be substance 2.20.

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AUTHOR CONTRIBUTIONS

Concept: S.F., A.G.; Design: S.F., A.G.; Control: A.G.; Sources: S.F., A.G.; Materials: S.F., T.B.; Data Collection and/or Processing: S.F., Y.Z.; Analysis and/or Interpretation: S.F., A.G.; Literature Review: A.G.; Manuscript Writing: S.F., A.G.; Critical Review: Y.Z., T.B.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

HYPERICUM SCABRUM L. EKSTRESİNİN ANTİOKSİDAN, ENZİM İNHİBİTÖR AKTİVİTESİNİN DEĞERLENDİRİLMESİ VE YPSK ANALİZİ

EVALUATION OF ANTIOXIDANT, ENZYME INHIBITOR ACTIVITY OF HYPERICUM SCABRUM EXTRACT AND HPLC ANALYSIS

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ÖZ

Amaç: *Diyabet ve obezite günümüzün en yaygın sağlık problemleri arasında yer almaktadır. Bu problemlerin tedavisi için yeni ilaç kaynaklarına ihtiyaç duyulmaktadır. Toplumda geleneksel olarak kullanımı ile bilinen Hypericum scabrum L. bitkisi bu araştırmanın konusu olmuştur. Araştırma kapsamında H. scabrum bitkisinin toprak üstü kısımlarından hazırlanan etanol (%80) ekstresinin, antioksidan, antidiyabetik, anti-obezite, antikolesterol aktiviteleri ile fitokimyasal içeriğinin incelenmesi amaçlanmıştır.*

Gereç ve Yöntem: *H. scabrum bitkisinin toprak üstü kısımlarından hazırlanan etanol (%80) ekstresinin toplam fenol, toplam flavonoit miktar tayini yapılmış ve fitokimyasal içerik değerlendirilmiştir. Aynı zamanda ekstre içeriğinde bulunan fenolik ve flavonoit bileşenler ters faz Yüksek Performanslı Sıvı Kromatografisi YPSK ile araştırılarak miktar tayini gerçekleştirilmiştir. Ekstrenin antioksidan etkisi DPPH radikal süpürücü etki, metal bağlama kapasite, demir indirgeme gücü, ABTS radikal süpürücü etki, toplam antioksidan kapasite testleriyle değerlendirilmiştir. Ekstrenin antidiyabetik etkisinin araştırılmasında α-glukozidaz ve α-amilaz enzim inhibitör aktiviteleri incelenmiştir. Anti-obezite, lipit metobolizma üzerinde etkilerinin araştırılmasında sırasıyla pankreatik lipaz ve kolesterol esteraz enzim inhibitör aktiviteleri incelenmiştir.*

Sonuç ve Tartışma: *Ekstre 2 mg/ml konsantasyonda %90.16 ± 0.13 DPPH radikal süpürücü etki ve %97.53 ± 0.22 ABTS radikal süpürücü etki göstermiştir. Ekstre 2 mg/ml konsantrasyonda α-glukozidaz enzimi üzerinde %98.80 ± 0.51 inhibitör etki göstermiş olup, bu değer standart madde olarak kullanılan akarboza (%99.13 ± 0.11) yakın bulunmuştur. Ekstre 2 mg/ml konsantrasyonda kolesterol esteraz enzimi üzerinde %68.63 ± 1.05 inhibitör etki göstermiştir. Ters faz YPSK analiz sonucuna göre ekstrenin 1.047 ± 0.000 g/100 g ekstre klorojenik asit içerdiği bulunmuştur. Bu*

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bulgulara göre ekstrenin antidiyabetik etkili doğal bir kaynak olabileceği ve bu etkinin incelenmesi için daha ileri çalışmalara ihtiyaç duyulduğu sonucuna varılmıştır.
Anahtar Kelimeler: *Antidiyabetik, antihiperlipidemik, antioksidan, anti-obezite, Hypericum scabrum*

ABSTRACT

Objective: *Diabetes and obesity are among the most common health problems today. New drug sources are needed to treat these problems. Hypericum scabrum L., which is known for its traditional use among the public, has been the subject of this research. Within the scope of the research, it was aimed to investigate the antioxidant, antidiabetic, anti-obesity, anticholesterol activities, and phytochemical content of ethanol (80%) extract prepared from the aerial parts of the H. scabrum.*
Material and Method: *The total phenol, total flavonoid contents, and phytochemical content of the ethanol (80%) extract prepared from the aerial parts of the H. scabrum were evaluated. Additionally, the phenolic and flavonoid compounds in the extract were investigated with reverse phase HPLC and their quantitative analysis was carried out. The antioxidant effect of the extract was evaluated with DPPH radical-scavenging effect, metal chelating capacity, ferric reducing power, ABTS radical scavenging effect, and total antioxidant capacity tests. In the investigation of the antidiabetic effect of the extract, α-glucosidase and α-amylase enzyme inhibitory activities were investigated. To investigate the effects of anti-obesity and lipid metabolism, pancreatic lipase enzyme inhibitory activity and cholesterol esterase enzyme inhibitory activity were investigated, respectively.*
Result and Discussion: *The extract showed 90.16 ± 0.13% DPPH radical scavenging and 97.53 ± 0.22% ABTS radical scavenging activity at 2 mg/ml concentration. The extract had an inhibitory activity of 98.80 ± 0.51 % on the α-glucosidase enzyme at a concentration of 2 mg/ml, and this value was close to the acarbose result used as the standard substance (99.13 ± 0.11%). The extract showed inhibitory activity of 68.63 ± 1.05% on the cholesterol esterase enzyme at a concentration of 2 mg/ml. According to the results of reversed-phase HPLC analysis, it was found that the extract contained 1.047 ± 0.000 g/100 g extract chlorogenic acid. According to the results, it was concluded that the extract may be a natural source with antidiabetic effects and further studies are needed to investigate this effect.*
Keywords: *Antidiabetic, antihyperlipidemic, antioxidant, anti-obesity, Hypericum scabrum*

GİRİŞ

Diyabet, dünya genelinde milyonlarca insanı etkileyen kronik bir problem olmakla birlikte toplumların önemli sağlık sorunlarından biridir. Diyabetle mücadele için önleyici stratejilere ihtiyaç duyulmaktadır. Uluslararası Diyabet Vakfı'na göre dünyada 415 milyon kişiye diyabet teşhisi konulmuştur ve bu sayının 2040 yılına kadar 600 milyonun üzerine çıkacağı tahmin edilmektedir. Obezite, diyabet öncesinde ve diyabet oluşumunda büyük rol oynamaktadır [1]. Obez kişilerde, insülin duyarlılığı ve β-hücre fonksiyonunun modülasyonu azalmaktadır [2]. Diyabet döneminde kolesterol metabolizmasının farklı olduğu ve diyabette kolesterol sentezinin daha yüksek olduğu bilinmektedir. Bu durum diyabetin kolesterol metabolizması üzerinde etkisinin olduğunu göstermektedir [3].

Diyabette reaktif oksijen türlerinin üretiminin arttığı ve antioksidan savunma kapasitesinin bozulduğu bildirilmiştir. Artan serbest radikal üretiminin ve oksidatif stresin, hastalığın gelişiminde merkezi bir olay olduğu öne sürülmüştür. Bu nedenle, antioksidanların diyabetin iyileştirilmesinde önemli bir rol oynayabileceği üzerinde durulmaktadır. Antioksidan etkili bileşiklerin diyabet yönetimi üzerinde etkileri hakkında birçok rapor bulunmaktadır. Antioksidan kullanımının oksidatif stresi azaltarak diyabetik komplikasyonları hafiflettiği bildirilmektedir [4].

Hypericaceae familyasında yer alan *Hypericum* cinsi 450'den fazla türden oluşmaktadır. *Hypericum* cinsi sıcak, ılıman, subtropikal ve tropikal bölgelerde dağılım göstermektedir [5]. *Hypericum* türleri, yaklaşık iki yüz yıldır farklı tıbbi özelliklere sahip tıbbi bitkiler olarak bilinmektedir [6]. Fitokimyasal içeriği nedeniyle *Hypericum* türlerine son zamanlarda artan bir ilgi vardır [7]. Halk tıbbında ve modern tıpta sıklıkla kullanılmaktadır [6]. Geleneksel Türk tıbbında sedatif, antihelmintik ve antiseptik etkileri nedeniyle uzun yıllardır kullanılmaktadır. Ayrıca bitkinin yağı depresyon, yanık,

yara, çürük ve çeşitli ağrı biçimlerini tedavi etmek için kullanılmıştır [8]. Literatürde *Hypericum* türlerinin antimikrobiyal, antifungal, antiviral, antioksidan, antidepresan ve antikonvülzan aktiviteleri ile ilgili raporlar yayınlandığı bildirilmiştir [7].

Bu türlerden biri olan *Hypericum scabrum* L., Türkiye'de kuru kayalık yamaçlarda ve açık ormanlık alanlarda bulunmaktadır [9]. *H. scabrum* halk arasında kepir otu, kızılcık otu, mayasıl otu olarak bilinmektedir [8,10]. *H. scabrum* uçucu yağının ana bileşenleri α-pinen, timol ve karvakroldür [11]. *H. scabrum* çiçeklerinin temel yağ asit içeriği incelendiğinde ise alfa-linolenik (%48.60), linoleik (%32.53) ve oleik (%11.45) asitlerin olduğu görülmüştür [12]. Fitokimyasal araştırmalar, *H. scabrum* içeriğinde benzoilfloroglusinol türevlerinin bulunduğunu göstermiştir [11,13]. *H. scabrum* içeriğinde apigenin-7-*O*-glukozit, amentoflavon, hiperisin, psödohiperisin, hiperosid, klorojenik asit, rutin, kersetin ve kersitrin bulunmuştur [14].

H. scabrum L. bitkisinin toprak üstü kısımları geleneksel ilaçların hazırlanmasında yaygın olarak kullanılmaktadır [11]. Türk halkı tarafından spazm giderici, yatıştırıcı, iltihap önleyici, ishal, egzama ve hemoroide karşı, çeşitli mantar hastalıklarında mantar önleyici olarak kullanılmaktadır [8,10,15]. Ayrıca bitkinin toprak üstü kısımlarından hazırlanan merhemler sedef hastalığına karşı kullanılmaktadır [14]. Türkiye'de *H. scabrum* çiçeklerinden hazırlanan infüzyonlar halk arasında diyabet tedavisinde kullanılmaktadır [16]. Çin, Tacikistan ve Moğolistan'da diyabetin önlenmesi ve tedavisi için hazırlanan geleneksel ilaçlarda *H. scabrum* kullanılmaktadır [17].

Yapılan araştırmalara göre *H. scabrum* ekstrelerinin yüksek antioksidan etkiler gösterdiği belirtilmiştir. Yüksek antioksidan aktiviteye sahip olan bileşikler serbest radikallerin neden olduğu oksidatif hasara karşı korumada yardımcı olduğu bilinmektedir. Dolayısıyla antioksidan potansiyele sahip bileşikler kardiyovasküler hastalıklarda ve diyabet problemlerinde faydalıdır [18].

Bu araştırmada, *H. scabrum* toprak üstü kısımlarından hazırlanan etanol (%80) ekstresinin, antioksidan, antidiyabetik, antiobezite ve antikolesterol aktiviteleri incelenmiştir. Antioksidan aktivite incelenirken DPPH radikal süpürücü etki, ABTS radikal süpürücü etki, metal bağlama kapasite, demir indirgeme gücü ve total antioksidan kapasite değerlendirilmiştir. Antidiyabetik aktivitenin incelenmesinde *α*-glukozidaz ve *α*-amilaz inhibitör aktiviteleri araştırılmıştır. Anti-obezite ve antikolesterol üzerindeki etkiler için sırasıyla pankreatik lipaz ve pankreatik kolesterol esteraz enzimleri üzerindeki inhibisyon değerlendirilmiştir. *H. scabrum* toprak üstü kısımlarının kimyasal içeriğinin araştırılmasında total fenol ve total flavonoit içeriği incelenmiştir. Ayrıca ekstrenin Yüksek Performanslı Sıvı Kromatografisi (YPSK) ile standardizasyonu araştırılmıştır. *H. scabrum* toprak üstü kısımlarından hazırlanan ekstrenin antioksidan, antidiyabetik, anti-obezite ve antikolesterol aktivitelerinin beraber incelendiği araştırmaya literatürde rastlanmamış olup ilk defa bu araştırmada 4 aktivite birlikte incelenmiştir.

GEREÇ VE YÖNTEM

Bitki Materyali ve Ekstraksiyon

Materyal olarak kullanılan *H. scabrum* bitkisinin toprak üstü kısımları Akşehir-Gelendost Yolu bölgesinden Haziran 2014'te toplanmıştır. Bitki Gazi Üniversitesi Fen Fakültesi Botanik Anabilim Dalı'ndan Doç. Dr. Mehmet Ufuk Özbek tarafından teşhis edilmiştir. Bitki örneği Gazi Üniversitesi Eczacılık Fakültesi Herbaryumu'na GUEF 3448 numarası ile kaydedilmiştir. Toplanan bitki kısımları gölgede kurutulmuş ve daha sonra öğütme işlemi gerçekleştirilmiştir. 10 gram tartılan *H. scabrum* toprak üstü kısımların üzerine 200 ml %80 etanol ilave edilmiş ve çalkalayıcı üzerinde 24 saat maserasyon işlemine tabi tutulmuştur. Süre bitiminde ekstreler filtre kağıdından süzülmüş ve örnek üzerine tekrar %80 etanol ilave edilerek aynı işlemler 3 kez yapılmıştır. 3 işlem sonunda elde edilen süzüntüler birleştirilerek alçak basınç altında 45°C'de rotavaporda (Heidolph, Almanya) kuruluğa kadar yoğunlaştırılmıştır. Kuru ekstreler tartılarak verimleri (ağrılık/ağırlık) hesaplanmıştır. Ekstreler kimyasal içerik, antioksidan aktivite testleri, enzim inhibitör aktivite testleri ve YPSK deneylerinde kullanılmak üzere +4°C'de saklanmıştır.

Ekstrenin Kimyasal İçeriği

Toplam Fenol Miktarı

H. scabrum bitkisinin toprak üstü kısımlarından hazırlanan %80 etanol ekstresinin üzerine %10 Folin-Ciocalteu reajanı eklenerek oda sıcaklığında 5 dakika boyunca inkübe edilmiştir. İnkübasyon işlemi tamamlanınca sodyum karbonat (Na_2CO_3) çözeltisi ilave edilerek oda sıcaklığında 30 dakika bekletilmiştir. Süre bitiminden sonra 735 nm dalga boyunda mikroplak okuyucu ile absorbans değerleri ölçülmüştür. Ekstrelerin toplam fenol içeriği gallik asit eşdeğeri (GAE) mg/g ekstre şeklinde ifade edilmiştir [19]. Belirtilen tüm işlemler 3 kez tekrarlanmıştır.

Toplam Flavonoit Miktarı

Hazırlanmış olan ekstrelerin üzerine sırasıyla %95 etanol, 1 M sodyum asetat çözeltisi, %10 $AlCl_3$ çözeltisi ilave edilmiştir. Karışımın son hacmi 1000 µl olacak şekilde distile su ile tamamlanmıştır. Bu karışım daha sonra 30 dakika boyunca oda sıcaklığında inkübe edilmiştir. İnkübasyon işlemi bittikten sonra 415 nm dalga boyunda mikroplak okuyucu ile absorbans değeri ölçülmüştür. Elde edilen bulgular kersetin eşdeğeri (KE) mg/g ekstre olarak gösterilmiştir [20]. Belirtilen tüm işlemler 3 kez tekrarlanmıştır.

Antioksidan Aktivite Testleri

DPPH Radikal Süpürücü Etki

%80 etanol çözücüsü ile hazırlanan ekstrelerinin üzerine 1 mM DPPH (1,1-difenil-2-pikrilhidrazil) çözeltisi ilave edilmiştir. Daha sonra karışım 30 dakika boyunca oda sıcaklığında ve karanlıkta inkübasyona bırakılmıştır. İnkübasyon işlemi tamamlandıktan sonra 520 nm dalga boyunda mikroplak okuyucu ile absorbans değeri ölçülmüştür. Bu deneyde askorbik asit standart madde olarak kullanılmıştır. Belirtilen tüm işlemler 3 kez tekrarlanmıştır. Ekstrelerin ve standart maddenin DPPH radikal süpürücü etkisi aşağıda verilen formüle göre hesaplanmıştır [21].

$$\% \text{ Etki} = [(\text{Absorbans kontrol} - \text{Absorbans örnek}) / \text{Absorbans kontrol}] \times 100$$

Metal Bağlama Kapasitesi

Ekstrelerin üzerine 2 mM $FeCl_2$ çözeltisi eklendikten sonra karışım 5 dakika boyunca oda sıcaklığında bekletilmiştir. Bu işlemin ardından toplam hacim 130 µl olacak biçimde 5 mM ferrozin çözeltisi eklenmiştir. Metal bağlama kapasitesi deneyinde standart madde olarak etilen diamintetraasetik asit (EDTA) kullanılmıştır. Daha sonra 562 nm dalga boyunda mikroplak okuyucu ile absorbans değeri ölçülmüştür. Belirtilen tüm işlemler 3 kez tekrarlanmıştır. Sonuçlar % metal bağlama kapasitesi şeklinde aşağıda verilen formüle göre hesaplanmıştır [22].

$$\% \text{ Kapasite} = [(\text{Absorbans kontrol} - \text{Absorbans örnek}) / \text{Absorbans kontrol}] \times 100$$

Demir İndirgeme Gücü

Demir indirgeme gücü deneyinde 50 µl örnekler pH 7.2'de hazırlanmış olan sodyum fosfat tamponu (0.1 mol/l) ile muamele edilmiştir. Karışıma potasyum ferrisiyanür (%1) eklendikten sonra karışım 37 °C'de bir saat üzerine cam kapak kapatılarak bekletilmiştir. Bir saatlik sürenin bitiminden sonra %10'luk (ağırlık/hacim) hazırlanan trikoloroasetik asit çözeltisi eklenmiştir. Bu işlemin ardından mikroplak okuyucusu ile 700 nm dalga boyunda karışımın ve standart maddenin ilk absorbans değerleri ölçülmüştür. İndirgeme gücü deney sonuçları standart madde olan kersetin ile mukayese edilmiştir. Daha sonra karışım üzerine %0.1 $FeCl_3$ çözeltisi eklenerek 700 nm dalga boyunda ikinci absorbans değerleri ölçülmüştür. Absorbans değerleri arasındaki fark alınarak indirgeme gücü hesaplanmıştır. Belirtilen tüm işlemler 3 kez tekrarlanmıştır [23].

ABTS Radikal Süpürücü Etki

ABTS çözeltisi (7 mM) üzerine potasyum persülfat çözeltisi (2.45 mM) ilave edilmiştir. Karışım çözeltisi, karanlık ortamda 20°C'de 16 saat boyunca bekletilmiştir. Daha sonra pH 7.4 fosfat tamponu hazırlanmıştır. Ekstre, standart madde üzerine ABTS ve fosfat tamponu eklenmiştir. Ekstreler standart

madde olan gallik asit ile karşılaştırılmıştır. Daha sonra 734 nm dalga boyunda mikroplak okuyucu ile absorbans değeri ölçülmüştür. Belirtilen tüm işlemler 3 kez tekrarlanmıştır. Sonuçlar % etki şeklinde aşağıda verilen formüle göre hesaplanmıştır [23].

% Etki = [(Absorbans kontrol–Absorbans örnek) / Absorbans kontrol] × 100

Fosfomolibden Testiyle Toplam Antioksidan Kapasite

Ekstrelere molibdat reaktifi eklenmiş ve 90°C'de 90 dakika inkübe edilmiştir. İnkübasyon işlemi tamamlandıktan sonra karışım soğumaya bırakılmıştır. Daha sonra ekstrelerin absorbans değeri 695 nm dalga boyunda mikroplak okuyucu ile ölçülmüştür. Toplam antioksidan kapasite sonuçları ise askorbik asit eşdeğeri (AAE) mg/g ekstre olarak bulunmuştur [24].

Enzim İnhibitör Aktivite Testleri

***α*-Glukozidaz İnhibitör Aktivite**

Deneyde kullanılmak üzere öncelikle 0.5 M, pH 6.5 fosfat tamponu hazırlanmıştır. *α*-Glukozidaz tip IV enzimi hazırlanan fosfat tamponunda çözülmüştür. Substrat olarak kullanılacak olan *p*-nitrofenil-α-D-glukopiranozit (PNG) benzer şekilde fosfat tamponunda çözülerek 20 mM konsantrasyonda hazırlanmıştır. Ekstreler üzerine pH 6.5 fosfat tamponu eklendikten sonra hazırlanan *α*-glukozidaz tip IV enzimi de eklenerek bir karışım elde edilmiştir. Bu karışım 37°C'de 15 dakika boyunca inkübasyona bırakılmıştır. İnkübasyon işlemi tamamlanınca karışım üzerine 20 mM PNG substratı eklenmiş ve 37°C'de 35 dakika boyunca etüvde bekletilmiştir. Bekleme işlemi bittikten sonra ekstrelerin 405 nm dalga boyunda mikroplak okuyucu ile absorbans değerleri ölçülmüştür. Standart madde olarak akarboz kullanılmış ve belirtilen tüm işlemler 3 kez tekrarlanmıştır [25].

***α*-Amilaz İnhibitör Aktivite**

Deneyde tampon olarak sodyum fosfat tamponu (pH 6.9) kullanılmıştır. Renk reajanı olarak 3,5-dinitrosalisilik asit (DNS) çözeltisi kullanılmıştır. DNS çözeltisi hazırlanırken 5.31 M sodyum tartarat çözeltisi (2M NaOH ile hazırlanmıştır), 96 mM DNS kullanılmıştır. Enzim olarak *α*-amilaz tip I kullanılmıştır. Bu deneyde standart madde olarak akarboz, substrat olarak %2.5 nişasta çözeltisi kullanılmıştır. Aynı zamanda deneyde maltoz kalibrasyon grafiği oluşturulmuştur. Ekstre ve standart madde üzerine sodyum fosfat tamponu (pH 6.9) ve *α*-amilaz enzimi eklenerek oda sıcaklığında 5 dakika inkübe edilmiştir. İnkübasyon işleminden sonra substrat olarak nişasta çözeltisi eklenerek oda sıcaklığında 15 dakika boyunca karışım bekletilmiştir. Daha sonra karışım üzerine DNS renk reajanı eklenerek karışım 80°C'de 40 dakika boyunca etüvde bekletilmiştir. Süre sonunda karşım üzerine distile su eklenerek 540 nm dalga boyunda mikroplak okuyucu ile ekstrelerin absorbans değeri ölçülmüştür. Belirtilen tüm işlemler 3 kez tekrarlanmıştır [26].

Pankreatik Lipaz İnhibitör Aktivite

Deneyde domuz pankreasından elde edilen *α*-amilaz tip II enzimi kullanılmıştır. Enzim hazırlanırken 10 mM 4-morfolinpropansülfonik asit ve 1 mM EDTA çözeltisinden hazırlanan pH 6.8 olan tampon çözeltisi kullanılmıştır. Deneyde kullanılmak üzere 100 mM Tris-HCl ile 5 mM $CaCl_2$ karışımından oluşan ve pH 7.0 olan Tris tamponu hazırlanmıştır. Standart madde olarak orlistat, substrat olarak 5 mM 4-nitrofenil butirat kullanılmıştır. Tris tamponu üzerine ekstreler eklendikten sonra karışıma enzim çözeltisi ilave edilmiştir. Oluşan karışım 37°C'de 15 dakika boyunca etüvde bekletilerek inkübasyon işlemi gerçekleştirilmiştir. İnkübasyondan sonra 5 mM 4-nitrofenil butirat eklenerek 37°C'de 30 dakika boyunca bekletilmiştir. Daha sonra 405 nm dalga boyunda mikroplak okuyucu ile ekstrelerin absorbans değeri ölçülmüştür. Belirtilen tüm işlemler 3 kez tekrarlanmıştır [27].

Kolesterol Esteraz İnhibitör Aktivite

Pankreatik kolesterol enzimi 0.1 M pH 7.0 sodyum fosfat tamponu ile hazırlanmıştır. Deneyde kullanılmak üzere 12 mM taurokolik asit hazırlanmıştır. Substrat olarak 5 mM 4-nitrofenil butirat, standart madde olarak simvastatin kullanılmıştır. 96 kuyucuklu mikroplak içerisine pH 7.0'de hazırlanan

sodyum fosfat tamponu ve 12 mM taurokolik asit eklenmiştir. Bu karışımın üzerine 5 mM 4-nitrofenil butirat ve ekstre eklendikten sonra 5 dakika boyunca oda sıcaklığında inkübasyon işlemi gerçekleştirilmiştir. İnkübasyondan sonra karışıma 0.1 µg/ml enzim eklenerek 405 nm dalga boyunda 15 dakika boyunca mikroplak okuyucu ile kinetik okuma gerçekleştirilmiştir [28].

Yüksek Basınçlı Sıvı Kromatografisi

Ekstrenin fitokimyasal içeriği ters faz-YPSK (HP Agilent 1260 seri LC sistem) kullanılarak araştırılmıştır. Kolon olarak ACE 5 C18 (150 x 4.6 mm, 5 µm) kullanılmıştır. Analiz boyunca kolon 25°C sıcaklığına ayarlanmıştır. Analizde akış olarak gradient akış tercih edilmiştir. Analiz başlatılırken mobil faz sistemi; Asetonitril:H_2O:Formik asit (80:20:0.5) karışımından oluşan %5 Solvan A ve H_2O:Formik asit (100:0.5) karışımından oluşan %95 solvan B'ye ayarlanmıştır. Bu akış toplamda 53 dakikada gerçekleşmiş olup %100 A olduğu zaman analiz sonlanmıştır. Analiz akış hızı 0.8 ml/dakika ve enjeksiyon hacmi 20 µl olarak ayarlanmıştır. Bu analizde Diode Array (DAD) dedektörü kullanılmış olup araştırılacak moleküllerin maksimum dalga boyları göz önüne alınarak analiz için 260, 280, 320 ve 350 nm dalga boyları seçilmiştir. Analizde ekstrenin fenolik içeriğinin aydınlatılmasında ferulik asit, gallik asit, *trans*-sinnamik asit, klorojenik asit, protokateşik asit, rosmarinik asit, vanilik asit, *p*-kumarik asit, siringik asit, sinapik asit, elajik asit, kafeik asit, epikateşin, kateşin standartların oluşan fenolik bir karışım kullanılırken, ekstrenin flavonoit içeriğinin aydınlatılmasında ise apigenin-7-*O*-glukozit, rutin, umbelliferon, mirsetin, naringenin, apigenin hesperidin, luteolin, kersetin standartlarından oluşan bir flavonoit karışımı kullanılmıştır.

SONUÇ VE TARTIŞMA

Ekstraksiyon işlemleri sonucunda alçak basınç altında 45°C'de rotavaporda kuruluğa kadar yoğunlaştırılan ham ekstre veriminin %20.26 (ağırlık/ağırlık) olduğu görülmüştür. Toplam fenol içerik miktarının 140.39 ± 10.20 gallik asit eşdeğeri mg/g ekstre olduğu bulunurken kalibrasyon denklemi y = 6.3667x – 0.0118, r^2= 0.9999 olmuştur. Yapılan toplam flavonoit içerik miktar tayini deney sonucunda ekstrenin flavonit içeriğinin 49.65 ± 4.15 kersetin eşdeğeri mg/g ekstre olduğu görülürken, kalibrasyon denklemi y = 2.1694x – 0.0067, r^2= 0.9999 olarak bulunmuştur.

Ekstre üzerinde farklı antioksidan aktivite testleri yapılmıştır. Bu yapılan testlere göre *H. scabrum* bitkisinin toprak üstü kısımlarından hazırlanan %80 etanol ekstresinin 1 mg/ml konsantrasyonda toplam antioksidan kapasitesi 196.04 ± 19.70 askorbik asit eşdeğeri mg/g ekstre olduğu bulunmuş olup kalibrasyon denklemi y = 1.8309x – 0.1606, r^2=0.9981 olmuştur. Hazırlanan ekstrelerin DPPH radikal süpürücü etki sonuçları incelendiğinde tüm konsantrasyonlarda yüksek radikal süpürücü etki gözlenmiş olup, ekstrenin 2 mg/ml konsantrasyon haricinde diğer tüm konsantrasyonlarda (0.25 mg/ml, 0.5 mg/ml, 1 mg/ml) standart madde olarak kullanılan askorbik asitten daha yüksek etki gösterdiği dikkat çekmiştir. Metal bağlama kapasitesi deney sonuçları incelendiğinde ekstre en yüksek bağlama kapasitesini 2 mg/ml konsantrasyonda %45.98 ± 8.88 olarak göstermiştir. Metal bağlama kapasitesi deneyinde kullanılan standart madde EDTA ise tüm konsantrasyonlarda %100 bağlama kapasitesi göstermiştir. İndirgeme gücü absorbans değerleri incelendiğinde ekstrenin konsantrasyonu azaldıkça absorbans değerlerinin de azaldığı görülmüştür. ABTS radikal süpürücü etki sonuçları incelendiğinde ekstrenin 2 mg/ml konsantrasyonda süpürücü etkisi %97.53 ± 0.22 bulunurken aynı konsantrasyonda standart madde olarak kullanılan gallik asitin süpürücü etkisi % 98.10 ± 0.80 bulunmuştur. Bu sonuca göre ekstrenin 2 mg/ml konsantrasyonda standart maddeye yakın bir radikal süpürücü etki gösterdiği şeklinde yorumlanmıştır. *H. scabrum* bitkisinin toprak üstü kısımlarından hazırlanan %80 etanol ekstresinin antioksidan aktivite test sonuçları Tablo 1'de gösterilmiştir.

Bitki ekstresinin α-glukozidaz, α-amilaz, pankreatik lipaz ve kolesterol esteraz enzimleri üzerinde inhibitör etkileri araştırılmıştır. Ekstrenin diyabet üzerindeki etkisi hem α-glukozidaz hem de α-amilaz inhibitör aktivite değerlendirilmiştir. Antidiyabetik etki için α-glukozidaz enziminde konsantrasyona bağlı olarak artan bir inhibitör aktivite bulunmuştur. *H. scabrum* toprak üstü kısımlarının %80 etanol ekstresi 2 mg/ml konsantrasyonda α-glukozidaz enzimi üzerinde %98.80 ± 0.51 inhibitör aktivite göstermiştir. Standart madde olarak kullanılan akarboz aynı enzim üzerinde %99.13 ± 0.11 inhibitör aktivite göstermiştir. Belirtilen konsantrasyonda ekstre ve standart maddenin enzim üzerinde benzer

inhibisyon değeri gösterdiği bulunmuştur. Antidiyabetik etkinin incelendiği α-amilaz enzim inhibisyonu deney modelinde ise ekstre 2 mg/ml konsantrasyonda %7.08 ± 1.79 enzim inbisyonu oluştururken bu deney modelinde standart madde olarak kullanılan akarboz aynı konsantrasyonda %92.75 ± 0.68 inhibisyon oluşturmuştur. Ekstrenin diğer konsantrasyonları için α-amilaz üzerinde herhangi bir inhibitör aktivite saptanmamıştır. *H. scabrum* toprak üstü kısımlarından hazırlanan %80 etanol ekstresi 2 mg/ml konsantrasyonda pankreatik lipaz enzimi üzerinde %28.03 ± 0.39 inhibitör etki göstermiş olup daha düşük konsantrasyonlarda inhibitör etki göstermemiştir. Pankreatik lipaz inhibitör aktivite araştırması sırasında standart madde olarak kullanılan orlistat 2 mg/ml konsantrasyonda %64.53 ± 0.32 inhibitör etki göstermiştir. Ekstrenin kolesterol esteraz enzimi üzerinde doz bağlı olarak artan inhibitör etkisi olduğu izlenmiştir. Ekstre standart madde olarak kullanılan simvastatin ile karşılaştırıldığı zaman ekstrenin 0.25 mg/ml konsantrasyonu hariç diğer tüm konsantrasyonlarda simvastatinden daha güçlü inhibitör aktivite göstermesi dikkat çekmiştir. Elde edilen bulgulara göre *H. scabrum* toprak üstü kısımlarından hazırlanan %80 etanol ekstresi incelenen 4 farklı enzim modeli üzerinde en yüksek inhibitör etkiyi α-glukozidaz enzimi üzerinde gösterdiği görülmüştür. Aynı zamanda kolesterol esteraz enzim inhibisyonu modelinde incelenen konsantrasyonlar birbiriyle kıyaslandığı zaman 0.25 mg/ml konsantrasyonu haricinde diğer tüm konsantrasyonlarda %50'nin üzerinde inhibitör etki olduğu anlaşılmıştır.

H. scabrum toprak üstü kısımlarından hazırlanan %80 etanol ekstresinin dört farklı enzim modeli üzerindeki göstermiş oldukları inhibitör aktivite sonuçları Tablo 2'de gösterilmiştir.

Tablo 1. *H. scabrum* ekstresinin antioksidan aktivite test sonuçları

Ekstre/Standart Madde Adı	Ekstre/Standart Madde Konsantrasyonu	DPPH Radikal Süpürücü Etki % Etki ± S.S*	Metal Bağlama Kapasitesi % Kapasite ± S.S	Demir İndirgeme Gücü Absorbans ± S.S	ABTS Radikal Süpürücü Etki % Etki ± S.S
***H. scabrum* toprak üstü kısımlarından hazırlanan %80 etanol ekstresi**	0.25 mg/ml	89.62 ± 0.29	17.02 ± 7.76	0.784 ± 0.08	23.62 ± 3.01
	0.5 mg/ml	90.79 ± 0.70	23.26 ± 4.81	1.380 ± 0.02	37.52 ± 1.29
	1 mg/ml	90.92 ± 0.25	22.55 ± 3.18	2.533 ± 0.20	83.78 ± 3.18
	2 mg/ml	90.16 ± 0.13	45.98 ± 8.88	3.810 ± 0.04	97.53 ± 0.22
Standart	0.25 mg/ml	88.36 ± 0.67 [a]	100.00 [b]	3.744 ± 0.16 [c]	100.00 [d]
	0.5 mg/ml	89.38 ± 0.61 [a]	100.00 [b]	3.931 ± 0.00 [c]	99.54 ± 1.04 [d]
	1 mg/ml	89.66 ± 0.35 [a]	100.00 [b]	3.930 ± 0.00 [c]	98.94 ± 0.26 [d]
	2 mg/ml	90.50 ± 0.59 [a]	100.00 [b]	3.840 ± 0.14 [c]	98.10 ± 0.80 [d]

*Standart sapma [a]Askorbik asit, [b]EDTA, [c]Kersetin, [d]Gallik asit

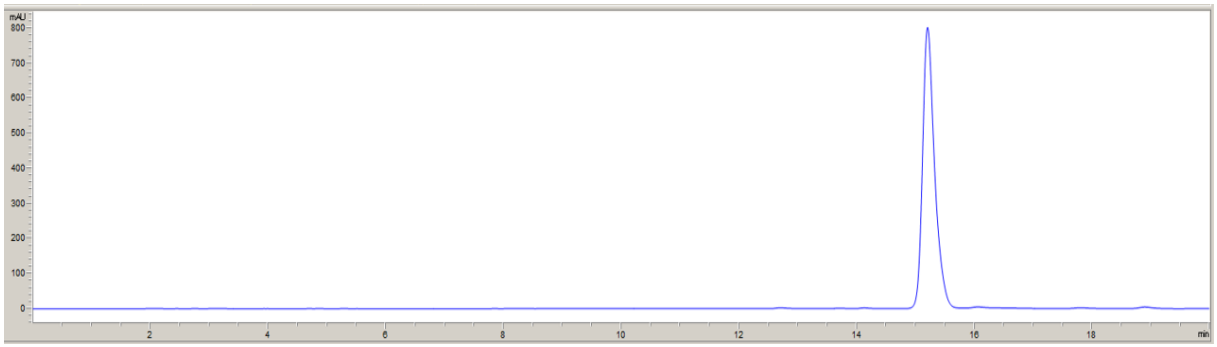
Tablo 2. *H. scabrum* ekstresinin enzim inhibitör aktivite test sonuçları

Ekstre ve Standart Maddeler		Enzim üzerinde % inhibitör Etki ± S.S*			
Ekstre/Standart Madde Adı	Ekstre/Standart Madde Konsantrasyonu	*α*-Amilaz	*α*-Glukozidaz	Pankreatik lipaz	Kolesterol esteraz
***H. scabrum* toprak üstü kısımlarından hazırlanan %80 etanol ekstresi**	0.25 mg/ml	-	54.84 ± 4.28	-	39.86 ± 2.54
	0.5 mg/ml	-	84.03 ± 1.18	-	52.37 ± 0.23
	1 mg/ml	-	95.43 ± 0.93	-	59.72 ± 2.80
	2 mg/ml	7.08 ± 1.79	98.80 ± 0.51	28.03 ± 0.39	68.63 ± 1.05
Standart	0.25 mg/ml	68.68 ± 1.05 [a]	98.55 ± 0.14 [a]	62.97 ± 0.88 [b]	40.67 ± 4.12 [c]
	0.5 mg/ml	85.30 ± 0.46 [a]	99.31 ± 0.29 [a]	62.86 ± 1.40 [b]	47.88 ± 5.11 [c]
	1 mg/ml	91.00 ± 0.43 [a]	99.57 ± 0.02 [a]	63.84 ± 1.17 [b]	52.21 ± 0.12 [c]
	2 mg/ml	92.75 ± 0.68 [a]	99.13 ± 0.11 [a]	64.53 ± 0.32 [b]	53.18 ± 3.36 [c]

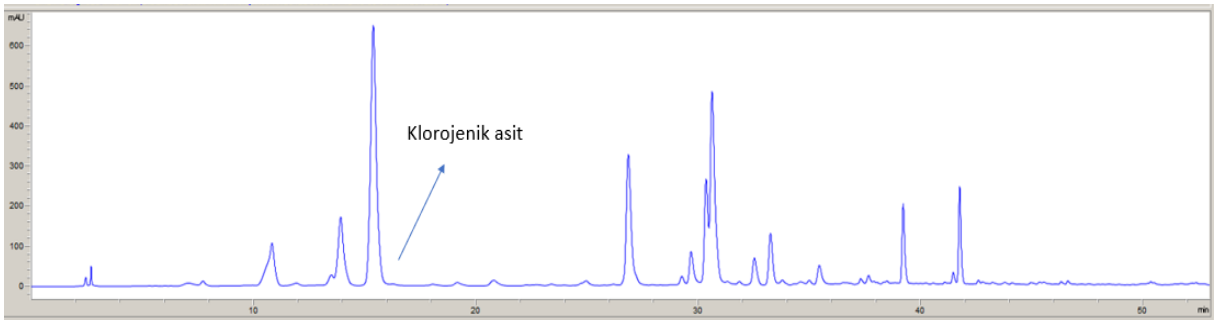
*S.S: Standart Sapma, [a]Akarboz, [b]Orlistat, [c]Simvastatin, -: Aktivite saptanmadı

Ekstrenin fitokimyasal profilini belirlemek için YPSK-ters faz sistemi kullanılmıştır. Ekstrenin flavonoit ve fenolik bileşik içeriği nicel ve nitel olarak incelenmiştir. Analizde *H. scabrum* toprak üstü

kısımlarından hazırlanan %80 etanol ekstresinin fenolik içeriğinin aydınlatılmasında ferulik asit, gallik asit, *trans*-sinnamik asit, klorojenik asit, protokateşik asit, rosmarinik asit, vanilik asit, *p*-kumarik asit, siringik asit, sinapik asit, elajik asit, kafeik asit, epikateşin, kateşin standartların oluşan fenolik bir karışım kullanılırken, flavonoit içeriğinin aydınlatılmasında ise apigenin-7-*O*-glukozit, rutin, umbelliferon, mirsetin, naringenin, apigenin hesperidin, luteolin, kersetin standartlarından oluşan bir flavonoit karışımı kullanılmış, sonuç olarak ekstrenin içerisinde araştırılan referans maddelerden sadece klorojenik asidin varlığı tespit edilmiştir. Klorojenik asit miktar tayini ile ilgili referans madde ve ekstre kromatogramları Şekil 1 ve Şekil 2'te gösterilmiştir.



Şekil 1. Standart madde olarak kullanılan klorojenik asit bileşiğinin 320 nm'de kromatogramı



Şekil 2. *H. scabrum* ekstresinin 320 nm dalga boyunda kromatogramı

Ekstre içeriğinde varlığı tespit edilen klorojenik asitin miktar tayini yapılmış olup, miktarı 1.047 ± 0.000 g/100 g ekstre olarak bulunmuştur. Miktar tayini sonuçları Tablo 3'te verilmiştir.

Elde edilen fitokimyasal bulgulara göre ekstrenin standardizasyonun klorojenik asit üzerinden yapılabileceği düşünülmüştür.

Tablo 3. Ekstrede varlığı tespit edilen klorojenik asit miktarı, kalibrasyon denklemleri ve retansiyon zamanları (Rt)

Madde	Tespit Edilen Miktar (g/100 g ekstre)	Tespit Edildiği Rt (Dakika)	Kalibrasyon Denklemi	Katsayı (r^2)
Klorojenik asit	1.047 ± 0.000	15.192	y=114.78x+24.966	0.9998

Seyrekoglu ve arkadaşları (2022), *H. scabrum* toprak üstü kısımlarından hazırlanan etanol:su (3:7) ekstresinin antioksidan etkisini araştırmıştır. Ekstrenin toplam fenolik içeriğin 148.31 ± 4.57 gallik asit eşdeğeri mg/g ekstre olduğu bulunmuştur. Ekstrenin DPPH radikal süpürücü aktivitesi 3.65 ± 0.40 (IC_{50}, µg/ml) olmuştur. Ekstreler güçlü DPPH ve toplam antioksidan aktivite sergilemiştir [29].

Türkiye'nin Güneydoğu bölgesinde Mardin ve Diyarbakır'dan toplanan *H. scabrum* bitkisinin antioksidan aktivitesi incelenmiştir. Bitkinin toprak üstü kısımlarından %70 etanol ile hazırlanan ekstreler kullanılmıştır. DPPH radikal süpürücü aktivite deneyinde bütil hidroksi toluen (BHT) pozitif kontrol olarak kullanılmış ve BHT 250 μg/ml konsantrasyonda %93 oranında yüksek radikal süpürme kabiliyeti göstermiştir. *H. scabrum* ekstresinin DPPH radikal süpürücü etkisinin BHT'den daha düşük olduğu bulunmuştur. Ekstrelerin ve standart maddelerin artan konsantrasyona bağlı olarak indirgeme gücü artmıştır. Standart olarak kullanılan BHT ve α-tokoferol tüm ekstrelerden daha yüksek indirgeme gücü göstermiştir. Metal bağlama kapasitesi incelendiğinde standart madde olarak kullanılan EDTA'nın tüm konsantrasyonlarda etkisi yaklaşık %100 olmuştur. *H. scabrum* ekstresi 150 mg/ml konsantrasyonda %39 bağlayıcı etki göstermiştir. *H. scabrum* ekstresinin toplam fenol içerik miktarı 262 μg/mg gallik asit eşdeğeri ekstre olmuştur [18].

H. scabrum bitkisinin fitokimyasal içeriğinin YPSK analizi ile araştırıldığı bir çalışmada bitkinin toprak üstü kısımları toplanarak, çiçek, yaprak ve gövde kısımlarına ayrılmıştır. Yapılan YPSK analiz sonucunda klorojenik asit, rutin, hiperosit, apigenin-7-*O*-glukozit ve kersetin birikimleri açısından yapraklar, çiçeklere ve gövdelere göre daha üstün bulunmuştur. Bitkinin çiçek kısımlarının ise daha yüksek düzeyde kemferol, kersitrin ve amentoflavon içerdiği belirtilmiştir [14]. Farklı bir çalışmada *H. scabrum* bitkisinin hiperisin ve psödohiperisin içerip içermediği bitkinin kök, gövde, yaprak ve üreme kısımlarında YPSK kullanılarak araştırılmıştır. Kökte hiperisin bulunmamıştır, yapraklar ve üreme kısımları büyüme evrelerine bağlı olarak her iki bileşiğin farklı düzeylerde olduğu belirtilmiştir [30].

32 bitki türünün *a*-glukozidaz enzimi üzerinde etkisinin incelendiği bir çalışmada *H. scabrum* bitkisi de araştırmaya konu olmuştur. Buna göre *H. scabrum* bitkisinin metanol ile hazırlanan ekstresi *a*-glukozidaz enzimi üzerinde %60'dan daha yüksek oranda inhibitör aktivite göstermiştir [31].

Türkiye'de yetişen *Hypericum olympicum, H. pruinatum* ve *H. scabrum* türlerinin enzim inhibitör potansiyelleri araştırılmıştır. Bitkilerin toprak üstü kısımlarından hazırlanan metanol ekstreleri incelenmiştir. Elde edilen sonuçlara göre, *H. olympicum* (61.32 ± 3.27 mg orlistat eşdeğeri (OE)/g) ve *H. pruinatum* (39.88 ± 2.51 mgOE/g) ekstreleri, lipaz enzimine karşı kayda değer bir inhibitör etki gösterirken, incelenen tüm ekstreler *a*-amilaza karşı orta düzey bir aktivite göstermiştir. *H. olympicum* (20.06±0.22 mg akarboz eşdeğeri (AE)/g) *H. pruinatum* (20.45±0.11 mgAE/g) ve *H. scabrum* (20.62±0.46 mgAE/g) ekstrelerinin her üçü de α-glukozidaz enzimine karşı güçlü bir aktivite sergilemiştir [32].

Yüksek yağlı diyetle beslenen sıçanlarda *H. scabrum* ekstrelerinin serum lipid profili ve obezite üzerindeki hipolipidemik etkileri incelenmiştir. *H. scabrum* ekstreleri, trigliserit, toplam kolesterol ve düşük yoğunluklu lipoprotein (LDL) kolesterolü değerlerini önemli ölçüde iyileştirmiştir. Yüksek yağ tüketen grupta yer alan sıçanlara *H. scabrum* ekstresinin verilmesi yüksek yoğunluklu lipoprotein (HDL) kolesterolü arttırmıştır. Bu araştırma ile *H. scabrum* ekstresinin yüksek yağ tüketimi ile görülen komplikasyonları azalttığı görülmüştür. Dolayısıyla *H. scabrum* ekstresinin hiperlipidemi ve obezite tedavisi için faydalı bitkisel bir ilaç olarak kullanılabileceği belirtilmiştir [33].

Tarafımızca yapılan araştırma; *H. scabrum* bitkisinin anitoksidan aktivitesinin, kimyasal içeriğinin, enzim inhibitör etkilerinin (*a*-glukozidaz, *a*-amilaz, pankreatik lipaz, kolesterol esteraz) birlikte değerlendirildiği ilk araştırmadır. Antidiyabetik etkinin incelendiği *a*-glukozidaz enzim sisteminde ekstrelerin standart olarak kullanılan akarboza benzer oranlarda inhibitör etki göstermesi dikkat çekmiştir. Fakat ekstreler diğer antidiyabetik etkinin incelendiği *a*-amilaz enzim sistemi üzerinde yüksek inhibitör etki göstermemiştir. Kolesterol esteraz enzimi üzerinde ekstreler standart madde olarak kullanılan simvastatin ile benzer etkiler göstermiş olup, bu durum daha önce *in-vivo* araştırmalardada tespit edilen antihiperlipidemik etki ile uyum göstermektedir. Daha önce yapılmış enzim inhibisyonu çalışmalarına bakıldığında her iki çalışmada da yüksek α-glukozidaz aktivitesinin görülmesi çalışmamızı destekler niteliktedir [31,32]. *α*-Amilaz ve panreatik lipaz inhibisyonları değerlendirildiğinde ise diğer çalışmada Konya'dan yaz aylarında toplanan bitkiden hazırlanan ekstrenin α-amilazı orta derecede inhibe ettiği belirtilmiş, pankreatik lipaz enzimi üzerine ise etki göstermemiştir [32], fakat çalışmamızda ekstrenin α-amilaz enzimini düşük bir oranda, lipaz enzimini ise orta derecede inhibe ettiği görülmüştür. Bunun sebebinin bitkinin fitokimyasal içeriğinde değişime sebep olabileceğinden dolayı, toplama zamanı arasındaki farklılıktan kaynaklanabileceği düşünülmüştür. Tarafımızca yapılan çalışmada olduğu gibi yapılan diğer araştırmalarda da ekstrenin

yüksek DPPH radikal süpürücü etkisi olduğu görülmüştür. Diğer antioksidan aktivite (metal bağlama, indirgeme gücü) sonuçları ve toplam fenolik içerik miktarları literatürde yer alan araştırmalar arasında tam uyum göstermemektedir. Bu farklılığın nedeninin bitkinin yetişme şartı, yetiştiği iklim, yükseklik ve toprak mineral dengesi farklılıklarından kaynaklı olabileceğini akla getirmiştir.

Bilindiği üzere diyabette reaktif oksijen türlerinin üretimini artmakta, antioksidan savunma kapasitesi olumsuz yönde etkilenmekte ve hastalığın seyri olumsuz yönde evrilmektedir. Dolayısıyla diyabet tedavisinde ve diyabet semptomlarının azaltılmasında antioksidan etkili doğal kaynaklar önemli bir rol üstlenebilir. Tüm bu bilgiler ışığında *H. scabrum* toprak üstü kısımlarından hazırlanan etanol ekstresinin antidiyabetik ve antihiperlipidemik etkili doğal bir kaynak olabileceği bu etkilerin daha ileri *in-vivo* ve klinik araştırmalarla desteklenmesine ihtiyaç olduğu sonucuna varılmıştır.

YAZAR KATKILARI

Kavram: H.N.G., S.B., D.D.O.; Tasarım: H.N.G., S.B., D.D.O.; Denetim: H.N.G., S.B., D.D.O.; Kaynaklar: H.N.G., S.B., D.D.O.; Malzemeler: H.N.G., S.B., D.D.O.; Veri Toplama ve/veya İşleme: H.N.G., S.B., D.D.O.; Analiz ve/veya Yorumlama: H.N.G., S.B., D.D.O.; Literatür Taraması: H.N.G., S.B., D.D.O.; Makalenin Yazılması: H.N.G., S.B., D.D.O.; Kritik İnceleme: H.N.G., S.B., D.D.O.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

ENDEMİK *ASPERULA LILACIFLORA* SUBSP. *PHRYGIA* (BORNM.) SCHÖNB. -TEM. (RUBIACEAE) ÜZERİNDE FARMASÖTİK BOTANİK ARAŞTIRMA

THE PHARMACEUTICAL BOTANICAL INVESTIGATION ON ENDEMIC ASPERULA LILACIFLORA SUBSP. PHRYGIA (BORNM.) SCHÖNB. -TEM. (RUBIACEAE)

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ÖZ

Amaç: *Bu çalışmada Rubiaceae familyasına ait endemik Asperula lilaciflora subsp. phrygia'nın farmasötik botanik açıdan araştırılması amaçlanmıştır.*
Gereç ve Yöntem: *Eskişehir-İnönü'den toplanan bitki morfolojik araştırmalarda değerlendirilirken, tüy ve polen morfolojisi SEM'de incelenmiştir. Anatomik çalışmalarda %70'lik alkole alınan örneklerin manuel kesitlerinde, yapıları aydınlatılmış ve fotoğrafları çekilmiştir. Toplam fenolik madde miktarı Folin-Ciocalteu reaktifi ile test edilmiştir. Bitki ekstresinin antioksidan aktivitesi ise DPPH ve ABTS süpürücü etki tayini ile belirlenmiştir.*
Sonuç ve Tartışma: *Bitkinin morfolojik, anatomik, tüy, polen ve kimyasal özellikleri ilk kez bu çalışmayla ortaya koyulmuştur. Morfolojik çalışmada taksonun ayrıntılı deskripsiyonu verilmiş, çizimler ve fotoğraflarla desteklenmiştir. Gövde ve yapraklarda yoğun hispit tüyler tanımlanmıştır. Polenler hekzakolpat olup, eliptik şekillidir. Gövdeler, dört köşeli-yuvarlak, kollateral tipte ve ergastik madde ile rafit kristalleri içermektedir. Yapraklar monofasiyal tiptedir. Toplam fenolik madde miktarı 1 g A. lilaciflora subsp. phrygia ekstresinde 31 mg gallik asite eşdeğer (GAE) olarak hesaplanmıştır. Antioksidan aktivitesi DPPH IC_{50}=0.38 mg/ml, ABTS IC_{50}=0.37 mg/ml-0.90 mg/ml olarak bulunmuştur.*
Anahtar Kelimeler: *Anatomi, antioksidan, Asperula, morfoloji, palinoloji*

ABSTRACT

Objective: *This study aimed to investigate the aspects of pharmaceutical botany of endemic Asperula lilaciflora subsp. phrygia in Rubiaceae family.*

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Material and Method: *While the plant from Eskişehir-İnönü was studied morphologically, it was also examined by SEM for trichome and pollen morphology. In anatomical studies, the structures of the samples taken in 70% alcohol were illuminated in manual sections and their photographs were taken. The total amount of phenolic substances was tested with the Folin-Ciocaalteu reagent. The antioxidant activity of the plant extract was determined by DPPH and ABTS scavenging effect assays.*

Result and Discussion: *The morphological, anatomical, trichome, pollen, and chemical properties of the plant were revealed for the first time in this study. A detailed description of the taxon was given in the morphological study, supported by drawings and photographs. The stem and leaves are densely hispid hairy. Pollen is hexacolpate and elliptical. The stems are tetragonal-round, collateral type and contain ergastic material, raphide crystals. Leaves are monofacial. The total amount of phenolic substances in 1 g of extract was calculated as 31 mg of gallic acid equivalent (GAE). Antioxidant activity was found to be DPPH IC_{50}=0.38 mg/ml, ABTS IC_{50}=0.37 mg/ml-0.90 mg/ml.*

Keywords: *Anatomy, antioxidant, Asperula, morphology, palynology*

GİRİŞ

Rubiaceae familyası üyeleri boyamacılıkta kullanılmakta ve köklerin boyama özelliğinden dolayı, bu familyaya "Kökboyasıgiller" adı verilmektedir [1]. Kozmopolit olan bu familya, dünyada yaklaşık 620 cins ve 13000 kadar tür ile familyalar arasında dördüncü sırada yer almaktadır [2,3]. Familyadaki bitkilerin antimikrobiyal, antienflamatuar, antimalaryal, antioksidan gibi birçok etkiye sahip olduğu bilinmektedir [4]. Ayrıca bazı türlerin ekonomik değerinin olmasıyla beraber; *Cinchona* L., *Coffea* L., *Uncaria* Schreber gibi bazı cinslerden droglar elde edilmekte ve tedavi amaçlı da kullanılmaktadır. *Asperula* L. da bu familyaya dâhil Türkiye'de bulunan cinslerden biri olup, bazı tıbbi etkiler göstermektedir. Halk arasında lenf ve kan temizlenmesinde, böbrek rahatsızlıklarında, cilt hastalıklarında ve kanser tedavisinde kullanılmaktadır; ayrıca sedatif etki gösterdiği de bilinmektedir [5-7].

Asperula cinsi Dünya üzerinde alt tür ve varyeteleri içeren 232 takson ile temsil edilmektedir [8]. Türkiye'de ise cinse ait 58 takson bulunmaktadır. Bu türlerin 26 tanesi endemik olup, yüksek bir endemizm oranı göstermektedir [9]. *Asperula lilaciflora* L. küme şeklinde yarı çalımsı bir bitki olup florada beş alttüre ayrılmaktadır. *A. lilaciflora* subsp. *phrygia* (Bornm.) Schönb.-Tem çiçek durumunun dallanması ve korolla tüpünün uzunluğu ile diğer alttürlerden ayrılmaktadır. Endemik olan bu attür, meşe ve çam ormanlarında, steplerde 300-1400 m yüksekliklerde yetişmektedir [10].

Asperula cinsi ile ilgili, literatürde çeşitli çalışmalar bulunmaktadır. *A. taurina* L. subsp. *caucasica* (Pobed.) Ehrend. türünün kök anatomisinde rafit kristalleri tespit edilmiştir [1]. *A. odorata* L. türünün kuru ekstraktının antihipoksik ve sedatif aktivitesi test edilmiştir [7]. Çankırı'da bulunan bir takson morfolojik incelemeler sonucu yeni bir tür (*A. cankiriense* B.Şahin & Sağıroğlu) olarak rapor edilmiştir [8]. *A. arvensis* L. türünün flavonol glikozidleri analiz edilmiştir [11]. Endemik olan *Asperula* cinslerinin agar difüzyon ve sıvı dilüsyon metotlarıyla aktimikrobiyal aktiviteleri test edilmiştir [12]. Bazı endemik *Asperula* taksonlarının kimyasal bileşenleri, toplam flavanoid ve α-tokoferol (E-vitamini) miktarları ve antioksidan aktiviteleri belirlenmiş olup, çalışılan taksonlar arasında radikal süpürücü etkisi en yüksek *A. brevifolia* Vent. olarak bulunmuştur [13]. 5 farklı *Asperula* taksonunun karyotip çalışması yapılarak, kromozom sayıları ortaya çıkarılmıştır [14]. *A. lutea* subsp. *rigidula* (Halacsy) Ehrend. türünde temel yağ asidi bileşenlerinin %32.7 palmitik asit, %20.1 linolenik asit ve %10.5 linoleik asit olduğu belirlenmiştir [15]. Anadolu'nun güney-doğusunda Şırnak Uludere'den toplanan, *Asperula* cinsinin bazı türleri arasında yapılan morfolojik inceleme sonucunda *A. anatolica* M. Ozturk, yeni tür olarak Türkiye Florası'na kazandırılmıştır [16]. *A. lilaciflora* bitkisinin toprak üstü kısımlarından yeni bir flavonol glikozit (lilasifloroside) ve iridoid (asperulogenin) izole edilmiştir [17]. Endemik *A. daphneola* O. Schwarz kök ve gövde anatomik kesitlerinde rafit kristalleri tespit edilmiş olup yapıları ayrıntılı açıklanmıştır. Ayrıca kromozom sayısına bakılmış ve 2n=20 olarak tespit edilmiştir [18]. *Asperula* cinsinin filogenetik ilişkisi tekrardan yorumlanmıştır [19]. *A. taurina* subsp. *caucasica* (Pobed.) Ehrend. taksonunun toprak üstü kısımlarının fitokimyasal, antikolinesteraz ve antioksidan özellikleri belirlenmiştir. Fitokimyasal çalışma sonucunda ise kersetin 3-O-β-galaktozit

izole edilmiş; asetilkolinesteraz inhibitör etki ise göstermediği tespit edilmiştir [20]. *A. odorata*'nın hidroalkolik ekstresinin fenolik ve flavonoid içeriğine sahip olduğu ve fareler üzerinde anti-depresan etki gösterdiği bildirilmiştir [21]. Çalışılan taksonların polen şekillerinin prolat-sferodial ve oblat-sferodial olduğu bulunmuştur [22].

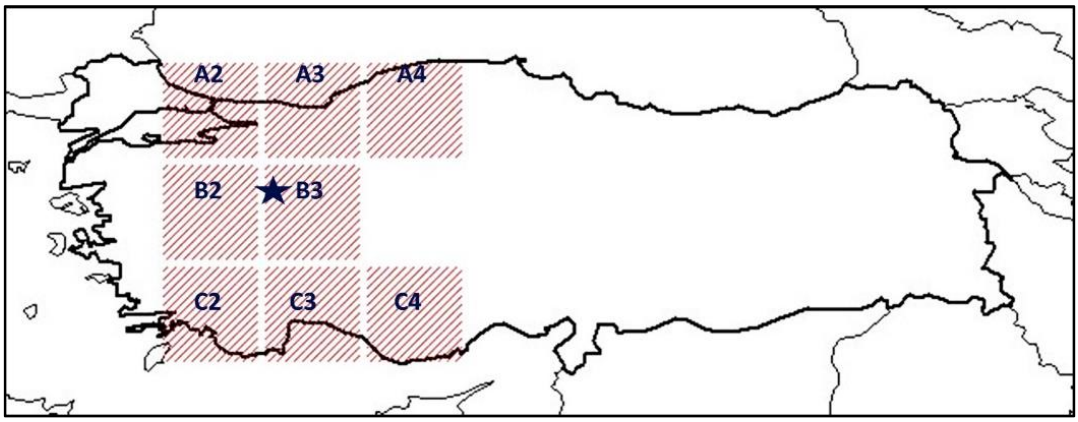
Ülkemizde yayılış gösteren endemik *A. lilaciflora* Boiss. subsp. *phrygia* alttürüne literatür taramasında genellikle flora çalışmalarında rastlanılmıştır. Taksona ait detaylı anatomik, morfolojik, palinolojik ve kimyasal çalışmanın bulunmaması nedeniyle; bu çalışmada endemik *A. lilaciflora* subsp. *phrygia*'nın farmasötik botanik açıdan ayrıntılı olarak ilk kez araştırılması amaçlanmıştır.

GEREÇ VE YÖNTEM

Bitkisel Materyaller

Bitkisel materyal çiçekli ve meyveli dönemlerinde (2020 Mayıs-Temmuz) Eskişehir-İnönü ilçesinden (30˚10'1.555" E- 39˚44' 21.496"N) toplanmış ve numaralandırılıp herbaryum örneği haline getirilmiştir (ESSE: 15611) (Şekil 1). Bitkinin yayılış gösterdiği alanlar ve bitkinin toplandığı bölge Şekil 2'de gösterilmektedir [23].

Şekil 1. **(a)** Bitki genel görünüş **(b)** Herbaryum örneği



Şekil 2. Davis'in kareleme sistemine göre, taksonun yayılış alanları ve *örneğin toplandığı yer

Morfolojik ve Anatomik Çalışmalar

Morfolojik çalışmada taksonun ayrıntılı deskripsiyonu verilmiş, çizimler ve fotoğraflarla desteklenmiştir (Şekil 3,4,5,6). Çizimler ve fotoğraflar için WILD TYP 181300 stereo mikroskobu ve resim çizme tüpü kullanılmıştır. Mikromorfolojik çalışmalar için bitkilerin gövde, yaprak, korolla, ovaryum yüzeylerindeki tüy ve yapılar taramalı elektron mikroskobu (SEM-TM3030 Plus Tabletop Microscope-HITACHI) ile görüntülenmiştir. Morfolojik terminolojide Baytop (1998)'un İngilizce-Türkçe Botanik Kılavuzu [24] isimli eseri kullanılmıştır.

Anatomik çalışmalarda kullanılmak üzere %70'lik alkolde muhafaza edilen gövdeden enine kesitler; yaprakların ise hem enine, hem de yüzeysel kesitleri alınmıştır. Tüm kesitler saydamlaşması için kloralhidrata maruz bırakılmıştır, daha sonra Sartur reaktifi ile boyanarak anatomik yapıları aydınlatılmıştır.

Palinolojik Çalışmalar

Polenler, ok uçları yardımıyla çıkarılıp, örnek tutucu (stab) üzerine çift taraflı bant ile yapıştırılmıştır. Hazırlanan örnekler altın ile kaplanmış ve masa üstü taramalı elektron mikroskobunda (TM3030 Plus Tabletop Microscope - HITACHI) ayrıntılı olarak incelenmiş ve fotoğraflanmıştır. Polen morfolojisinde, Walker ve Doyle [25] terminolojisinden yararlanılmıştır.

Bitki Ekstreleri ve Kimyasal Çalışmalar

Bitkilerin oda sıcaklığında kurutulmuş toprak üstü kısımları iri taneli toz parçaları haline getirilmiştir. Toz numuneden 20 g tartılarak erlenmayere konulmuştur. Üzerine %70'lik 100 ml etanol konularak, 1 gün boyunca çalkayıcı ile oda sıcaklığında masere edilmiştir. Süre sonunda karışım süzülüp, numune üzerine tekrar 100 ml etanol ilave edilmiştir. Aynı işlem üç kez yapılmıştır. Süzüntüler bir kap içerisinde toplanıp dolaba konulmuş, daha sonra saklanan süzüntülerdeki etanol evaporatörde 30 dakika boyunca 41^{o}C'de kontrollü bir şekilde uçurulmuştur. Çözücü uzaklaştırma işleminden sonra, numunedeki suyun kristalleşmesi için dondurucuya yerleştirilmiştir. Kristal halindeki su ise liyofilizatör ile uzaklaştırılmıştır. Elde edilen ekstre ağzı kapalı şişelerde saklanmıştır.

Toplam fenolik madde miktarı tayini için Folin-Ciocalteu yöntemi kullanılmıştır. 20 mg bitki ekstresi 2 ml metanolde çözündürülerek 10 mg/ml derişimli çözelti hazırlanmıştır. Standart olan gallik asitten de farklı derişimlerde (0.1 mg/ml, 0.35 mg/ml, 0.5 mg/ml, 0.7 mg/ml, 1 mg/ml) çözeltiler hazırlanmıştır. Hazırlanan çözeltilerden 20 µl mikroplak kuyucuklarına konularak üzerine 1560 µl distile su ve 100 µl Folin-Ciocalteu reaktifi eklenmiştir. Reaktifin homojen dağılması için 1-2 dakika bekledikten sonra, 300 µl %20'lik sodyum karbonat (Na_2CO_3) eklenerek reaksiyon başlatılmıştır. Karanlıkta 2 saat oda sıcaklığında bekledikten sonra absorbans değerleri 760 nm'de ölçülmüştür.

DPPH süpürücü etki tayini için MeOH'de (metanol) çözülmüş bitki örneğinden seri seyreltme yapılmıştır. Radikal uzaklaştırma aktivitesi, %50 inhibisyon konsantrasyonu denklemi (IC_{50} = $[(A_0-A_1)/A_0)] \times 100$) kullanılarak hesaplanmıştır. Denkleme göre; IC_{50}: %50 inhibe eden konsantrasyon, A_0: kontrol absorbans, A_1: numune absorbansıdır [14]. 96-kuyucuklu mikrotitrasyon plak kuyucuğuna 200'er µl MeOH'de çözülmüş bitki örneği (10 mg/ml derişimli) aktarılmıştır. Diğer kuyucuklara 100'er µl MeOH konulmuştur. İlk kuyucuktan 100 µl numune alınarak, sırasıyla 10 seri seyreltme işlemi yapılmıştır. Seyreltilmiş örnekler üzerine 100 µl $DPPH^{\bullet+}$ çözeltisinden eklenip 30 dakika karanlıkta bekletilmiştir. Spektrofotometrede 517 nm'de ölçüm yapılmıştır.

ABTS süpürücü etki tayini için, 7 miliMolar (mM) $ABTS^{\bullet+}$ ve 2.5 mM sodyum persülfat ($Na_2S_2O_8$) ile karıştırılmıştır. Karışım 12-16 saat karanlıkta bekletilmiş ve mavi-yeşil renkli radikal oluşumu sağlanmıştır. Hazırlanan radikal çözeltinin absorbans değerinin 734 nm'de 0.7- 0.8 olması için, etanol ile seyreltme yapılmıştır. Bitki ekstresinden 0.1 mg/ml ve 10 mg/ml derişiminde çözeltiler elde edilmiştir. Daha sonra ise, standart olarak kullanılacak 3 mM Troloks'dan farklı derişimler (2.5 mM, 2 mM, 1.5 mM, 1 mM, 0.5 mM ve 0.1 mM) hazırlanmıştır. 0.1 mg/ml ve 1 mg/ml yoğunluğa sahip gallik asit ise pozitif kontrol olarak kullanılmıştır. Hazırlanan numunelerden 10 µl mikroplak kuyucuklarına sırasıyla konulmuş ve üzerine 990 µl $ABTS^{\bullet+}$ eklenmiştir. 30 dakika sonunda 734 nm'de absorbansı ölçülmüştür. Troloksa eşdeğer antioksidan kapasite hesabı için, standartın kalibrasyon eğrisi kullanılmıştır.

SONUÇ VE TARTIŞMA

Morfolojik Sonuç ve Tartışma

Bitki çok gövdeli, tabanda yoğun yapraklı, yarı çalımsıdır (Şekil 3a). Çiçekli gövdeler 16-35(-39) cm, yükselici - dik, dört köşeli, ya da hafifçe yuvarlak, yoğun puberulent ya da hispit tüylü üst kısımlarda tüysüz (Şekil 4a,b).

Yapraklar her nodta dört adet ve çevrel dizilişli (Şekil 5a), linear-subulat (Şekil 5b,c). Taban yapraklar 2-10 × 0.2-1 mm, yoğun hispit tüylü (Şekil 5d), tepede aristat, şeffaf arista 0.5-1 mm; üst yapraklar 10-20 × 0.2-1 mm, tüy daha az ya da eksik, şeffaf arista 1 mm, kenarlar düz, tabanda trunkat ve belirgin geriye kıvrık.

Çiçek durumu basit ya da hemen hemen dallanmış, panikula çiçek durumunda bir araya gelen (Şekil 3b) çiçekler dalların ucunda simoz şeklinde (Şekil 4c), simözlarda bir araya gelen çiçeklerin sayısı 3-12, çiçek durumunun sapı 8-47 mm.

Brakte simözlarda, 2 adet (Şekil 6d), 2.4-4.5 × 1-1.6 mm, ovat-üçgen, alt kısım yanlarda kulakçıklı, düz veya belirgin şeffaf kenarlar genellikle düzensiz dişli (Şekil 6e,f). Tepede aristalı, aristalar 0.5-1 mm, şeffaf, hafif geriye kıvrık, tüysüz.

Brakteoller 2-3.6 × 0.4-0.8 mm (Şekil 6g,h), lanseolat, kenarlar düz, alt kısımlarda içe kıvrık, genellikle düzensiz dişli, tepede aristalı. Şeffaf aristalar 0.5-1 mm, tüysüz.

Kaliks eksik.

Korolla gamopetal, huni ya da lobları yayık boru şeklinde, 5-10 mm (Şekil 5e,f), loblarda pembemsi beyaz, tüp kısmında pembe (Şekil 3b). Loblar 4 parçalı (Şekil 5g), lanseolat, akuminat, 2-3 × 0.8-1.2 mm, tüp 5-7.5 mm, tüpten 2 ya da 3 kat kadar daha kısa, çok nadiren tüp ve lob neredeyse eşit, tüysüz.

Pistil açık pembe, hemen hemen ikili eşit dallanmış yuvarlak stigmalı, stilus 6-6,5 mm. Ovaryum 0.5-1.2 × 0.5-0.8 mm, dış yüzeyi yoğun papilli (Şekil 6b).

Stamenler 4 adet, 1-1.3 mm, loblar ile almaşık dizili, dorsifiks. Anterler linear (Şekil 6a), sarımsı-koyu kahverengi, filamentler çok kısa.

Meyve iki merikarpa ayrılan şizokarp (Şekil 6c), ovat-orbikular, genişçe oblong, 2-3×1-2 mm, genç merikarplar pembemsi, olgun merikarplar kahverengi (Şekil 3c), dış yüzey yoğun papilli.

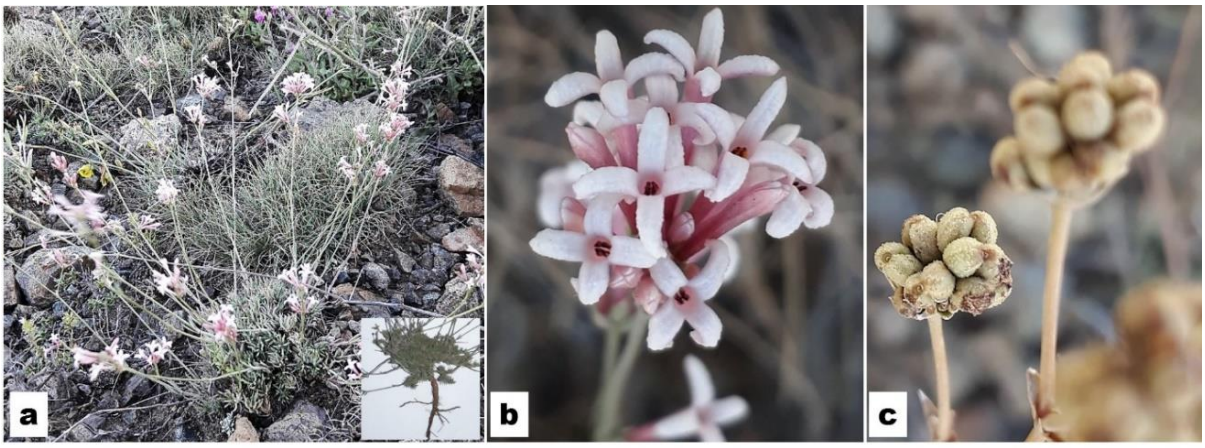
Not: Bitki kuruduğunda korolla ve üst gövdelerin bazıları belirgin mor.

Çiçeklenme zamanı: Haziran-Temmuz.

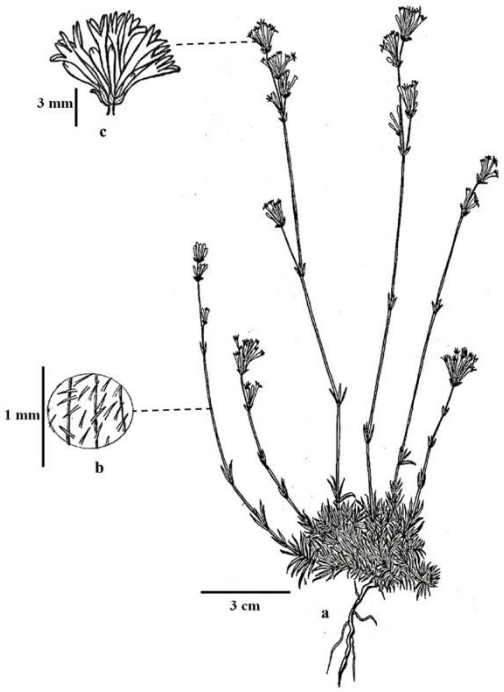
Habitatı: Tepe stepleri, meşe ve çam ormanlarıdır.

Yüksekliği: 300-1400 m

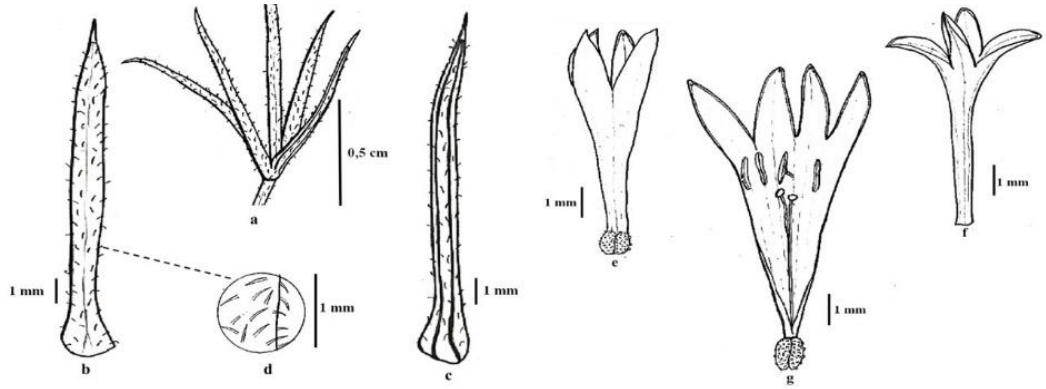
Endemik



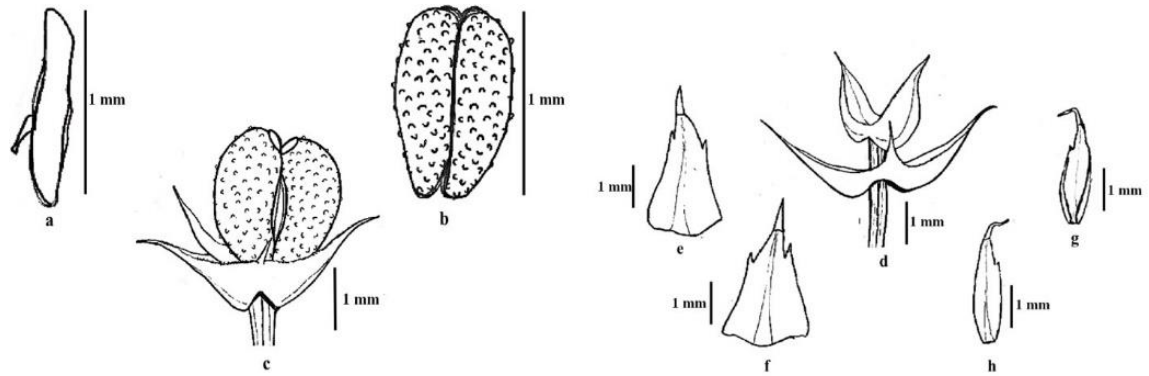
Şekil 3. (a) Bitki genel görünüş**,** taban yapraklar ve kök **(b)** çiçekler **(c)** meyveler



Şekil 4. Çizimler **(a)** bitki genel görünüş **(b)** gövde tüyü **(c)** çiçek durumu



Şekil 5. Çizimler **(a)** yaprakların dizilişi **(b)** yaprak üst yüzey **(c)** yaprak alt yüzey **(d)** yaprak tüyleri **(e)** ovaryumlu korolla **(f)** ovaryumsuz korolla **(g)** korolla iç yüzey



Şekil 6. Çizimler **(a)** anter **(b)** ovaryum **(c)** merikarplar (meyve) **(d)** çiçeği saran brakte **(e)** brakte iç yüzey **(f)** brakte dış yüzey **(g)** brakteol iç yüzey **(h)** brakteol dış yüzey

Davis'in kareleme sistemine göre, bu çalışmada B3 karesinden toplanan ve genel yapısı Şekil 3'teki gibi olan endemik *A. lilaciflora* subsp. *phrygia* alt türünün morfolojik sonuçları, Davis [10]'in "Flora of Turkey and the East Aegean Islands" kaynağı ile kıyaslanarak farklılıklar kısaca özetlenmiştir (Tablo 1).

Tablo 1. *A. lilaciflora* subsp. *phrygia* morfolojik özelliklerinin flora ile karşılaştırılması

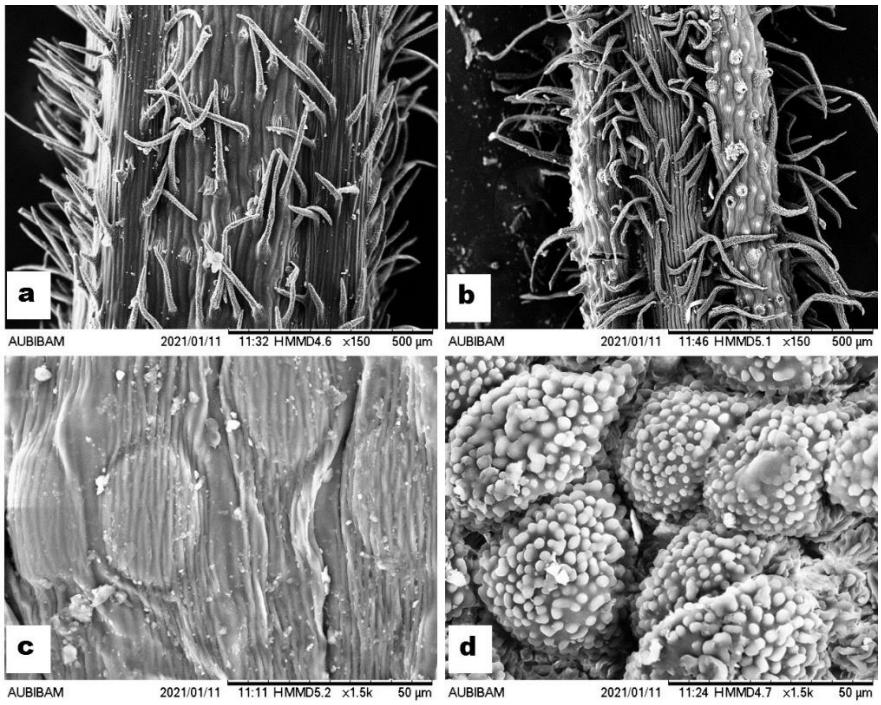
Özellikler		***A. lilaciflora* subsp. *phrygia***	**Türkiye Florası**
Gövde boyu		16-35(-39) cm	(10-)15-35 cm
Yaprak boyutu		Taban yaprak 2-10 × 0.2-1 mm, üst yaprak 10-20 × 0.2-1 mm	Taban yaprak 2-7 × 0.6-1 mm, üst yaprak (-8)10-20 × 0.3-0.75 mm
Yaprak arista boyu		0.5-1 mm	1 mm
Brakte boyu		2.4-4.5 × 1-1.6 mm	1.5-6 mm
Korolla boyu		5-10 mm	(4-)5-8 mm
Korolla rengi		Loblarda pembemsi beyaz, tüpte ise pembe	Açık pembe-pembe
***Yaprak**	**Sayısı**	4, çevrel dizilişli	-
	Kenarı	Düz	-
***Çiçek durumu**	**Çiçek sayısı**	Simozlarda 3-12	-
	Sap uzunluk	8-47 mm	-
***Brakte**	**Arista boyu**	0.5-1 mm, şeffaf hafif geriye kıvrık	-
***Brakteol**	**Boyut**	2-3.6 × 0.4-0.8 mm	-
	Şekil	Lanseolat	-
	Kenar	Düz ya da düzensiz dişli	-
	Tepe	Aristat	-
	Arista	0.5-1 mm, şeffaf	-
***Stamen**	**Sayısı**	4	-
	Şekli	Dorsifiks	-
	Konumu	Petale bağlı, loblar ile almaşık	-
***Anter**	**Boyu**	1-1.3 mm	-
	Şekli	Linear	-
	Rengi	Sarımsı-koyu kahve	-
***Filament**	**Boy-Yapı**	Kısa, Korollaya yapışık	-
***Ovaryum**	**Boyu**	0.5-1.2 × 0.5-0.8 mm	-
	Rengi	Koyu kahverengi	-
***Stilus**	**Boyu**	6-6.5 mm	-
	Şekli	İkili eşit dallanmış	-
	Rengi	Açık pembe	-
***Stigma**	**Şekli**	Küresel	-
	Sayısı	2	-
***Meyve**	**Şekli**	Ovat-orbikular	-

Bitki boyu, çalışmamızda (16-35 (-39) cm) Türkiye Florası'ndan ((10-)15- 35 cm) daha fazla belirlenmiştir. Gövde şekli ve tüy yapısı flora ile uyumlu çıkmıştır. Floraya göre çalışmamızdaki taban yapraklar daha uzun, üst yapraklar ise benzerdir. Yaprak enleri ise aynı ya da daha dardır. Florada brakte 1.5-6 mm, sonuçlarımızda 2.4-4.5 × 1-1.6 mm olup daha küçüktür. Brakte şekli ovat-üçgen olup floradan farklı bulunmuştur. Florada korolla loblarının boyu 1.5-3 mm olup, tüp boyu loblara eşit ya da 3 kat uzun iken; çalışmamızda lob boyu 2-3 × 0.8-1.2 mm ve tüp boyu loblardan 2 ya da 3 kat uzun çıkmıştır. Bununla birlikte yaprak, çiçek durumu, brakte, brakteol, stamen, pistil ve meyveye ait tabloda belirtilen özellikler ilk kez bu çalışmayla verilerek florada bu yöndeki eksiklikler giderilmiştir.

Mikromorfolojik Sonuç ve Tartışma

Bitkiye ait tüy özellikleri ve yoğunlukları Tablo 2'de verilmiştir. Alt gövde yoğun tüylü, üst gövde tüysüz, gövde üzerinde çok sayıda stoma bulunur. Örtü tüyleri tek hücreli, basit, genelde uzun-yatık hispit, 101 µm-174 µm ve yoğun kütikula kabarcıklıdır. Yaprak alt ve üst yüzeyi yoğun örtü tüylü olup gövde ile benzer tüy yapısını sahiptir (Şekil 7a,b). Yaprağın iki yüzünde de stoma olup, üst yüzde daha

fazladır. Korolla dış yüzeyi düzensiz çizgili ve yer yer elipsoid-oblong şekiller içerirken (Şekil 7c), iç yüzey düzensiz epidermal katlanmaların oluşturduğu yoğun şekilsiz kabartılar halindedir. Ovaryumun tüm yüzeyi sıkı dizilimli, çok sayıda, irili ufaklı, konveks şekilde papiller ile kaplıdır (Şekil 7d). Papillerin üzerinde açık renkte küçük yoğun ikincil papil kabarcıklar yer alır.



Şekil 7. SEM görüntüleri **(a)** gövde tüyleri **(b)** yaprak tüyleri **(c)** korolla yüzeyi **(d)** ovaryum yüzeyi

Tablo 2. Vejetatif ve generatif organların tüy özellikleri ve yoğunlukları

	Özellikler	**Konum**	**Yoğunluk**
Gövde	101 µm-174 µm Hispit Tek hücreli	Üst	±
		Alt	++
Yaprak	50.3 µm-265 µm Hispit Tek hücreli	Üst yüz	+
		Alt yüz	+
Korolla	Tüysüz	Tüp	-
		Lob	-
Ovaryum	Yuvarlak ve konveks, Papillalı		++

(-) yok, (±) seyrek, (+) yoğun, (++) çok yoğun

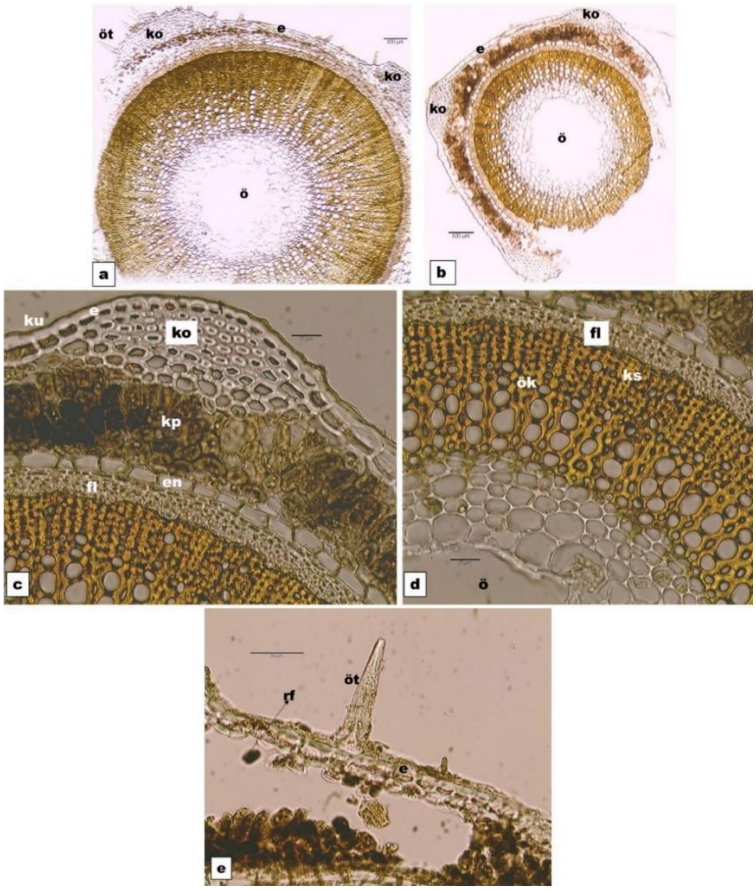
Metcalfe ve Chalk'ın [26] *Asperula* ve bazı cinslerde rapor ettiği tek hücreli uzun tüyler ve uç kısımdaki kıvrımlı yapı bizim sonuçlarımızla uyumludur. Şahin vd.'nin yaptıkları bir çalışmada *A. cankiriense*'de en üst yapraklar seyrek kısa setalı veya tüysüz olarak rapor edilirken [8], *A. anatolica* yaprak üst ve alt yüzeylerinde tüysüz, kenarlarda ve ana yüzeyde sadece seyrek olarak kısa setalı tüylerin olduğu bildirilmiştir [16].

Anatomik Sonuç ve Tartışma

Gövde

Gövde enine kesitlerde alt gövdeler dört köşeli-yuvarlak; üst gövdeler belirgin dörtgendir (Şekil 8a,b). Dış kısımda dikdörtgen ya da kare şekilli, tek sıra, üst ve alt çeperleri daha kalın olan epiderma

hücreleri bulunur. Epidermanın üzeri düz kalın bir kütikula tabakası ile örtülüdür. Alt gövdelerde seyrek, basit tek hücreli örtü tüylerine rastlanmıştır (Şekil 8e). Köşelerde epidermanın altında 3-7 sıra, enine oval hücreli kollekimatik doku yer alır. Hemen altında korteksi oluşturan, 2-4 sıra, büyük, yuvarlak-oval hücrelerin meydana getirdiği parankimatik doku yer alır. Yoğun ergastik maddeler ve rafit kristali içeren (Şekil 8e) parankima dokusu üst gövdelerde neredeyse korteksin tamamında koyu renkte ve halka şekliyle dikkat çekicidir. Alt gövdelerde ise ergastik maddeler içeren parankima dokusu daha azdır. Endoderma tek sıra, belirgin, büyük enine dikdörtgenimsi hücrelerden oluşur (Şekil 8c). Endoderma üst gövdelerde gövdeyi halka şeklinde çevrelerken, alt gövdelerde parankima hücreleriyle yer yer kesintiye uğramıştır. İletim demetleri gövdeyi tümüyle sarar. Floem tabakası 4-5 sıra, basık düzensiz şekilli hücrelerden oluşur Floem altında, belirsiz ya da 1-2 sıra ezilmiş hücrelerden oluşan kambiyum yer alır. Trake ve trakeidlerden oluşan ksilemde, trakeler büyük oval-yuvarlak şekilde, trakeidler ise küçük düzensiz şekillidir. Öz kolları 1 (-2) sıradır. Öz bölgesini dolduran hücreler ksilemin altında küçük, öze doğru büyük, poligonal, ince çeperli, hücreler arasında boşluklu parankimatik hücrelerden oluşur. Merkeze doğru parçalanan parankima hücrelerinden dolayı orta kısım boştur (Şekil 8d).



Şekil 8. *A. lilaciflora* subsp. *phrygia* enine kesitleri **a)** alt gövde genel görünüş **b)** üst gövde genel görünüş **c-d-e)** yakın görünüm ku:kütikula, e:epidermis, ko:kollenkima, kp:korteks parankiması, en:endodermis, fl:floem, ks:ksilem, ök:öz kolları, ö:öz, öt:örtü tüyü, rf:rafit kristali (Ölçek a-b=100 µm, c-d=25 µm, e=50 µm)

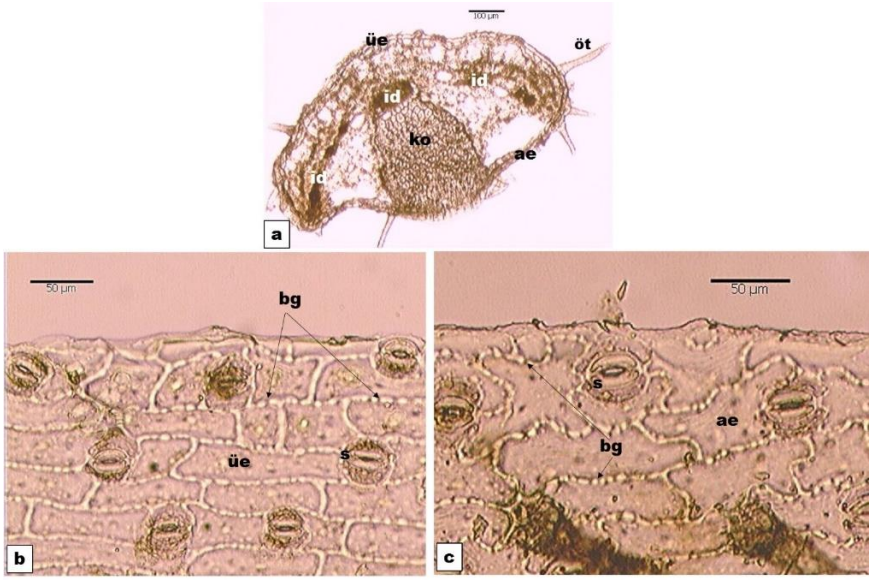
Yaprak

Yaprak enine kesitte genel görünüm ovaldir. Geriye kıvrık olan yapraklar alt yüzeyde hafif girintilidir (Şekil 9a). Yapraktan alınan enine kesitte; alt ve üst epidermis hücreleri tek sıralı, dikdörtgenimsi olup, alt ve üst çeperle yan çeperlere göre daha kalındır. Yüzeysel kesitlerde üst-alt epiderma hücreleri uzun dikdörtgen şekilli, hemen hemen eşit uzunlukta ve çok sayıda basit geçitli; alt

epidermalar, üst epidermadan daha dalgalı çeperlidir (Şekil 9b,c). Alt ve üst epiderma üzerinde kalın, düz ya da hafif dalgalı bir kütikula tabakası ve tek hücreli, konik-uzun, basit örtü tüyleri bulunur. Epidermanın iki yüzünde yer alan stoma (amfistomatik), parasitik tiptedir (Şekil 9b). Epiderma hücreleri ile stomalar aynı seviyede (mezomorf) olup, üst yüzde stoma sayısı, alt yüze oranla daha fazladır. Alt epidermada kollenkimanın sağında ve solunda yer alan stoma altı boşlukları dikkat çekecek kadar geniştir. Yaprak monofasiyaldir. Üst epidermanın altında mezofili oluşturan 2 sıra, sıkı dizilimli, uzun, silindrik, bol kloroplast içeren palizat parankiması yan yüzlerde tek sıra halinde devam eder. Alt epidermada palizat parankimasının üzerinde 3-4 sıra boyuna silindirik ya da oval, gevşek dizilimli sünger parankiması bulunur. Mezofil tabakasında yer yer rafit kristalleri görülmektedir. Orta damarın altında dikeyde 15-19 sıra, yatayda 6-15 sıra oval-yuvarlak hücreli kollenkimatik doku yer alır. Kollenkima tabakasının üzerinde demet kını ile çevrelenmiş büyük bir iletim demeti, bu demetin sağında ve solunda ise 4-5 adet küçük iletim demetleri vardır. İletim demetlerinde ksilem üst epiderma, floem ise alt epiderma yönündedir.

Metcalfe ve Chalk'a göre *Asperula* cinsinin gövdeleri kollenkimatik köşe çıkıntılarının olduğu poligonal şekle sahiptir ve mantar görülmez. Endoderma iyi gelişmiş, bununla birlikte skleranchimatik periskl bulunmaz. Ksilem dar, silindirik bir yapıdadır ve trakeal elemanlarının çapları küçüktür. Rubiaceae familyası genellikle dorsiventral yaprak yapısına sahip iken, bazı üyelerinde sentrik (*Asperula*) ya da homojen (*Borreria*) bir yapı görülebilmektedir. Bazı cinslerin türlerinde tüyler küçük kristaller içerebilmektedir. Stoma neredeyse sadece yaprak alt yüzde bulunsa da, *Asperula* ve bazı cinslerin türlerinde üst yüzünde de yer alır ve rubiaseus (parasitik) tiptedir. Epiderma hücreleri düz ya da dalgalı çeperlidir. Familya üyelerinde kristaller genellikle yaprak ve gövdelerde yoğun olup kum kristali, rafitler, küme kristaller şeklindedir ve kristallerin tipi cinslerin ve türlerin tanımlanmasında son derece önemlidir. Rafitler ya yalnız olarak ya da küme kristallerle birlikte *Asperula* dâhil birçok cinste bulunmaktadır. *Asperula* gövdelerinde kristaller sadece rafitlerden oluşmaktadır [26]. Metcalfe ve Chalk'ın Rubiaceae familyası ve *Asperula* cinsi için yapmış olduğu gövde, yaprak anatomik özellikler ve kristal tanımlamaların birçoğu çalışma sonuçlarımızla benzerdir.

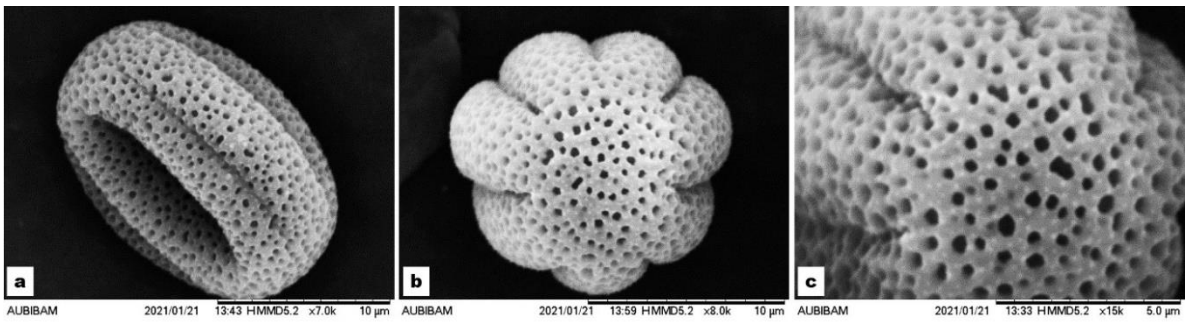
Asperula daphneola türünün anatomik çalışmasında gövde, ince bir tabaka periderm ve altında ince bir parankimatik hücrelerden oluşan kortekse sahip ve kortekste rafit kristalleri bulunmaktadır. İzolateral olan yaprakta orta damar altında büyük kollenkimatik doku, demet kını hücreleri ile çevrelenmiş iletim demetleri, alt ve üst epidermisin altında palizat parankiması yer aldığı belirtilmektedir. Mezofilde rafit kristallerinin varlığı rapor edilmektedir [18]. Bu sonuçlar bizim sonuçlarımızla genel olarak uyumludur.



Şekil 9. (a) Yaprak enine kesit **(b)** yaprak üst yüzey **(c)** yaprak alt yüzey öt: örtü tüyü üe: üst epidermis id: iletim demeti ko: kollenkima ae: alt epidermis s: stoma bg: basit geçit

Palinolojik Sonuç ve Tartışma

Palinolojik çalışmaların sonucuna göre Şekil 10'daki polen tanelerinin özellikleri monad, radyal simetrili, izopolar, hegzakolpattır (ya da heptakolpat). Ekvatoral görüntüleri eliptik (Şekil 10a), polar görüntüleri hemen hemen daire (Şekil 10b) şeklindedir. Skulptur skabrat-perforattır (Şekil 10c). Polen ölçümlerindeki maksimum ve minimum değerler ve polen şekli ise Tablo 3'de özetlenmiştir.



Şekil 10. SEM görüntüleri **(a)** ekvatoral görünüm **(b)** polar görünüm **(c)** yüzeysel görünüm

Tablo 3. Palinolojik özellikler ve maksimum-minimum değerler

Özellikler	Polen	
Ornamentasyon	Skabrat-Perforat	
Apertür tipi	Kolpus	
Polen şekli	Öprolat	
Apertür sayısı	6-7	
Kolpus genişlik	< 1 µm	
Kolpus boyu	**Max.**	**Min.**
	15.2 µm	11.9 µm
Polar uzunluk (P)	**Max.**	**Min.**
	21.2 µm	17.3 µm
Ekvatoral uzunluk (E)	**Max.**	**Min.**
	14.8 µm	11.4 µm
P/E değeri	**Max.**	**Min.**
	1.51 µm	1.43 µm

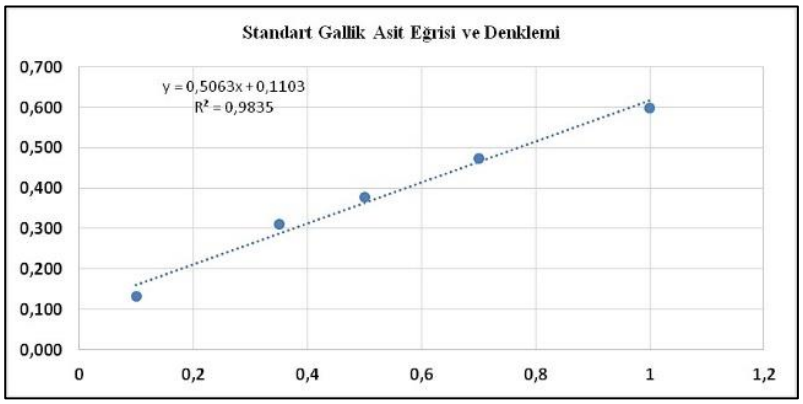
Polen çalışmaları bitki taksonomisinde son derece önemlidir ve bitkilerin tanımlanmasında kullanılabilmektedir. *Asperula* türleri ile ilgili yapılmış bazı polen çalışmalarına rastlanılmıştır. *A. cankiriensis*'de polen şekli oblat-sferoidal ve prolat- sferoidal, polar eksen 17.03-20.53 µm, ekvatoral eksen 18.41-20.02 µm, ornemantasyonu skabrat-perforat, kolpus sayıları 6-8, kolpus boyu 12.41-15.40 µm, kolpus eni 0.95-1.38 µm arasında bulunmuştur [8]. Bu sonuçlar ile bizim sonuçlarımız kıyaslandığında kolpus sayısı ve ornemantasyon tipi uyumluluk göstermektedir.Yeni tür olarak tanımlanan *A. anatolica* türünün polen özellikleri taksonun yakın akrabası *A. comosa* Schönb.-Tem. ile karşılaştırılmıştır [16]. İki taksonun P ve E değerleri bizim sonuçlarımızdan daha fazla, P/E oranı ise daha düşük rapor edilmiştir. Ayrıca kolpus boy ve enlerinin sahip olduğu değer aralığının da daha geniş olduğu bildirilmiştir. Her iki türe ait kolpus sayısı, çalışmamız ile benzerdir. *A. daphneola* türünün polen özelliklerinde P değeri 18.47±0.78 µm, E değeri 17.37±0.67 µm, P/E değeri1.06, kolpus uzunluğu 9.60±0.50 µm, kolpus eni 0.90±0.10 µm, kolpus sayısı 6, ornemantasyonu ise granulat tespit edilmiştir [18]. Bulgular göz önüne alındığında, kolpat sayısı ve kolpus genişliği çalışmamız ile benzer olup, polen şeklinin prolat-sferoidal olması sonuçlarımız ile farklılık göstermektedir.

Asperula cinsinin *Thlipthisa* seksiyonunundaki tüm taksonların palinolojik özellikleri ışık mikroskobu ve SEM ile incelenmiştir. Çalışmanın sonuçlarına göre; 6-7-8 kolpus sayısı, 1 µm'den

küçük kolpus eni ve perforat yüzey süslemesi, polenlerimiz ile uyumlu iken; P/E oranı daha düşük, kolpus uzunluğu ise benzer aralıklarda bulunmuştur [27].

Antioksidan Aktivite Sonuçları ve Tartışma

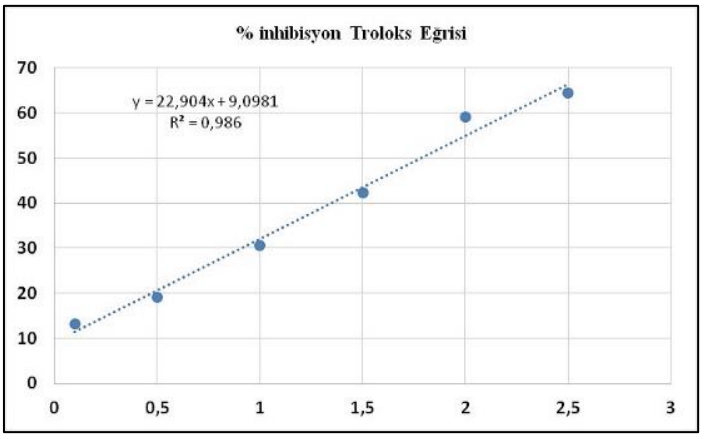
Yapılan toplam fenolik madde miktarı temel olarak renk reaksiyonuna dayanmaktadır. Oksidan molekül, antioksidandan elektron alır. Bu durum renk değişimine neden olur. Renk değişiminin derecesi, antioksidan derişimiyle orantılıdır (Mo(VI)(sarı) + e-(antioksidan) → Mo(V)(mavi)) [28]. Ekstrenin içerdiği fenolik madde miktarı, mg cinsinden standart gallik asite eşdeğer olarak hesaplanmıştır. Bu değerler standart eğri denkleminden bulunmuştur (Şekil 11). 1 g ekstredeki fenolik madde miktarı 31 mg GAE (Gallik asite eşdeğer) bulunmuştur.



Şekil 11. Hesaplamada kullanılan Gallik asit eğrisi ve denklemi

DPPH radikali süpürücü etki tayininde sonuçlar; %50 inhibisyon konsantrasyonu (IC_{50}) şeklinde verilmiştir ve standart gallik asit ile kıyaslanmıştır. Gallik asit IC_{50} değeri 0.0011±0.0002 mg/ml hesaplanmıştır. *A. lilaciflora* subsp. *phrygia* ekstresi için, IC_{50}= 0.38±0.035 mg/ml olarak belirlenmiştir.

ABTS radikal süpürücü metotu, antioksidan bileşikler tarafından ABTS'nin rengini kaybetmesi temeline dayanır [28]. ABTS radikali süpürücü etki tayininde, veriler ile kalibrasyon eğrisi çizilerek denklemi oluşturulmuştur (Şekil 12). Yapılan hesaplamalar sonucu ekstrelerin, Troloksa eşdeğer konsantrasyonları (mM TEAC) verilmiştir. 0.1 mg/ml derişimli ekstrede 0.37±0.07 mM TEAC, 10 mg/ml derişimde 0.90±0.18 mM TEAC hesaplanmıştır. Standart olarak kullanılan Gallik asitin 0.1 mg/ml derişiminde 1.44±0.45 mM TEAC; 1 mg/ml derişiminde ise 2.63±0.07 mM TEAC çıkmıştır. Bu durumda ekstrenin konsantrasyonları arttıkça, radikal süpürücü etkilerinin de arttığı gözlenmiştir.



Şekil 12. Hesaplamada kullanılan eğri ve denklemi

Literatüre baktığımızda, *Asperula* türleriyle yapılmış kimyasal çalışmalar çoğunlukla bileşen tespitine dayanmaktadır. Antioksidan ve biyoaktivite analizleri ise az sayıda bulunmaktadır. Bir çalışmada 5 farklı endemik *Asperula* cinsinin (*A. brevifolia, A. pseudochlorantha* var. *antalyensis* (Ehrend.) Minareci & K.Yıldız, *A. pseudochlorantha* var. *pseudochlorantha* Ehrend., *A. purpurea* subsp. *apiculata* (Sibth. & Sm.) Ehrend. ve *A. serotina)* antioksidan aktiviteleri çalışılmıştır [13]. Çalışma sonucunda, *Asperula* taksonlarının antioksidan aktivite göstermesi, sonuçlarımız ile uyumlu bulunmuştur. Loizzo ve arkadaşları, *A. glomerata* (M.Bieb) Griseb türünün total fenolik madde miktarını tespit etmiştir [29]. Fenolik bileşen miktarı 81.5±0.13 (mg/g), bizim sonucumuzdan oldukça yüksek hesaplanmıştır. *A. lilaciflora* türünün toprak üstü kısımları ile çalışılmış ve yeni bir flavonol glikozit ile iridoid tespit edilmiş ve flavonol glikozitin adı "lilaciflorasit"; iridoid adının ise "asperulojenin" olduğu belirtilmiştir [17]. İridoidler dikotil familyaları için taksonomik değere sahip olup, aynı zamanda bitkilere antioksidan özellik kazandırmaktadır [30]. *A. oppositifolia* Regel & Schmalh. türünün toprak üstü kısımlarının metanol ile elde edilen ekstrelerinin antioksidan aktivitesi test edilmiş ve yüksek konsantrasyonda yüksek inhibitör etki görülmüştür. Bu sonuç, bizim çalışmamız ile uyumlu olup bitkimizin de antioksidan ajanlar için yeni bir kaynak olabileceğini göstermektedir [31]. *Asperula involucrata* Wahlenb. türünün toprak üstü kısımları ile yapılan bir çalışmada üç yeni iridoid glikozitleri bulunmuştur. Kromatografi yöntemi kullanılarak, involucratosit A, B ve C adı verilen üç yeni bileşen tespit edilmiş ve molekül yapıları açıklanmıştır [32]. Farklı *Asperula* türlerinin (*A. antalyensis, A. brevifolia, A. pseudochlorantha, A. purpurea* subsp. *apiculata* ve *A. serotina*) metanol ve eter ekstraktlarının agar difüzyon ve sıvı dilüsyon metotlarıyla aktimikrobiyal aktiviteleri test edilmiştir. Testler sonucunda en aktif türler *A. brevifolia* ve *A. serotina* çıkmıştır [33].

Çalışmanın tümü ele alındığında morfolojik, anatomik, tüy ve polen sonuçlarının literatürde belirtilen özellikler ile benzeştiği; fakat bazı yönlerden ise farklılık gösterdiği bulunmuştur. Ayrıca takson ile ilgili morfolojik, mikromorfolojik, anatomik, palinolojik ve antioksidan özellikler ilk kez bu çalışmayla ayrıntılı bir şekilde incelenmiştir. Sonuçlarımızda *A. lilaciflora* subsp. *phrygia*'nın antioksidan aktivite göstermesi, yapılmış çalışmalar temel alındığında fenolik bileşenlerinde flavonoid ve iridoid yapıları taşıyabileceğini düşündürmüştür. Flavonoid, iridoid gibi fenolik bileşenlerinin tespiti ve antimikrobiyal aktivite testleri ileriki çalışmamızın konusu olarak öngörülmektedir.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EFFECTS OF ORCINOL ON PROLIFERATION AND APOPTOSIS OF SW480 HUMAN COLORECTAL CANCER CELLS

ORSİNOL'ÜN SW480 İNSAN KOLOREKTAL KANSER HÜCRELERİNDE PROLİFERASYON VE APOPTOZ ÜZERİNE ETKİLERİ

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ABSTRACT

Objective: *Colorectal cancer is a rapidly increasing disease worldwide, and almost half of the diagnosed patients die from this disease each year. The methods used in the treatment of colorectal cancer, including traditional treatment methods such as surgery, radiotherapy and current chemotherapy options, have low effectiveness and have many side effects. Because of all these problems, the importance of developing new agents for the treatment of colorectal cancer is increasing. Orsinol is a secondary metabolite isolated from lichens, and there are findings regarding the antioxidant, antimicrobial and antidepressant activity of the compound. In recent years, research on the anticancer activity of the compound has also started to take place in the literature. In this study, it was aimed to investigate the efficacy of orcinol on cell proliferation and apoptosis in human SW480 colorectal cancer cells.*

Material and Method: *Within the scope of the study, SW480 human colorectal cancer cells were used and cultured in DMEM medium. Orcinol was dissolved with dimethylsulfoxide to prepare a stock solution and applied to the cells in a concentration range of 1-25 mM. The effect of orcinol on cell viability was determined by MTT test. The apoptotic activity of the compound was evaluated with Annexin V binding assay using the Muse Cell Analyzer.*

Result and Discussion: *The results of MTT analysis showed that orcinol significantly decreased cell viability at 5 mM and above ($p<0.05$). While cell viability was 100.00±6.14% in the control group, it was determined as 12.50±0.65% in cells treated with 25 mM orcinol ($p<0.0001$). According to Annexin V binding analysis findings, the early apoptotic cell population was 12.06±1.22% in the 25 mM orcinol treated group, while it was 0.60±0.11% in the control group. The findings obtained from the study showed that that orcinol has a cytotoxic effect at high concentration on SW480 colorectal cancer cells, and further studies are needed to increase the efficiency of the compound and to elucidate its mechanism of action.*

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Keywords: *Apoptosis, colorectal cancer, orcinol, proliferation, SW480.*

ÖZ

Amaç: *Kolorektal kanser, her yıl dünya çapında hızla artan bir hastalıktır. Teşhis konulmuş hastaların neredeyse yarısı her yıl bu hastalıktan hayatını kaybetmektedir. Kolorektal kanserin tedavisi için uygulanan geleneksel tedavi yöntemleri olan cerrahi, radyoterapi ve mevcut kemoterapi seçenekleri de dahil olmak üzere tedavide kullanılan yöntemlerin etkinliği düşüktür ve çok fazla yan etkileri bulunmaktadır. Tüm bu problemler nedeni ile kolorektal kanserin tedavisi için yeni ajanlar geliştirmenin önemi giderek artmaktadır. Orsinol, likenlerden izole edilen bir sekonder metabolit olup, söz konusu bileşiğin antioksidan, antimikrobiyal ve antidepresan aktivitesine yönelik bulgular mevcuttur. Son yıllarda bileşiğin antikanser etkinliğine dair araştırmalar da literatürde yer almaya başlamıştır. Bu çalışmada, orsinolün insan SW480 kolorektal kanser hücrelerinde hücre proliferasyonu ve apoptoz üzerine etkinliğinin araştırılması amaçlanmıştır.*
Gereç ve Yöntem: *Çalışma kapsamında SW480 insan kolorektal kanser hücreleri kullanılmış ve DMEM besiyerinde kültüre edilmiştir. Orsinol, dimetilsülfoksit ile çözülerek stok çözeltisi hazırlanmış ve hücrelere 1-25 mM konsantrasyon aralığında uygulanmıştır. Orsinolün hücre canlılığı üzerine etkisi MTT testi ile belirlenmiştir. Bileşiğin apoptotik etkinliği Annexin V bağlanma analizi ile Muse Hücre Analiz cihazı kullanılarak değerlendirilmiştir.*
Sonuç ve Tartışma: *MTT analiz sonuçları, orsinolün 5 mM ve üzerinde hücre canlılığını doza bağlı olarak anlamlı şekilde azalttığını gösterdi ($p<0.05$). Kontrol grubunda hücre canlılığı 100.00±6.14% iken, 25 mM orsinol uygulanan hücrelerde canlılık 12.50±0.65% olarak belirlendi ($p<0.0001$). Annexin V bağlanma analizi bulgularına göre erken apoptotik hücre popülasyonu 25 mM orsinol uygulanan grupta 12.06±1.22% iken, kontrol grubunda 0.60±0.11% olarak belirlendi. Çalışmadan elde edilen bulgular, orsinol'ün, SW480 kolorektal kanser hücreleri üzerinde yüksek konsantrasyonda sitotoksik etkili olduğunu göstermiş olup, daha ileri çalışmalar ile bileşiğin etkinliğinin artırılmasına ve etki mekanizmasının aydınlatılmasına ihtiyaç duyulmaktadır.*
Anahtar Kelimeler: *Apoptoz, kolorektal kanser, orsinol, proliferasyon, SW480.*

INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease that occurs in the gastrointestinal tract, colon and rectum. According to Globocan 2020 data, while the number of new colorectal cancer cases in the world constitutes 10% of all cancers, the mortality rate due to colorectal cancer is 9.4% [1]. It has been reported that the incidence and mortality of colorectal cancer in women is approximately 25% lower than in men [2]. With continued increase in developing countries, the worldwide incidence of colorectal cancer is estimated to rise to 2.5 million new cases by 2035 [2,3]. According to cancer statistics, colorectal cancer ranks third in men and women in Turkey and is seen with a frequency of 23.1 per hundred thousand in men and 14.4 per hundred thousand in women [4]. Although mortality has decreased moderately with the effect of many therapeutic advances worldwide in recent years, the 5-year survival rate in metastatic patients is less than 10% [5].

The development of colorectal cancer is associated with many risk factors, including genetic predisposition, smoking, alcohol, excessive consumption of red meat and processed food, obesity, and decreased physical activity [6,7]. The methods used in the treatment of colon cancer, including the traditional methods of surgery, radiotherapy and current chemotherapy options, are generally low in efficacy and have many side effects. All these problems increase the importance of developing new therapeutically effective compounds in the treatment of colorectal cancer [8].

Natural products and their derivatives have higher efficiency and lower toxicity compared to chemical agents and are less likely to develop multidrug resistance. These products contain many bioactive anticancer compounds and can provide an effective alternative drug therapy for many cancers [9]. Some natural compounds have become productive sources of new anticancer drugs due to their therapeutic advantages, and about 50% of currently used anticancer drugs are directly or indirectly derived from natural products with various structures, including alkaloids, polysaccharides, polyphenols, diterpenoids and unsaturated fatty acids [10].

Orcinol is a natural organic phenolic compound that can be obtained from many lichen species,

including Roccella tinctoria and Lecanora [11]. Studies have revealed that orcinol exhibits antioxidant, antidepressant, adaptogenic and neuroprotective activities [12-15]. The strong radical scavenging activity of orcinol was also demonstrated in Raw 264.7 macrophage cells [16]. In addition, it has been demonstrated that polymer-lipid hybrid nanostructured lipid carrier systems loaded with orcinol glucosides have strong anticancer effects in colon, stomach and liver cancer cell lines, regarding that this system can be developed orally and be preferred in treatment as a good chemotherapeutic agent [17].

The present study aimed to evaluate the effect of orcinol cell viability and apoptosis of SW480 human colorectal cancer cells.

MATERIAL AND METHOD

Cell line and Cell Culture

Human colorectal cancer cell line SW480 (CCL-228) was obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biowest, USA) consisted of 10% fetal bovine serum (FBS) (Biowest, USA), 1% L-glutamine (Biowest, USA), and 1% penicillin/streptomycin (Biowest, USA) within a 5% CO2 humidified atmosphere at 37°C.

Orcinol (Sigma, USA) was dissolved in dimethylsulfoxide (DMSO) and the final DMSO concentration was less than 0.1%.

Cell Viability Assay

The effect of orcinol on the viability of SW480 cells was analyzed by MTT assay. The cells were seeded at a density of 9x103cells/well and treated with orcinol at 1, 5, 10, 15, 20 and 25 mM concentrations, and incubated for 24 h. The cells treated with DMSO were used as control. After incubation, the cells were treated with MTT solution (5 mg/mL) and incubated at 37°C for 2 h. The formazan crystals were dissolved in DMSO. The absorbance at 540 nm was determined by a microplate reader (Thermo, Germany). Data were represented as mean ± standard deviation (± SD) from two independent experiments.

Annexin V Binding Assay

SW480 cells were seeded at a density of 2×10^5 cells per well and incubated for 24 hr. The cells were then treated with 1, 5, 10, 20 and 25 mM of orcinol for 24 h. Following incubation, the cells were washed with PBS, and apoptotic cell amount was determined through Annexin V assay kit (Luminex, Germany) by Muse Cell Analyzer (Millipore, Germany).

Statistical Analysis

GraphPad Prism 6.0 version (GraphPad Software Inc.) was used for statistical analysis. The data of cell culture experiments were expressed as mean ±SD and One-way ANOVA test was performed for multiple comparisons.

RESULT AND DISCUSSION

Human colorectal cancer is the leading cause of cancer-related death in almost all developed countries and is the second most common type of cancer worldwide. Half of all patients diagnosed with colorectal cancer die as a result of the disease. Less than 10% of patients with metastatic colorectal cancer can survive for a maximum of five years after diagnosis [18]. Current colorectal cancer treatment is a surgical resection combined with chemotherapy using cytotoxic drugs and radiation therapy. Since the applied treatment is moderately successful for late-stage cancers, it increases the importance of preventive strategies and new approaches in the treatment of colorectal cancer [19,20]. In this context, studies on the effectiveness of natural origin products in cancer treatment are being carried out quite intensively [21,22].

Lichens are complex symbiotic associations consisting of a fungus and an algae. Currently, thousands of primary and secondary metabolites obtained from lichens have been identified [23]. The use of lichens in medicine is mainly based on the content of various biologically active substances with antimicrobial effects. Lichen metabolites show wide biological effects such as antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects [23-26]. Secondary metabolites of lichens also have strong antioxidant effects. These are substances with a high ability to scavenge toxic free radicals due to their phenolic groups [27].

Orcinol is one of the secondary metabolites of lichens and it has been reported that phenolic orcinol glucosides have potential antioxidant activity, antidepressant activity and immunomodulatory effects [28-30].

At present study, the cytotoxic and apoptotic effects of orcinol in human SW480 colorectal carcinoma cells were experimentally investigated. The cytotoxic effect of the orcinol compound at various concentrations was determined by MTT cell viability assay and the results showed that orcinol significantly decreased the viability of SW480 cells at 5 mM and higher concentrations. The cell viability decreased to 84.84±1.69% at 5 mM ($p<0.05$), while it was determined as 46.94±1.97%, 31.98±1.56%, 13.88±0.63% and 12.50±0.65% at 10, 15, 20 and 25 mM concentrations, respectively ($p<0.0001$) compared to control (Table 1, Figure 1).

Table 1. Viable cell amount % of SW480 colorectal cancer cells.

Concentration (mM)	Viable cell amount (%)	P value (vs control)
0	100.00±6.14	
1	108.79±5.19	0.0217
5	84.84±1.69	<0.0001
10	46.94±1.97	<0.0001
15	31.98±1.56	<0.0001
20	13.88±0.63	<0.0001
25	12.50±0.65	<0.0001

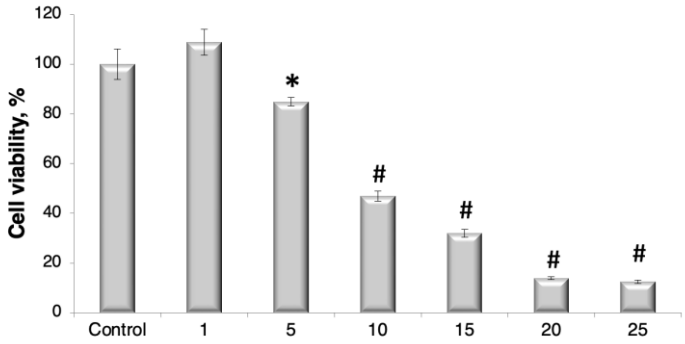

Figure 1. The effect of orcinol on viability of SW480 human colorectal cancer cells. The cell viability was determined by MTT assay. The cells were seeded at a density of 9x103 cell/well and then treated with 1-25 mM orcinol for 24 h. Following incubation MTT dye (5 mg/ml) was added into the wells and the formazan crystals were dissolved in DMSO. The absorbance at 540 nm was recorded. The results are expressed as percentage of live cells compared with untreated control. The data were given as mean±SD of three independent experiments. The differences are * $p<0.05$, # $p<0.0001$ vs control.

The apoptotic effect of orcinol on SW480 colorectal cancer cells were evaluated by Annexin V binding assay. The cell population % values and plot images of flow cytometry analyses were given in Figure 2,3, respectively in which the cell population were defined as percentage of live, early apoptotic (annexin V +), late apoptotic (annexin V +, 7-aminoactinomycine D +), and dead cells. Our results

showed that the early apoptotic cell population % increased to 12.06±1.22% at 25 mM orcinol treatment while it was 0.60±0.11% in control group ($p<0.0001$). The total apoptotic cell population were significantly higher at 5 mM and higher concentration of orcinol when compared to control ($p<0.05$).

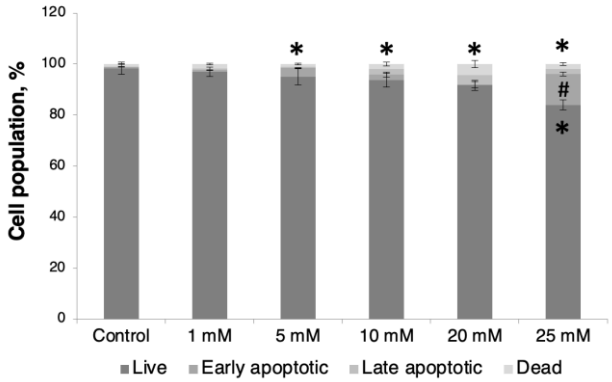

Figure 2. The cell population % of annexin V binding assay. The cells were seeded in 6-well plate and treated with orcinol at 1-25 mM concentrations for 24 h. Four different cell population were determined by Muse cell analyzer including live (annexin V-, 7-AAD-), early apoptotic (annexin V+, 7-AAD-), late apoptotic (annexin V+, 7-AAD+), dead (7-AAD+). Data are expressed as mean±SD (n=3) of cell population %. Significant differences are * * $p<0.05$, # $p<0.0001$ vs control.

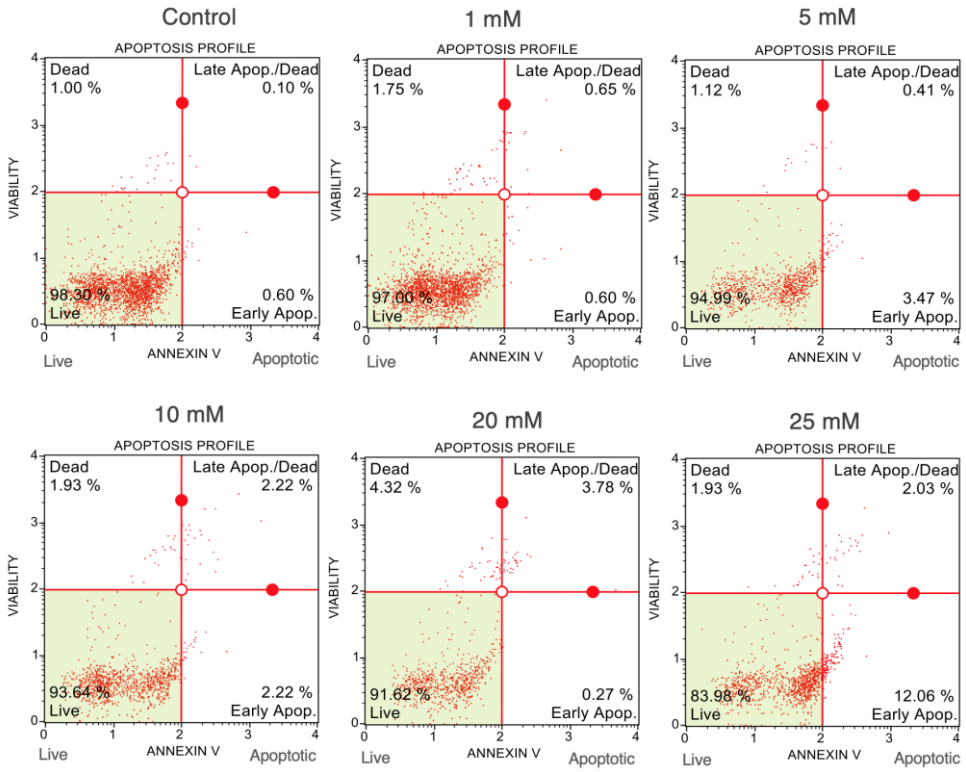

Figure 3. The plot graphs of annexin V binding assay. The cells were treated with 1-25 mM of orcinol and Muse cell analyzer (Merck Millipore) was used to determine the apoptotic cell population. Based on the Annexin V and 7-AAD positivity, the apoptotic and dead cell population % were determined, respectively. The live, early apoptotic, late apoptotic and dead cell population were detected through cytofluorometric separation on cell analyzer. The figure represents dot plots of three independent experiments.

There are various studies in the literature on the efficacy of orcinol in cancer cells. In a study, the potential cytotoxic activities of different compounds derived from phloroglucinol and orcinol in PC3 and DU145 prostate cancer cell lines, MDA-MB231 breast cancer cells, HT29 colon cancer cells and human dermal fibroblasts, a non-tumoral cell line, were evaluated. It has been determined that the cytotoxic activity of compounds derived from orcinol against cells and their effects on chromatin core condensation and/or fragmentation induction, mitochondrial membrane potential and caspase-3 activity are variable [31].

In another study investigating the immunomodulatory activity of thirteen lichen metabolites, including orcinol, orsellinic acid, and methyl orsellinate, it was determined that all of these metabolites had antioxidant activity, however, the immunomodulatory activity of orcinol was low [32]. A study evaluating the cytotoxicity of 5-n-alkylresorcinol homologues and its fraction on mouse fibroblast cell line L929, it was reported that orcinol showed low cytotoxicity among isolated compounds [33].

In conclusion, our study determined that orcinol had a dose-dependent cytotoxicity and apoptotic activity in SW480 human colorectal cancer cells. Further studies are required to increase the efficiency of the compound in question and to elucidate its mechanism of action.

AUTHOR CONTRIBUTIONS

Concept: F.B.A.; Design: F.B.A.; Control: F.B.A.; Sources: F.B.A.; Materials: B.Y., F.B.A.; Analysis and/or Interpretation: B.Y., F.B.A.; Literature Review: B.Y., F.B.A.; Manuscript Writing: B.Y., F.B.A.; Critical Review: F.B.A.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

RPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF SELECTIVE SEROTONIN REUPTAKE INHIBITORS IN TABLET FORMULATIONS

SEÇİCİ SEROTONİN GERİ ALIM İNHİBİTÖRLERİNİN TABLET FORMÜLASYONLARINDA EŞ ZAMANLI TAYİNİ İÇİN RPLC YÖNTEM GELİŞTİRİLMESİ VE VALİDASYONU

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ABSTRACT

Objective: *Selective serotonin reuptake inhibitor (SSRIs) compounds are the most used compounds in the treatment of depression. The determination of chromatographic separation and quantitative determination of these compounds is very important in the clinical use of these compounds and the success of biopharmaceutical studies. For this reason, this study, it was aimed to optimize the chromatographic conditions for the quantitative determination of SSRIs in tablet formulation by Reversed phase liquid chromatography method.*

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Material and Method: *The optimum separation condition for the studied compounds was determined based on the relationship between the retention values of the compounds and the pH and the content of the mobile phase. Chromatographic determination was made on the X Terra C18 column (250 x 4.6 mm I.D., 5 µm), which is widely used for the determination of hybrid-based and basic compounds. The validation of the developed method was carried out based on the parameters of linearity, precision, and accuracy.*

Result and Discussion: *The developed and validated method was successfully applied for the determination of active ingredients in the tablet dosage form. The experimental results of the amount of studied SSRIs in selected commercial tablets are in good agreement with the label claims. The calculated percent recoveries show that the sample preparation techniques developed for the quantification of the compounds studied are not affected by interferences. The evaluation of the obtained results showed that the developed method is suitable for the routine use of studied compounds.*

Keywords: *Antidepressant, method optimization, quantitative analysis, RPLC*

ÖZ

Amaç: *Seçici serotonin geri alım inhibitörleri (SSRI) depresyon tedavisinde en çok kullanılan bileşiklerdir. Bu bileşiklerin kromatografik ayrımları ve kantitatif tayinlerinin belirlenmesi, bileşiklerin klinik kullanımlarında ve yapılan biyofarmasötik çalışmaların başarısında oldukça önemlidir. Bu nedenle bu çalışmada tablet formülasyonunda SSRI'ların kantitatif tayini için kromatografik koşulların Ters faz sıvı kromatografi yöntemi ile optimize edilmesi amaçlanmıştır.*

Gereç ve Yöntem: *Çalışılan bileşikler için optimum ayırma koşulu, bileşiklerin alıkonma değerleri ile pH ve mobil faz bileşimi arasındaki ilişkiye göre belirlenmiştir. Kromatografik ayrım, hibrit bazlı ve bazik bileşiklerin tayini için yaygın olarak kullanılan X Terra C18 kolonu (250 x 4.6 mm I.D., 5 µm) kullanılarak gerçekleştirilmiştir. Geliştirilen yöntemin validasyonu; doğrusallık, kesinlik ve doğruluk parametrelerine göre yapılmıştır.*

Sonuç ve Tartışma: *Tablet formülasyonlarındaki etken maddelerin tayini için geliştirilen yöntem başarıyla uygulanmış ve valide edilmiştir. Elde edilen deneysel SSRI miktarları, ticari formülasyonlarda belirtilen miktarlar ile uyumlu olarak bulunmuştur. Hesaplanan geri kazanım yüzdeleri, çalışılan bileşiklerin kantitatif tayini için geliştirilen numune hazırlama tekniklerinin dış etkenlerden etkilenmediğini göstermektedir. Elde edilen sonuçlar değerlendirildiğinde, geliştirilen yöntemin çalışılan bileşiklerin rutin analizlerine uygun olduğu görülmüştür.*

Anahtar Kelimeler: *Antidepresan, kantitatif analiz, metot optimizasyonu, RPLC*

INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are frequently used in the treatment of psychiatric disorders such as depression, obsessive-compulsive disorder, and anxiety [1,2]. SSRIs have advantages over tricyclic antidepressants such as less pronounced anticholinergic side effects and no severe cardiotoxicity [3]. These drugs inhibit the uptake of serotonin across the plasma membrane before it is stored in specific organelles [4].

Sertraline, citalopram, and fluvoxamine, which belong to the SSRI group, are basic compounds due to the basic functional group in their chemical structure (Figure 1). These compounds with primary, secondary, and tertiary amine groups were apolar compounds with poor water solubility according to the log P values of 5.15, 3.76, and 2.80, respectively [5].

In recent years, the identification of drug candidates has often faced the problem that many new molecules in drug discovery are less water-soluble and more lipophilic. This problem can be solved by using water-organic solvent binary mixtures. In addition to the discovery and design of a compound used as a drug in pharmaceutical chemistry, it is important to develop effective analytical methods for chemical analysis and quality control. There are studies in the literature for the determination of sertraline, citalopram, and fluvoxamine alone or together with their degradation products in various samples [6-8]. Among these methods, reverse phase liquid chromatography (RPLC) is mostly preferred due to its advantages such as accuracy, precision, and repeatability of the measurements taken [9-12]. The primary purpose of RPLC studies is to ensure that the studied compounds are separated from each

other as soon as possible or to make simultaneous determinations provided that certain validation conditions (ICH parameters) are met [13].

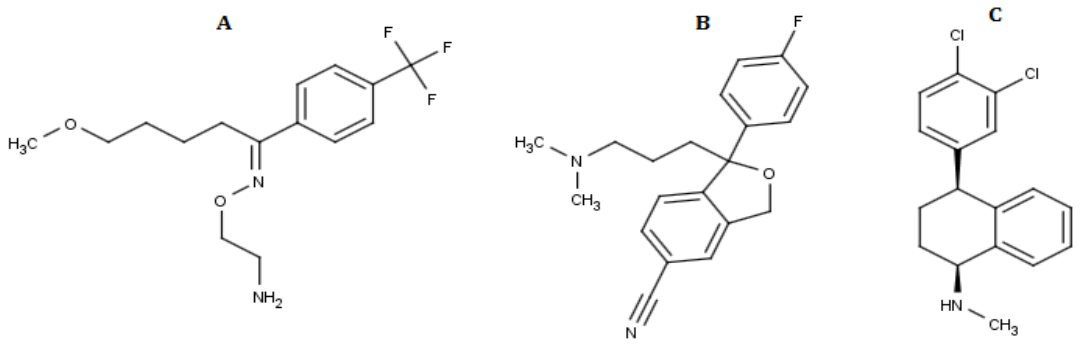

Figure 1. Chemical structure of studied compounds (A) fluvoxamine, (B) citalopram, and (C) sertraline [5]

In the HPLC method, the parameters known to affect the retention factor (*k*) values of the compounds are changed individually or randomly to determine the optimum separation condition in most studies. While this situation causes unnecessary time and material loss, in some cases, it is insufficient in determining the separation condition [14-18]. To determine the chromatographic working conditions, instead of this trial-and-error method, the chromatographic conditions (column temperature, mobile phase pH and organic solvent concentration, etc.) should be optimized in the developed method [15-19]. In this study, studies were found for the determination of the selected SSRIs sertraline, citalopram, and fluvoxamine by the RPLC method for the determination of the compounds alone or simultaneously [20-22]. In addition, there are few studies on the determination of optimum conditions of compounds with the experimental design method related to this group [23-25]. For the qualitative determination of the selected compounds in this study, the optimum chromatographic condition was determined by examining the change in *k* values depending on the mobile phase pH at the constant column temperature and the organic modifier concentration in the mobile phase. With this type of study, the simultaneous determination of compounds could be made in the shortest time possible. In addition, the method developed was validated according to International Conference on Harmonization (ICH) parameters and then quantitative determinations in drug formulations were performed.

MATERIAL AND METHOD

Apparatus

In this study, the qualitative and quantitative analyses of the compounds were made with a high-performance liquid chromatography device (Shimadzu Technologies, Japan). The system used consists of a UV detector (SPD-20A), pump (LC-20AD), column oven (CTO-20A), and degasser unit (DGU-20A3). Mobile phase pH measurements prepared for chromatographic determination were made using a Mettler Toledo pH/Ion analyzer (Schwerzenbach, Switzerland) and In Lab 413 Ag / AgCl pH electrode. Ultrapure water was supplied from the Direct-Q®3 UV (Millipore, Bedford, MA, USA) water purification system.

Chemicals

In this study, sertraline, citalopram, fluvoxamine, and uracil were obtained from Sigma-Aldrich (USA). Acetonitrile was used as an organic solvent in the preparation of the mobile phase, o-phosphoric acid, sodium hydroxide, ammonium bicarbonate, and ammonia were used as buffer components in the mobile phase, and potassium hydrogen phthalate was used as the primary standard reference in electrode calibration were supplied from Merck (Darmstadt, Germany). All chemicals used in the study are of analytical purity.

Chromatographic Study

In this study, the acetonitrile-water binary mixture containing 50% (v/v) acetonitrile was prepared as a mobile phase for the chromatographic determination of the compounds. o-phosphoric acid (85%, w/w) was added to the mobile phase medium at 25 mM and 1 M NaOH solution was added to reach the desired mobile pH. The pH values of the mobile phases were prepared between 6.0 and 10.5. o-phosphoric acid-sodium hydroxide and ammonia-ammonium bicarbonate were used as buffer compositions for the mobile phases. These solutions were used after degasification in an ultrasonic mixer. This work was carried out on an X Terra C18 column (250 x 4.6 mm I.D., 5 µm, Waters, USA), which is suitable for the analysis of hybrid-based and basic compounds. Separation was carried out at a column temperature of 30°C and a flow rate of 1.0 ml/min. The maximum absorbance wavelength of the studied compounds was determined as 210 nm with a UV detector.

Preparation of Standard and Calibration Solutions

Stock solutions of the analyzed compounds (100 µg/ml) were prepared by dissolving them in the mobile phase. For the calibration of the pH electrode, 0.05 mol/kg potassium acid phthalate solution was prepared in the working mobile phase binary mixture. The prepared solutions were protected from sunlight and stored at +4°C.

Calibration solutions of citalopram, fluvoxamine, and sertraline, whose linear working range was determined, were prepared in the optimum hydro-organic mixture at concentrations of 2-12 µg/ml, 4-32 µg/ml, and 1-10 µg/ml, respectively. The internal standard method was used for the calibration graph. The internal standard imipramine chosen for this was kept constant at 1 µg/ml throughout the entire study.

Analysis of Tablet Solutions

For quantitative determination of citalopram, fluvoxamine and sertraline tablet analysis was performed. In this method, ten tablets were finely powdered and weighed in an equivalent amount to 1 tablet. Then, the powder in the amount of one tablet was put into the volumetric flask and by adding the mobile phase, its volume was made up to 100 ml. To dissolve the active ingredients of the drugs determined in the prepared sample solutions, the solutions were kept in an ultrasonic bath for 20 minutes. The insoluble part in the prepared solution was removed by filtration. Finally, the solution obtained was prepared at different dilution rates according to the concentration in the calibration range specified for each compound [16,26,27].

Recovery Experiment

A recovery study was conducted to determine the accuracy of the proposed method. Samples were analyzed by adding a known amount of pure standard and selected internal standard to the tablet sample containing a fixed amount of active ingredient. The percent recovery was calculated using the concentrations of the active ingredient in the sample and the added standard solution.

RESULT AND DISCUSSION

In general, the analysis of basic compounds is difficult due to peak asymmetry arising from secondary interactions between column residues of silanols and the ionized form of the compound. In addition, compounds with apolar properties cause higher retention times, peak broadening, and unnecessary mobile phase consumption because they interact more strongly with the apolar column. For this reason, suitable columns should be preferred considering the chemical properties of the compounds in RPLC analysis [28,29]. In this study, an X Terra column with a wide pH working range (pH 1-12) suitable for the analysis of basic compounds from the new generation columns was selected.

A mobile phase optimization study was performed to determine the optimum separation condition in the quantitative determination of sertraline, citalopram, and fluvoxamine used in the treatment of depression by the RPLC method. With knowing the pK_a values of the compounds, it is possible to determine the pH values at which they are in molecular or ionized form. For this, pH values above and below 1.5 units of pK_a value are determined as working pH ranges. For this, by keeping constant

chromatographic conditions and the acetonitrile concentration in the mobile phase, the effect of pH change on the *k* values of the compounds was investigated. The t_0 value was determined by using the standard solution of uracil, which was used as the non-retained species in the column. The *k* values at each pH value (6.0-10.0) studied were calculated by using the t_R and t_0 values of the studied compounds in the hydro-organic mixture containing 50% acetonitrile. When the *k* values of the compounds are plotted against the mobile phase pH values, the sigmoidal behavior belongs to the basic functional group (Figure 2).

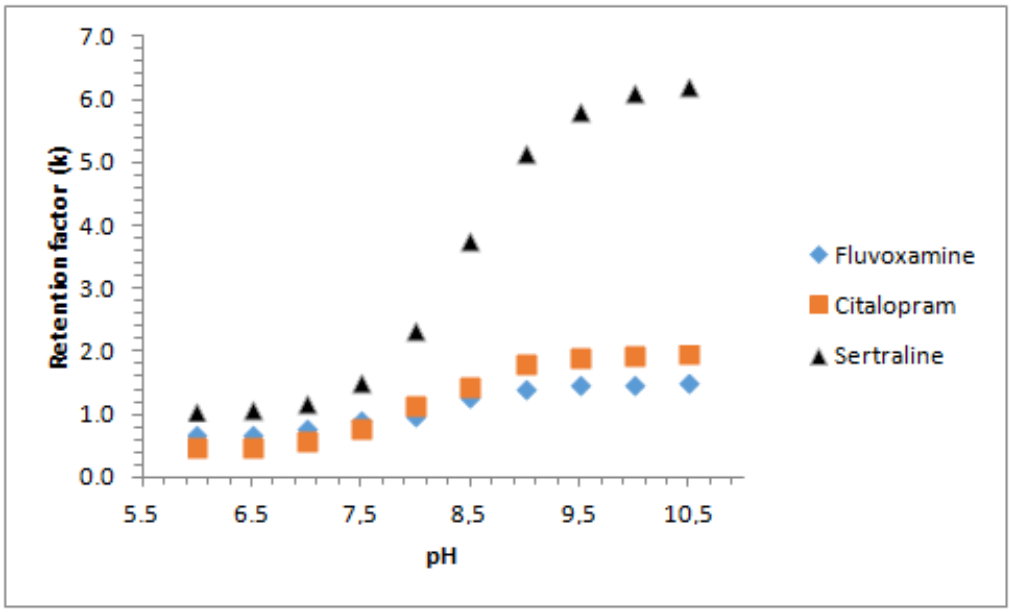

Figure 2. Sigmoidal behavior of studied compounds

In the optimization studies, it was aimed to determine the best chromatographic conditions in which the compounds were separated. The most basic method to find the optimum separation conditions for compounds containing ionizable functional groups in their structures is to examine the sequential change of chromatographic conditions. In particular, the effect of the pH of the mobile phase and the organic modifier concentration on the *k* value should be determined. Since compounds containing ionizable functional groups in their structure have different forms at different pH values, the affinity of these forms is different due to the second equilibrium requirement in the RPLC method. Therefore, the retention of these forms is also different. Furthermore, since the polarity of the mobile phase changes with the amount of organic modifier in the mobile phase, there are changes in the solvation of the compound. Because of the changes in the solvation of the compound, its affinity for the stationary phase changes, so the retention of the compound in the chromatographic column also changes [9,30,31].

The best condition that satisfies the desired chromatographic conditions is determined as the optimum separation condition. In addition, if the *k* values of the compounds are in the range of $1 \leq k \leq 5$, the selectivity factor (α) is greater than 1.15, and the peak resolution (R_s) value is greater than 1.5, optimum chromatographic separation occurs when these favorable conditions are met. The selectivity factor is calculated by dividing the retention factor (k_2) of the second peak by the retention factor of the first peak (k_1).

When the experimental data obtained are examined, the *k* value of the compounds under pH 8 is below 1. At pH 8 and 8.5, compound pairs have α values below 1.15 and R_s below 1.5. At pH 9, *k* values for peak pairs are above 1, and α and R_s values for peak pairs are above 1.15 and 1.5, respectively (Figure 3).

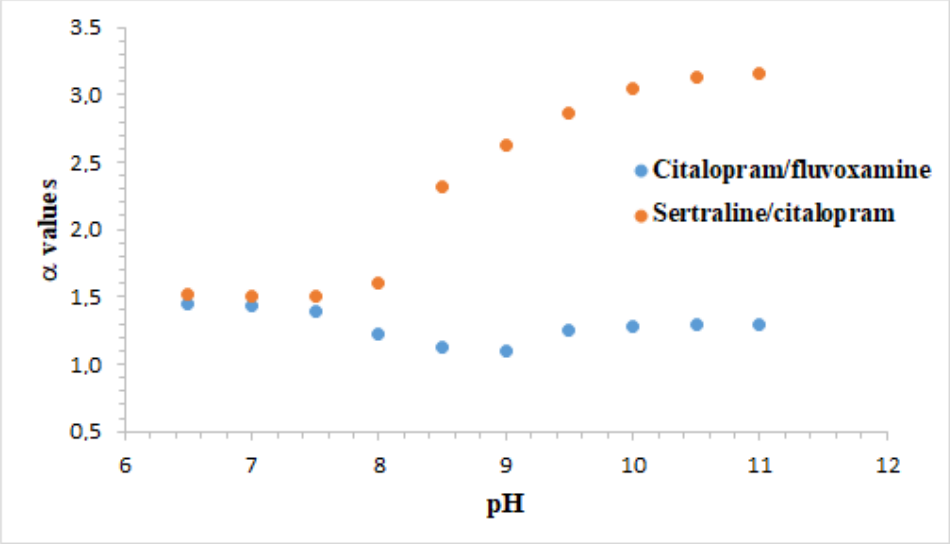

Figure 3. Variation in α values for compound pairs with mobile phase pH

For this, the pH value of the mobile phase was determined as pH 9.0 in the binary mixture containing 50% acetonitrile. The Purnell equation shows the relationship between the selectivity factor, peak resolution, and retention factor. For this reason, the R_s value between the two peaks must be calculated using this equation in the qualitative determination. The values calculated according to the Purnell equation under this mobile phase condition are given in Table 1.

Table 1. Calculated data of compounds at optimum separation condition

Compounds	k_2	α	k_2/k_2+1	$(\alpha-1)/\alpha$	$(\frac{1}{4})\sqrt{N}$	R_s
Fluvoxamine/citalopram	1.784	1.246	0.641	0.198	22.020	2.789
Citalopram/ imipramine (I.S)	3.713	2.082	0.788	0.520	24.477	10.022
Imipramine (I.S)/ sertraline	5.130	1.381	0.837	0.276	23.286	5.381

After the optimization of the liquid chromatographic method developed in the study, method validation was performed for the quantitative determination of the compounds. Analysis with the addition of an internal standard (IS) is common to eliminate systematic errors in analytical measurements [32,33]. The IS method is preferred to exclude systematic and random errors such as additives in drug formulations and volume errors during sample injection. When the internal standard is selected, it must be chromatographically separated from the compounds determined under optimal separation conditions. In this study, imipramine was selected as the IS. Under the selected optimal separation conditions, imipramine could be retained in this column because it was present in its molecular form. The chromatogram obtained under the optimal separation conditions is shown in Figure 4.

Once the optimal separation conditions were determined, the stability of the chromatographic system was determined according to the ICH guidelines [34]. For this purpose, chromatographic parameters were calculated by injecting the compounds into the HPLC system (Table 2).

The system suitability parameters results (Table 2) showed that the developed chromatographic method was suitable for the analysis and analytical method validation part.

A calibration curve was prepared to determine the linearity of the developed method. The regression parameters calculated when the peak area ratios of the compounds were plotted against the concentrations of the studied compounds are presented in Table 3.

A good correlation coefficient (0.999) from the graphs shows the appropriate linear concentration range for the three compounds. The precision of the developed method was determined by calculating the relative standard deviation (%RSD) of the compound concentrations calculated using the peak area ratios of five repetitive injections of the standard solution. For this purpose, intraday and interday studies were carried out. These studies were performed at two different concentrations for each compound and the results are shown in Table 4. It is seen that %RSD values calculated from Table 4 are less than 2%.

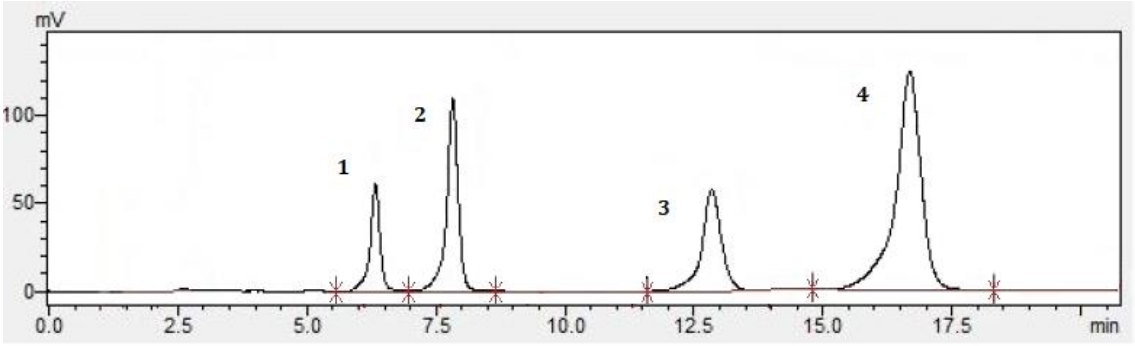

Figure 4. Chromatogram of standard mixture: 1) fluvoxamine, 2) citalopram, 3) imipramine (I.S), 4) sertraline

Table 2. System suitability parameters for studied compounds

Parameters	Fluvoxamine	Citalopram	Imipramine (I.S)	Sertraline	Recommended Value
Retention time (t_R)	6.299	7.678	12.695	16.513	
Retention Factor (k)	1.437	1.798	3.721	5.170	> 1
Tailing Factor (T_f)	1.250	1.176	1.389	1.682	≤ 2
Selectivity Factor (α)		1.575	1.248	2.212	> 1
Theoretical Plates (N)	6631.992	7950.845	9586.248	8640.986	>2000
Resolution (R_s)		2.875	9.971	9.971	>2
RSD% (for retention time)	0.216	0.590	0.141	0.885	≤1
RSD% (for peak area)	0.872	0.178	0.743	0.455	≤1

Table 3. The calibration data of studied compounds

Sample	Linearity Range (µg/ml)	Slope	Intercept	Correlation Coefficient (r)	Detection Limit (LOD) (µg/ml)	Quantitation Limit (LOQ) (µg/ml)
Fluvoxamine	4-32	0.265(0.003*)	-0.059(0.043*)	0.999	0.773	2.343
Citalopram	2-12	0.768(0.004)	-0.065(0.032)	0.999	0.147	0.446
Sertraline	1-10	2.916(0.011)	-0.085(0.065)	0.999	0.094	0.286

*Standard error

For the quantification of sertraline, citalopram, and fluvoxamine in tablet formulations, tablet solutions were prepared as described in the "Experimental" section, and the ratio of the peak area of the analyzed compounds to the peak area values of imipramine because of the analysis was evaluated in the corresponding calibration functions. Then, the amount of active compounds contained in the tablets was calculated (Table 5). Recovery studies were also performed to determine the accuracy of the method developed in the study. The calculated % recovery values are also shown in Table 5. Chromatograms showing the analysis of the tablet samples were given in Figure 5 for fluvoxamine, Figure 6 for citalopram, and Figure 7 for sertraline.

Table 4. Precision data of the developed method

Compounds	Theoretical concentration (µg/ml)	Intraday measured concentration, mean (µg/ml)	RSD (%)	Bias (%)	Interday measured concentration, mean (µg/ml)	RSD (%)	Bias (%)
Fluvoxamine	6	6.054	0.637	-0.894	6.083	0.787	-1.390
	16	16.036	0.689	-0.222	16.123	1.154	-0.768
Citalopram	4	4.019	0.752	-0.478	4.090	1.468	-2.247
	10	10.048	0.362	-0.482	9.946	1.493	0.540
Sertraline	2	2.016	0.645	-0.800	2.042	1.397	-2.075
	8	8.090	0.393	-1.126	8.213	0.447	-2.665

Table 5. Recovery results in drug formulation

Parameters	Fluvoxamine	Citalopram	Sertraline
Labeled claim (mg)	100	20	50
Amount found (mg)[a]	100.051±3.144[b]	20.341±0.327	49.919±0.938
RSD (%)	1.283	0.650	0.757
Bias (%)	-0.052	-1.706	0.162
Recovery (%)	100.051±1.137	101.705±2.006	99.838±0.755
RSD (%) of recovery	0.675	0.645	0.524

[a]Each value is the mean of 5 experiments, [b] confidence interval

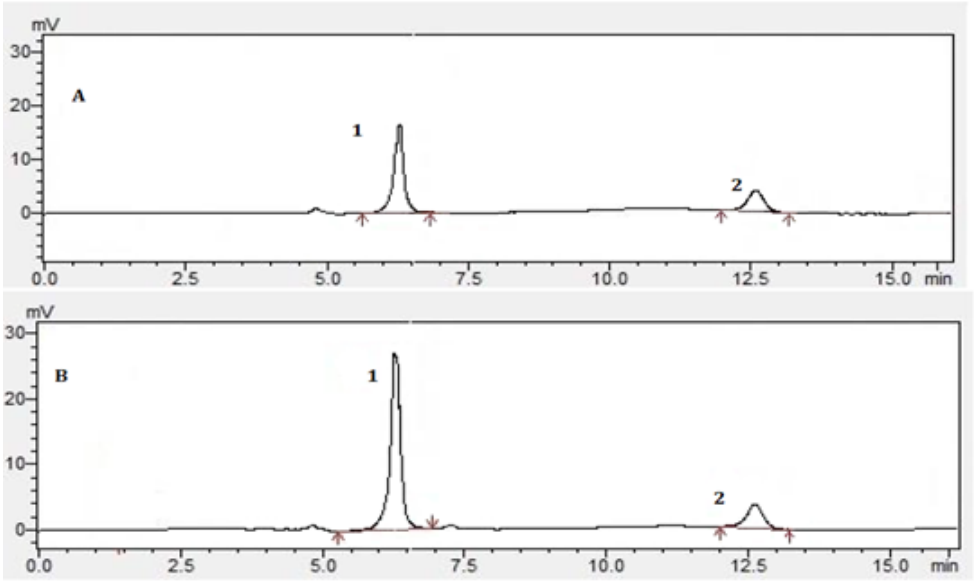

Figure 5. Chromatograms showing A) Faverin® tablet sample containing fluvoxamine (1) fluvoxamine (6 µg/ml) and (2) imipramine (I.S.) (1 µg/ml) B) tablet spiked with fluvoxamine (1) fluvoxamine (12 µg/ml) and (2) imipramine (I.S.) (1 µg/ml)

The experimental results of the amount of fluvoxamine, citalopram, and sertraline in selected commercial tablets are in good agreement with the label claims. The calculated percent recoveries show that the sample preparation techniques developed for the quantification of the compounds studied are not affected by interferences.

This is the first study of mobile phase optimization under specified chromatographic conditions. The retention time of the citalopram, fluvoxamine, and sertraline and the suitability of other chromatographic parameters were determined based on the combined effect of the percentage and pH of the organic solvent on the retention behavior of the compounds in RPLC. Moreover, the sufficient

reproducibility and good shapes of the peaks obtained throughout the liquid chromatographic study indicate the suitability of the selected column for this study. Method validation and quantitative determination of compounds in tablet formulations were performed under the determined optimal chromatographic conditions. The evaluation of the obtained results showed that the developed method is suitable for routine use.

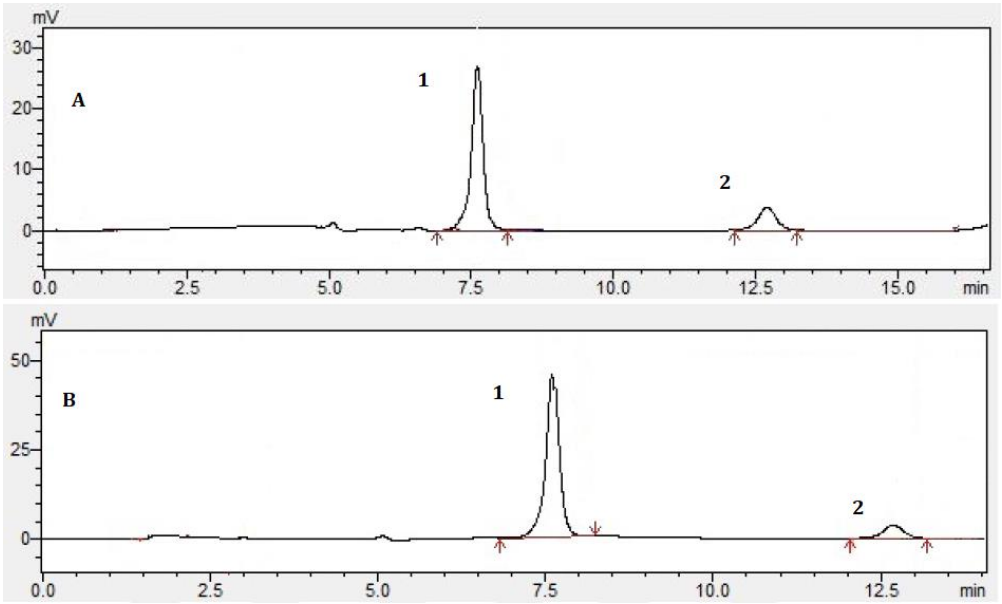

Figure 6. Chromatograms showing A) Citol® tablet sample containing citalopram (1) citalopram (4 µg/ml) and (2) imipramine (I.S.) (1 µg/ml) B) tablet spiked with citalopram (1) citalopram (8 µg/ml) and (2) imipramine (I.S.) (1 µg/ml)

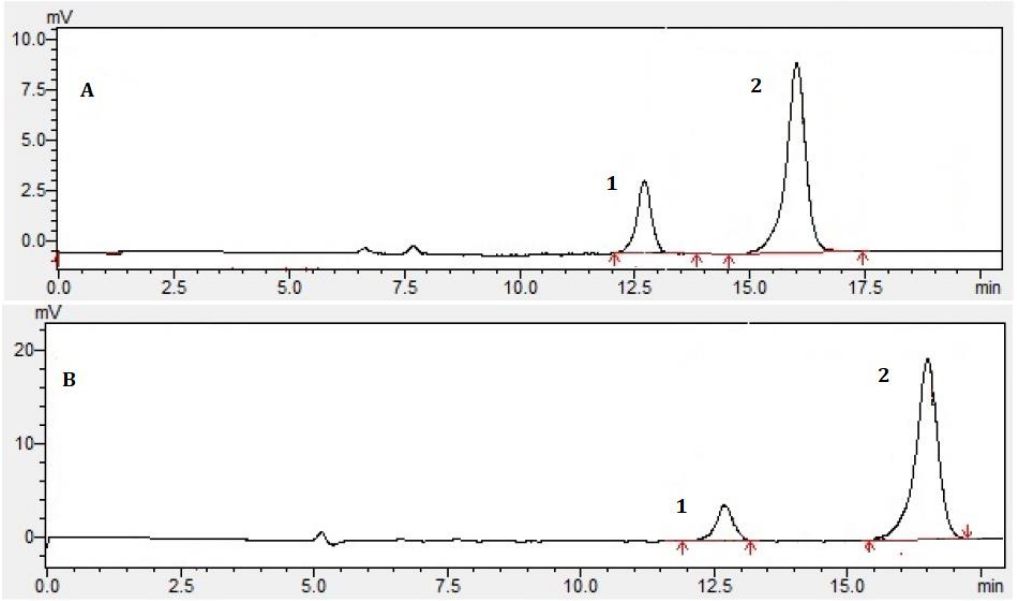

Figure 7. Chromatograms showing A) Lustral® tablet sample containing sertraline (1) imipramine (I.S.) (1 µg/ml) and (2) sertraline (2 µg/ml) B) tablet spiked with sertraline (1) imipramine (I.S.) (1 µg/ml) and (2) sertraline (4 µg/ml)

AUTHOR CONTRIBUTIONS

Concept: E.Ç.D., F.Ü., Y.D.D.; Control: E.Ç.D., Y.D.D.; Sources: E.Ç.D.; Data Collection and/or Processing: E.Ç.D., F.Ü., Z.Ü., Y.D.D.; Analysis and/or Interpretation: E.Ç.D., F.Ü., Y.D.D.; Literature Review: E.Ç.D., F.Ü., Z.Ü., Y.D.D.; Manuscript Writing: E.Ç.D.; Critical Review: E.Ç.D., Z.Ü., Y.D.D.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

TAURINE PREVENTS AGAINST 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN-INDUCED OXIDATIVE STRESS IN THE LIVER AND KIDNEY OF RATS

TAURİN, SIÇAN KARACIĞER VE BÖBREK DOKULARINDA 2,3,7,8-TETRAKLORODİBENZO-P-DİOKSİN KAYNAKLI OKSİDATİF STRESİ ÖNLER

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ABSTRACT

Objective: *The aim of the study was to investigate the preventive effects of taurine against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced organ damage in rats. The environmental toxin TCDD has a high toxicity in animal and human tissues. Taurine is an amino acid found in many organs, with multiple physiological roles including the protection of cells with its antioxidant and anti-inflammatory properties. In this context, our aim in this study was to investigate the potential preventive effect of taurine on oxidative stress and organ damage caused by TCDD in rat liver and kidney tissues. To evaluate these possible effects, we measured the levels of thiobarbituric acid reactive substances (TBARS), and glutathione (GSH), as well as the activity of superoxide dismutase*

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(SOD). In addition, immunohistochemical detection of caspase-3 expression, and the assessment of histopathological changes in tissue samples were performed.
Material and Method: *Adult male Wistar rats (250-300 g, 12-13 weeks, n = 32) were randomly allocated into four groups (n = 8/group): Control, TCDD, TAU, and TCDD+TAU. TCDD and/or taurine were administered via gavage in doses of 2 µg/kg/week and 200 mg/kg/day, respectively.*
Result and Discussion: *The results showed that TCDD caused oxidative stress in the liver and kidney tissues of rats by decreasing the levels of GSH and SOD activity and increasing the levels of TBARS. Taurine treatment significantly reduced TBARS levels ($p<0.05$), it significantly increased GSH levels and SOD activity ($p<0.05$) in the concurrent administration of TCDD and taurine. Taurine also reduced the histopathological changes caused by TCDD-induced oxidative stress in the liver and kidney tissues. Taurine prevented the apoptotic pathway by decreasing cysteine aspartate specific protease-3 (caspase-3). Taurine supplementation helps to regulate oxidative imbalance and reduces histopathological changes caused by TCDD-induced organ damage. This could be a novel approach to avoiding TCDD toxicity.*
Keywords: *Oxidative stress, rats, taurine, TCDD*

ÖZ

Amaç: *Çalışmanın amacı, sıçanlarda 2,3,7,8-tetrachlorodibenzo-p-dioksin (TCDD) kaynaklı organ hasarına karşı taurinin önleyici etkilerinin araştırılmasıdır. Çevresel toksin TCDD, hayvan ve insan dokularında yüksek toksisiteye sahiptir. Taurin, birçok organda bulunan bir amino asit olup, antioksidan ve antienflamatuar özellikleri ile hücrelerin korunmasında görev alır. Bu kapsamda, bu çalışmadaki amacımız, TCDD'nin sıçan karaciğer ve böbrek dokularında neden olduğu oksidatif stres ve organ hasarları üzerinde taurinin potansiyel önleyici etkisini araştırmaktır. Bu olası etkileri değerlendirmek üzere, tiyobarbitürik asitle reaksiyona giren reaktif maddeler (TBARS) ve glutatyon (GSH) düzeylerinin yanı sıra süperoksit dismutazın (SOD) aktivitesi ölçülmüştür. Ayrıca, kaspaz-3 ekspresyonunun immünohistokimyasal tespiti ve doku örneklerinde histopatolojik değişikliklerin değerlendirilmesi gerçekleştirilmiştir.*
Gereç ve Yöntem: *Yetişkin erkek Wistar sıçanları (250-300 g, 12-13 hafta, n = 32) rastgele dört gruba (n = 8/grup) ayrıldı: Kontrol, TCDD, TAU ve TCDD+TAU. TCDD ve/veya taurin gavaj yoluyla sırasıyla 2 µg/kg/hafta ve 200 mg/kg/gün dozlarında uygulandı.*
Sonuç ve Tartışma: *Bulgular, TCDD'nin GSH düzeyi ve SOD aktivitesini azaltarak ve TBARS düzeylerini artırarak sıçanların karaciğer ve böbrek dokularında oksidatif strese neden olduğunu göstermiştir. Taurin uygulaması, TCDD ve taurinin eşzamanlı uygulamasında TBARS düzeylerinde önemli ölçüde azalma ($p<0.05$), GSH düzeylerinde ve SOD aktivitesinde ise önemli ölçüde artış sağlamıştır ($p<0.05$). TCDD'nin karaciğer ve böbrek dokularında neden olduğu oksidatif kaynaklı histopatolojik değişiklikler de taurin uygulaması ile azalmıştır. Taurin, sistein aspartat spesifik proteaz-3'ü (kaspaz-3) azaltarak apoptotik yolağı engelledi. Taurin takviyesi, oksidatif dengesizliği düzenlemeye yardımcı olmakta ve TCDD'nin neden olduğu organ hasarının göstergesi olan histopatolojik değişiklikleri azaltmaktadır. Bu, TCDD toksisitesinden kaçınmak için yeni bir yaklaşım olabilir.*
Anahtar Kelimeler: *Oksidatif stres, sıçanlar, taurin, TCDD*

INTRODUCTION

The environmental contaminant 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD) is a high-toxic persistent organic pollutant that bioaccumulates and biomagnifies in animal fat and plant tissues [1]. Because TCDD is a fat-soluble chemical, it is easily bioconcentrated in animal fat stores and then in human tissue [2]. It has received a lot of attention in the recent literature especially with regard to its toxic effects such as hepatotoxicity [3], nephrotoxicity [4], immunotoxicity [5], and reproductive damage [6]. The aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor with a high affinity for TCDD, is known to mediate TCDD toxicity [7]. The activation of the AHR by TCDD affects the cellular redox homeostasis to induce an oxidative stress response, which is a crucial mechanistic component of many toxicologic processes [8]. TCDD-mediated oxidative damage has been observed in such as the liver [9], testis [10], and kidneys [11] in experimental animal studies. Taurine is a sulfur-containing intracellular amino acid that is involved in neuromodulation, thermoregulation, osmoregulation, calcium regulation, antioxidant defense, apoptosis, and vascular activities in tissues

like the brain, heart, muscle, and blood [12]. Taurine's antioxidant activity is one of its most essential properties, and taurine supplementation strengthens the antioxidant defense system by preventing the loss of antioxidant enzymes due to oxidative stress [13]. Thiobarbituric acid reactive substances (TBARS), which has cytotoxic characteristics, superoxide dismutase (SOD), and glutathione (GSH) are all key indicators of oxidative organ damage [14,15]. Taurine's effect on SOD activity, GSH content, and malondialdehyde (MDA) in mice liver and kidney have been shown to decrease oxidative stress after ethanol exposure [16]. It has been demonstrated that taurine enhances antioxidant defense mechanisms in the testis and epididymis of hypertensive rats [17]. The purpose of this study was to investigate the effect of taurine on TCDD-induced oxidative organ damage by evaluating at histopathological changes and oxidative parameters including TBARS, SOD activity, and GSH in the liver and kidney of rats.

MATERIAL AND METHOD

Experimental Protocol

Taurine was purchased from Carl Roth (Karlsruhe, Germany). TCDD was obtained from AccuStandard (>99% purity).

In this study, adult male Wistar rats (250-300 g) aged 12-13 weeks were used. All animals were fed rodent pellets and water on an ad-libitum and were housed in cages at room temperature (25 ºC) and humidity (50-55%) with a light-day cycle. Experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals under the approval of the Pamukkale University Animal Experiments Local Ethics Committee (Protocol no. PAUHDEK-2021/50). A total of 32 rats were randomly divided into four groups of eight each (n=8). All treatments were administered via oral gavage. 1: Control; only corn oil was administered. 2: TCDD; Once a week, rats were given TCDD suspended in corn oil at a dose of 2 ug/kg. 3: TAU; Taurine was administered at a dose of 200 mg/kg. 4: TCDD + TAU: Rats were given a combination of TCDD and Taurine (TCDD+TAU). The dose of TCDD was chosen based on previous studies [18,19]. Animals were sacrificed under anesthesia 30 days following treatment (xylazine, 8-10 mg/kg and ketamine hydrochloride 80-100 mg/kg intraperitoneally). For biochemical analysis, liver and kidney tissues were quickly dissected and stored at −80°C until analysis. Samples from these tissues were fixed in 10% formaldehyde solution for histopathological evaluation.

Biochemicals Assay

Tissue samples were homogenized with PCV Kinematica Status Homogenizer using phosphate buffered saline at pH 7.4. The resulting homogenates were sonicated for three cycles (40 s on ice after 20 s sonication) using the Bronson sonifier 450. Tissues were centrifuged at 1500 g for 10 minutes at 4°C, and supernatants were stored at -20°C for later analysis. The TBARS level was determined using Yagi's method [20]. The color produced by the reaction of MDA and thiobarbituric acid (TBA) is spectrophotometrically measured in this method. The resulting absorbance was measured spectrophotometrically at a wavelength of 532 nm. The TBARS level was given in nmol/g tissue. SOD activity was determined using the method developed by Sun et al. [21]. The TBARS level was given in nmol/g tissue. The inhibition of nitroblue tetrazolium reduction by xanthine-xanthine oxidase as a superoxide generator is associated with superoxide dismutase activity. After that, the formazan product was spectrophotometrically measured at 560 nm. The results are given in units of U/mg protein. Reduced GSH levels of tissue homogenates were measured at 412 nm spectrophotometrically [22]. The GSH level was given in nmol/ml protein. The protein content of tissue homogenates was determined using the Lowry method and bovine serum albumin as the standard [23].

Histopathological Examination

Tissue samples from the kidney and liver were fixed in 10% formalin for histopathological examination. Following routine tissue processing, the samples were embedded in paraffin. Sections of 5 mm thickness were taken from each block, affixed to slides and stained with Hematoxylen-Eosin (H&E). The sections were examined using a light microscope (Leica DFC 280). Histopathological

assessment of damage was calculated based on the severity of the damage to 0 (none), 1 (mild), 2 (moderate), 3 (severe).

Immunohistochemical Examination

Sections taken from kidney and liver tissues for immunohistochemical analysis were adhered to slides coated with poly-lysine. After rehydration, tissue samples were microwaved for 20 minutes in citrate buffer (pH 7.6). The sections were washed again with phosphate buffer after being left at room temperature for 20 minutes during the cooling stage. The sections were immersed in a 0.3% H_2O_2 solution for 7 minutes before being washed with phosphate buffer. The Caspase-3 kit protocol was applied to the prepared sections in accordance with the manufacturer's instructions. Accordingly, the primary rabbit-polyclonal was incubated with Caspase-3 antibody (Ab4051, Abcam) for 2 h. The biotinylated goat was rinsed in phosphate buffer and treated with an anti-polyvalent for 10 min. After that, it was incubated for 10 minutes at room temperature with streptavidin peroxidase. Following the completion of the chromogen substrate staining, the slides were treated for 1 min with Mayer hematoxylin and dehydrated by rinsing in tap water. Caspase-3 positive cell numbers were counted in 10 randomly selected fields from each section and score tables were prepared for immune positive cells between groups.

Statistical Analysis

SPSS 25.0 package program (Chicago, IL, USA) was used for statistical analysis. All values were presented as mean ± standard error of means (SEM). Statistical significance was accepted as $p<0.05$. ANOVA and post hoc Tukey tests were performed to compare data between experimental groups in biochemical parameters ($p<0.05$). Histological parameters were calculated using the SPSS computer program (SPSS 20, SPSS Inc., Chicago IL, USA) and the MedCalc 11.0 (Belgium) statistical programs. The results were presented as mean ± (SEM). Kruskal-Wallis and Connover tests were used for comparisons between groups.

RESULT AND DISCUSSION

Biochemical Results

As shown in Figure 1A, 1B, 1C GSH and SOD levels in liver tissue of rats administered TCDD were significantly decreased compared to the CONTROL group ($p<0.001$). The GSH level and SOD activity in the liver tissues of the TCDD+TAU group were significantly increased compared to the TCDD group ($p<0.001$). TBARS levels were associated with a significant increase in the liver tissues of TCDD-treated rats compared to CONTROL groups ($p<0.001$). Conversely, it was determined that TBARS levels in liver tissues decreased in the taurine-administered TCDD group ($p<0.05$). In addition, there was no statistically significant difference between TAU and CONTROL groups in terms of TBARS, SOD and GSH levels.

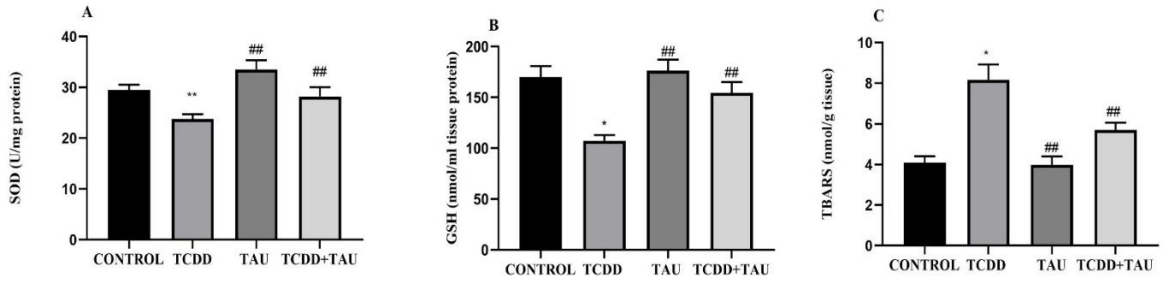

Figure 1. Liver tissue markers of oxidative stress (n=8, for each group). (A) SOD activity in liver tissue. (B) GSH content of liver, (C) TBARS level in liver. Significantly different from Control groups *(*; $p<0.05$,**; $p<0.001$)*. Significantly different from TCDD groups (#; $p<0.05$, ##; $p<0.001$).

As illustrated Figure 2, SOD activity and GSH level in kidney tissue were decreased in the TCDD group compared with the CONTROL group ($p<0.001$). When compared with the TCDD group, the SOD activity and GSH content increased significantly in the TCDD+TAU group ($p<0.001$). Results indicated a significant increase of TBARS level in TCDD administrated group compared with CONTROL group ($p<0.001$). Combined administration of TCDD and taurine was effective in reversing TBARS levels to similar values than those of CONTROL group. The levels of SOD, GSH, and TBARS in the TAU group were not statistically different from those in the CONTROL group ($p>0.05$).

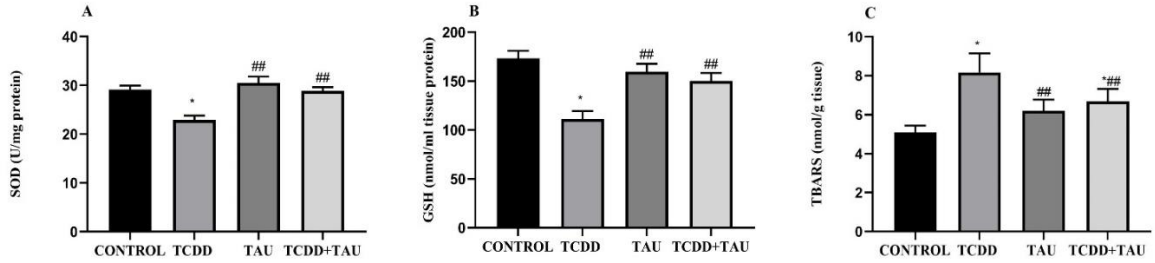

Figure 2. Kidney tissue markers of oxidative stress (n=8, for each group). (A) SOD activity in kidney tissue. (B) GSH content of kidney, (C) TBARS level in kidney. Significantly different from Control groups *(*; $p<0.05$,**; $p<0.001$)*. Significantly different from TCDD groups (#; $p<0.05$, ##; $p<0.001$).

Histological Evaluations

Histologically, the CONTROL (Figure 3A) and TAU (Figure 3B) groups appeared normal. In the TCDD group, liver tissue showed vascular congestion (black asteriks) (Figure 4A,4B), mononuclear cell infiltration (black arrows) (Figure 4A,4B,4D), apoptotic cell in vena centralis (blue arrow) (Figure 4C), eosinophilic stained and pyknotic nuclei cells (Figure 4B,4D,4E), sinusoidal dilatation (Figure 4C,4E). In TCDD + TAU group, liver damages were decreased compared with TCDD group. Little hemorrhage (Figure 5A), mononuclear cell infiltration (thick black arrow) (Figure 5B), and vascular congestion (black asteriks) (Figure 5B) were observed in TCDD + TAU group. Normal histological appearance in kidney tissue were detected in the CONTROL (Figure 6A) and TAU (Figure 6B) group.

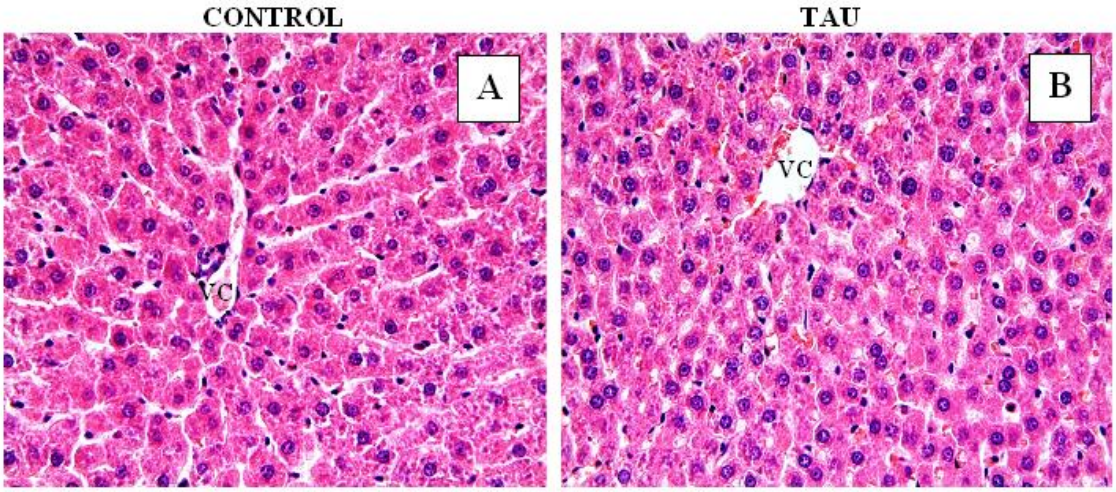

Figure 3. The liver tissue in the CONTROL (A) and TAU (B) groups appeared histologically normal. A, B: X40 (H&E).

The glomerular complex, renal tubules, and tubular epithelial cell membrane contour were all regular in the CONTROL and TAU groups. In TCDD group, glomerular degeneration (Figure 7A,7B) (white arrows), dilatation of the tubular lumen (Figure 7B,7C,7D), inflammatory cell infiltration (thick black arrows) (Figure 7A,7C), narrowing in the bowman area (Figure 7B,7C), epithelial atrophy and cell desquamation (Figure 7C,7D), casts in tubular lumen (Figure 7C) (black asteriks), hemorrhage between the tubules (Figure 7E) were observed. Kidney damage was reduced in the TCDD + TAU group compared to the TCDD group. Little hemorrhage between the tubules (Figure 8A), and cell desquamation (Figure 8B) were observed in TCDD + TAU group. In addition, histopathological damage score results in liver and kidney tissues of all groups are presented in Table 1. Histological alterations in all organs were more severe in the TCDD treatment group than in the TCDD and TAU administration group combined.

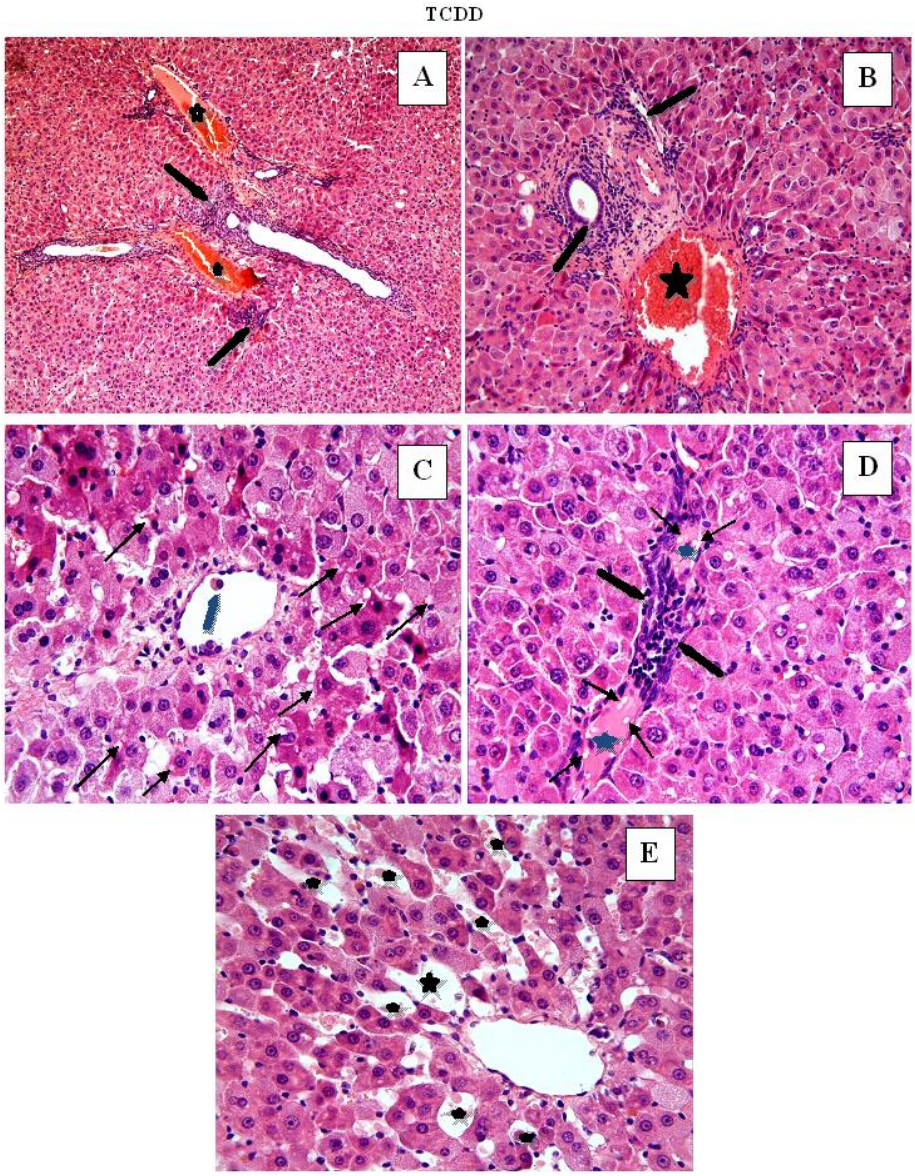

Figure 4. In TCDD group, vascular congestion (black asteriks) (A,B), mononuclear cell infiltration (black arrows) (A,B,D), apoptotic cell in vena centralis (blue arrow) (C), eosinophilic stained and pyknotic nuclei cells (B,D,E), sinusoidal dilatation (C,E), oedema (black asteriks) and vacuolisation (C,E) were observed in liver tissue. A: X10; B: X20; C, D, E: X40 (H&E).

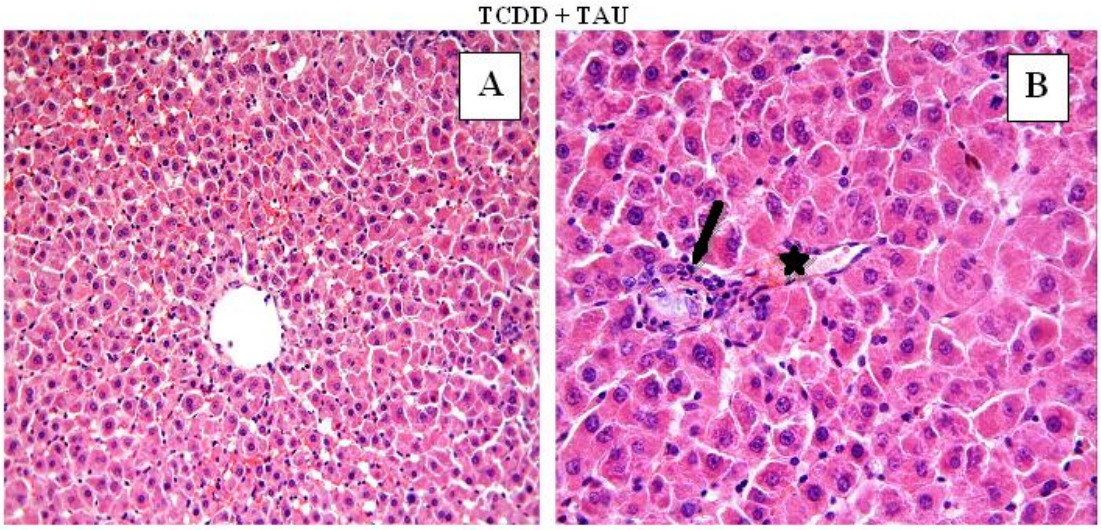

Figure 5. In comparison to the TCDD group, liver damage was decreased in the TCDD + TAU group. Little hemorrhage (A), mononuclear cell infiltration (thick black arrow) (B), vascular congestion (black asteriks), and (B) were observed in TCDD + TAU group. A: X20, B: X40 (H&E).

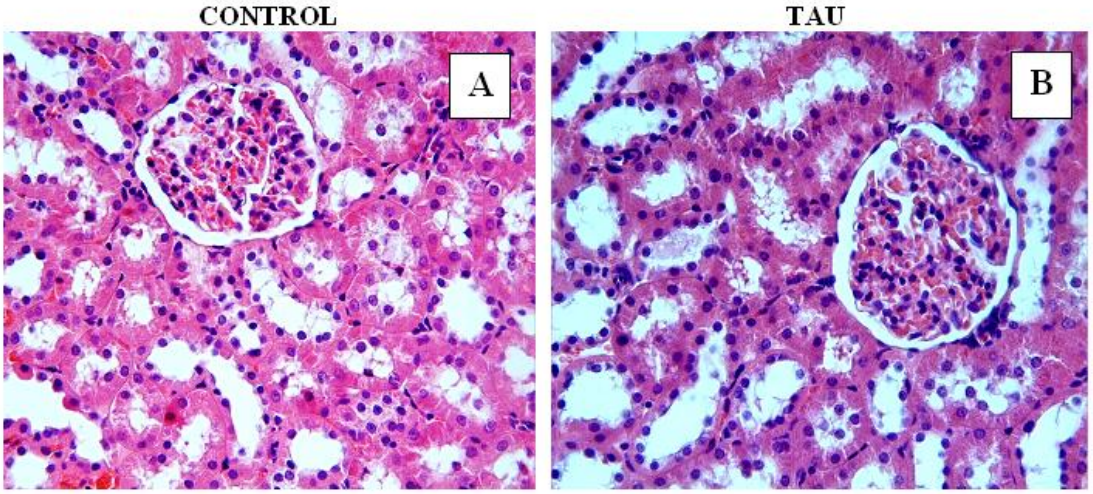

Figure 6. Normal histological appearance was detected in the CONTROL (A) and TAU (B) groups in the kidney tissue. CONTROL and TAU groups revealed regular morphology of glomerular complex, renal tubules with intact tubular epithelial cell membrane contour in CONTROL and TAU group. A, B: X40 (H&E).

Table 1. Histopathological score of groups. (Mean ± SEM)

Groups	Histopathologic Damage (Mean±SE)	
	Liver	Kidney
CONTROL	$0.47 \pm 0.09^{*}$	$0.57 \pm 0.09^{*}$
TCDD	$1.86 \pm 0.12^{\#}$	$2.04 \pm 0.87^{\#}$
TAU	$0.91 \pm 0.10^{*}$	$0.89 \pm 0.09^{*}$
TCDD+TAU	$1.17 \pm 0.11^{+}$	$1.57 \pm 0.11^{+}$

The mean differences between values with different superscripts in the same column were statistically significant (n=8) ($p \leq 0.001$). The mean differences between values with the same superscript in the same column were not statistically significant (n=8) ($p > 0.05$). *, #, and + superscripts express statistical significance of histopathological damage between groups in liver and kidney tissue. Each value is the mean ± standard error of the mean.

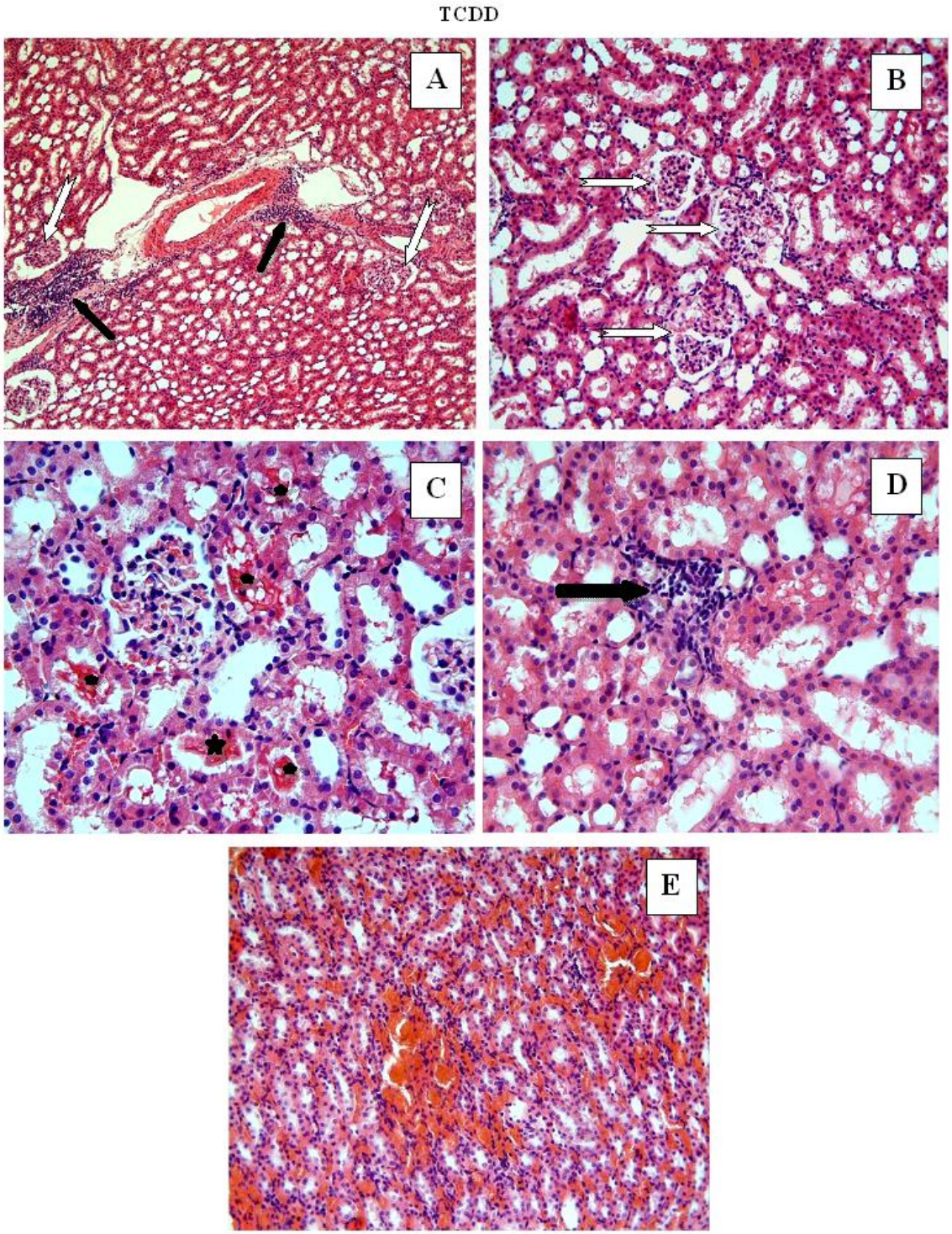

Figure 7. In TCDD group, glomerular degeneration (A,B) (white arrows), dilatation of the tubular lumen (B,C,D), inflammatory cell infiltration (thick black arrows) (A,C), narrowing in the bowman area (B,C), epithelial atrophy and cell desquamation (C,D), casts in tubular lumen (C) (black asteriks), hemorrhage between the tubules (E), were observed. A: X10; B,E: X20; C,D: X40 (H&E).

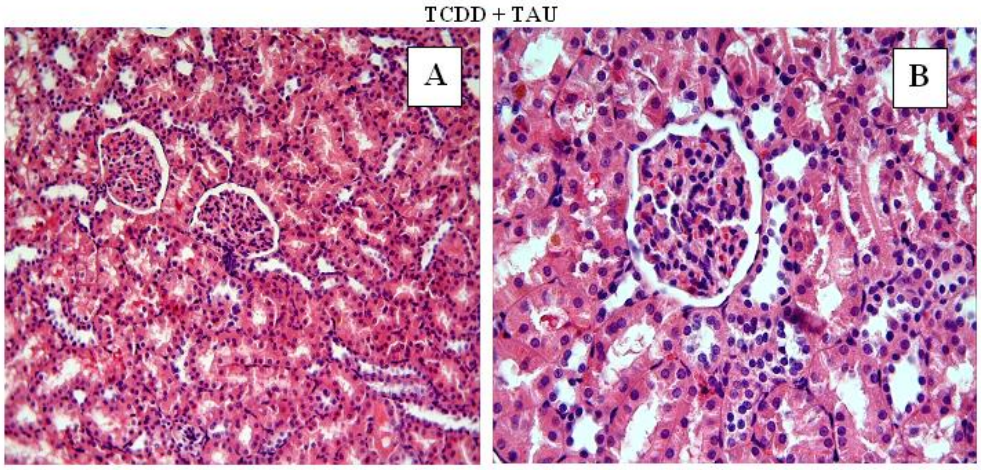

Figure 8. In TCDD + TAU group, kidney damages were decreased compared with TCDD group. Little hemorrhage between the tubules (A), and cell desquamation (B) were observed in TCDD + TAU group. A: X20; B: X40 (H&E).

Immunohistochemical Evaluations

We evaluated liver and kidney tissues for Caspase-3 activity. There were no positive stained cells in CONTROL (Figure 9A, 10A) and TAU (Figure 9D, 10D) groups. In TCDD (Figure 9B, 10B) and TCDD+ TAU (Figure 9C, 10C) groups, Caspase-3 positive stained cells were observed in both organs. In TCDD + TAU group, positive stained cells were decreased compared with TCDD group in liver and kidney tissues. The intensity of caspase-3 positive staining score in liver and kidney tissue is given in Tables 2 and 3, respectively.

In this study, taurine was assessed for its preventive effect in TCDD-induced hepatotoxicity and nephrotoxicity in male Wistar rats by analyzing histopathological and oxidative stress markers such as TBARS, SOD activity, and GSH tissue contents. Subchronic and chronic TCDD exposure causes an increase in the production of ROS, lipid peroxidation, and DNA damage in rats [24,25]. The AHR signal transduction pathway is known to be responsible for the majority of TCDD's *in vivo* toxic effects [26]. Histopathological examination and oxidative marker levels in our study showed that TCDD causes severe damage to tissues. TCDD significantly increased oxidative damage by increasing TBARS levels, decreasing GSH levels and SOD enzyme activity in the liver and kidney tissues.

Taurine is an important homeostasis mediator with multiple roles in protecting against oxidative stress [27]. Our findings show that taurine at a dose of 200 mg/kg/day alleviated the oxidative stress and organ damage induced by TCDD in rats. In a previous study, we have demonstrated that capsaicin has a curative effect by reducing oxidative stress in TCDD-induced organ damage in rats' kidney and liver [28]. SOD activity and GSH levels are critical in the detoxification of superoxide radicals, which protect cells from free radical damage [29]. TBARS are formed as a result of ROS peroxidizing fatty acids, and they cause irreversible cell damage [30]. In rat liver damage caused by TCDD, the antioxidants quercetin [31] and hesperidin [32] have been shown to reduce MDA levels while increasing GSH and SOD levels. Taurine has been shown to scavenge ROS and reduce lipid peroxidation, which helps to stabilize biological membranes [33]. TCDD significantly altered the levels of oxidative stress markers GSH, SOD, and TBARS in kidney and liver tissue, according to our findings. The TCDD group had significantly higher TBARS levels in the liver and kidney tissues than the CONTROL group; however, SOD and GSH levels were noticeably lower. In contrast to the TCDD group, the TCDD+TAU group had significantly lower TBARS levels, while GSH and SOD levels were higher. Similar to our results, it has been reported that TCDD significantly increased lipid peroxidation and decreased antioxidant activities, and that melatonin treatment significantly protected subjects from TCDD-induced cardiotoxicity in rats [34]. It has been shown that taurine treatment protected against oxidative damage in erythrocytes of tertiary butyl hydroperoxide-exposed mice by increasing GSH levels and decreasing

MDA and ROS levels [35]. Taurine's ability to reduce oxidative stress has also been reported in male albino rats with testicular dysfunction [36].

Our histopathological findings on the effects of TCDD in the tissues were consistent with biochemical results. Vascular congestion, apoptotic cells in the vena centralis, and eosinophilic stained pyknotic nuclei cells in the liver tissues have all been observed in rats exposed to TCDD. In addition, epithelial atrophy and cell desquamation, mononuclear cell infiltration, hemorrhage between the tubules, tubular lumen dilatation, casts in the tubular lumen, and glomerular degeneration in the kidney tissues. Taurine significantly reduced the severity of histopathological changes in the liver and kidney tissues when compared to the TCDD exposure group. Similarly, it has been shown that TCDD causes histopathological changes in the liver [37] and kidney [38], and reducing oxidative stress with antioxidant substances including beta-glucan and thymoquinone has been reported to have curative effects. Taurine has been shown to reverse caspase-3 activity as well as histological damage in treated rats' brain, testis, and epididymis [39]. Apoptotic cells were identified in this study using the immunohistochemical method. Taurine treatment inhibited Caspase-3 activity, according to immunoblotting analysis. Caspase-3-positive cells were not found in either the CONTROL or TAU groups' kidney or liver tissues. Taurine not only reduced histopathological damage but also the number of apoptotic cells, confirming Taurine's ability to reduce the toxic effects of TCDD. Previous studies found that taurine administration significantly reduced tissue damage and the number of apoptotic cells by suppressing increased oxidative stress via its antioxidant effect [40,41].

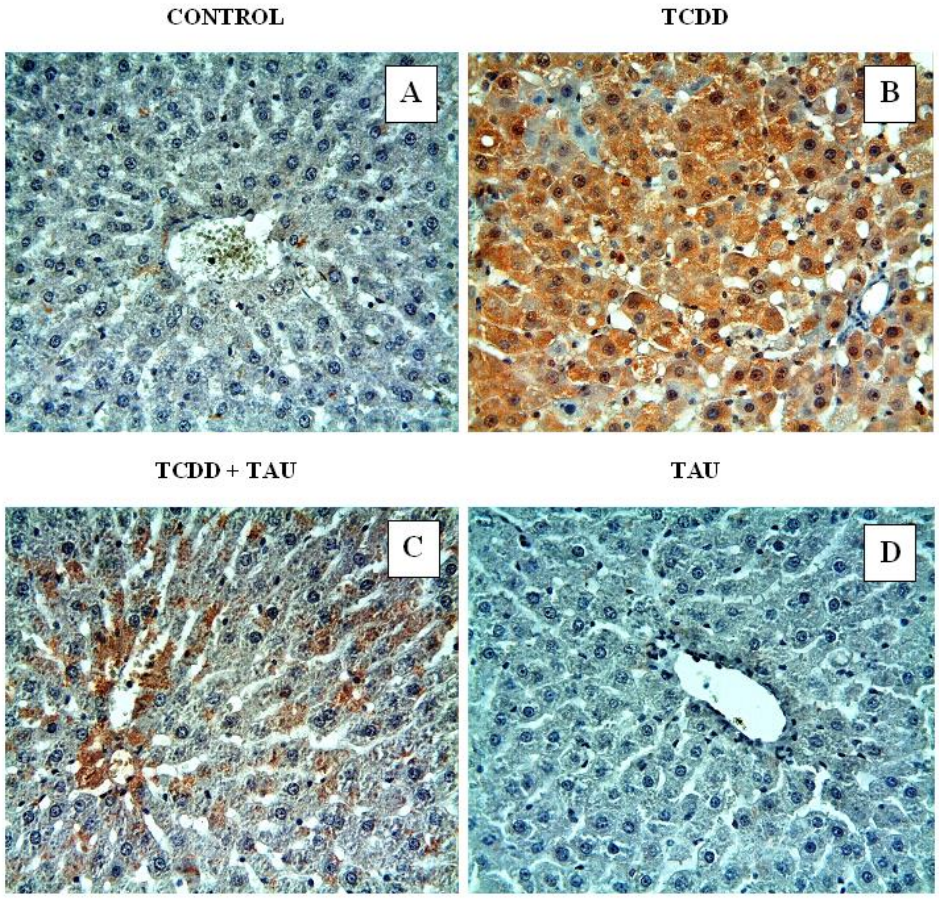

Figure 9. Liver tissue immunostaining for Caspase-3. In the CONTROL (A) and TAU (D) groups, no positively stained cells were observed. In TCDD (B) and TCDD + TAU (C) groups, Caspase-3 positive stained cells were observed. In TCDD + TAU group, positive stained cells were decreased compared with TCDD group (A-D: X40).

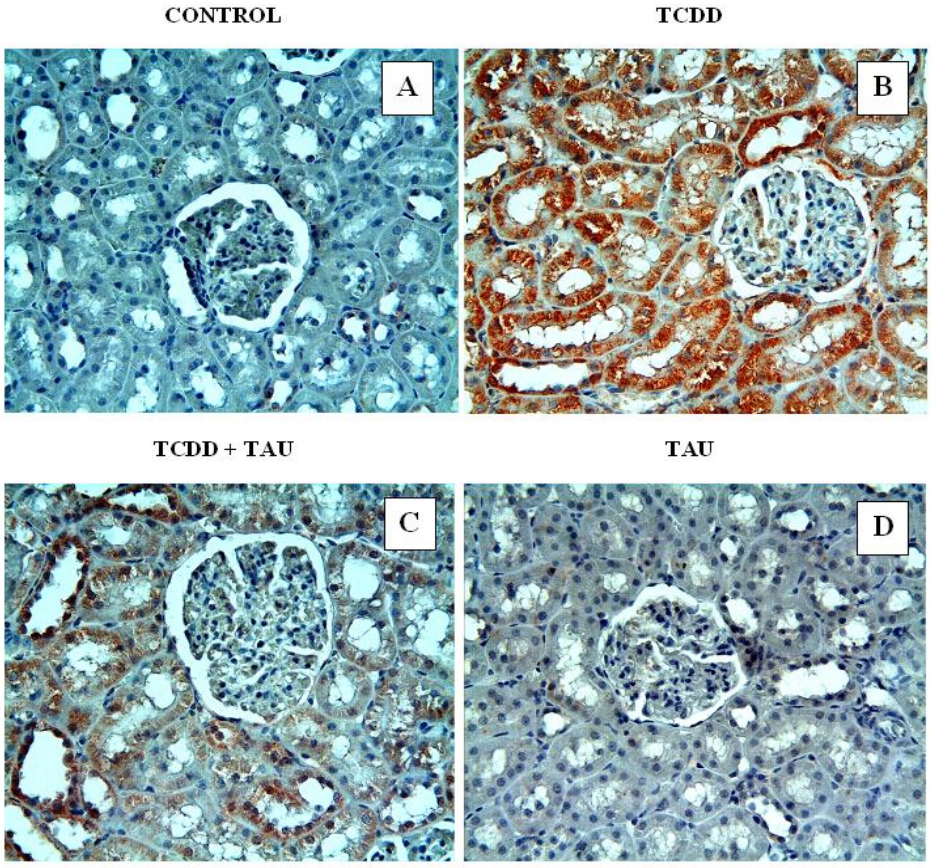

Figure 10. Kidney tissue immunostaining for Caspase-3. There were no positive stained cells in CONTROL (A) and TAU (D) groups. In TCDD (B) and TCDD + TAU (C) groups, Caspase-3 positive stained cells were observed. Positive stained cells were decreased in the TCDD + TAU group compared to the TCDD group (A-D: X40).

Table 2. Intensity of Caspase-3 positive staining score in liver tissue (Mean ± SE).

Groups	Caspase-3 positive staining score
CONTROL	$0.43 \pm 0.07^{*}$
TCDD	$16.7 \pm 0.86^{\#}$
TCDD + TAU	$10.4 \pm 0.39^{+}$
TAU	$1.15 \pm 0.17^{*}$

The mean differences between values with different superscripts in the same column were statistically significant (n=8) ($p \leq 0.001$). The mean differences between values with the same superscript in the same column were not statistically significant (n=8) ($p > 0.05$). *, #, and + superscripts indicate statistical significance of Caspase-3 positive staining score in liver tissue. Each value is mean ± standard error of the mean.

Table 3. Intensity of Caspase-3 positive staining score in kidney tissue (Mean ± SE).

GROUPS	Caspase-3 positive staining score
CONTROL	$0.78 \pm 0.10^{*}$
TCDD	$17.6 \pm 1.06^{\#}$
TCDD + TAU	$10.8 \pm 0.75^{+}$
TAU	$1.92 \pm 0.20^{*}$

The mean differences between values with different superscripts in the same column were statistically significant (n=8) ($p \leq 0.001$). The mean differences between values with the same superscript in the same column were not statistically significant (n=8) ($p > 0.05$). *, #, and + superscripts indicate statistical significance of Caspase-3 positive staining score in kidney tissue. Each value is mean ± standard error of the mean.

In conclusion, our findings show that taurine supplementation markedly reduces liver and kidney damage in male rats exposed to TCDD, improving both oxidative imbalance and histopathological alterations. Taurine supplementation could be used as an adjunctive therapy in the presence of TCDD toxicity due to its ability to reduce oxidative stress and apoptosis in liver and kidney organ damage caused by TCDD.

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AUTHOR CONTRIBUTIONS

Concept: M.F.D., O.Ç.; Design: M.F.D., O.Ç.; Control: O.Ç., B.Ç.; Sources O.Ç., N.B.T.; Materials: N.B.T., A.T.; Data Collection and/or Processing: M.F.D., M.N.Z.; Analysis and/or Interpretation: N.B.T., A.T., B.Ç.; Literature Review: A.T., B.Ç.; Manuscript Writing: M.F.D., M.N.Z.; Critical Review: M.F.D., O.Ç., N.B.T., A.T.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

Ethics committee approval was obtained from Pamukkale University Animal Experiments Ethics Committee on 13.01.2022 (Protocol no. PAUHDEK-2021/50).

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

TOXIC EFFECTS OF AROCLOR 1254 ON RAT HEART AND THYROID AND MODIFYING ROLE OF SELENIUM STATUS

AROCLOR 1254'ÜN SIÇAN KALBİ VE TİROİT ÜZERİNDEKİ TOKSİK ETKİLERİ VE SELENYUM DÜZEYLERİNİN DEĞİŞTİRİCİ ROLÜ

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ABSTRACT

Objective: *Polychlorinated biphenyls (PCBs) were very widely used in industrial products in past. These chemicals were banned in the 1970s due to their toxic effects. PCBs can still affect human health, as they are persistent in the environment. Aroclor 1254 (A1254) is a commercial PCB congener which was used in electrical transformers, fluorescent lighting fixtures and old appliances such as televisions or refrigerators. In this study, we aimed to evaluate the toxic effects of A1254 on heart and thyroid in male Sprague-Dawley rats. In addition, the modifying role of selenium status was also evaluated.*

Material and Method: *8-week-old male Sprague-Dawley (SD) rats were used in the experiment. The animals were separated randomly into 6 groups (n=6) as control; selenium supplemented (SeS); selenium deficient (SeD); A1254 exposed (A); selenium supplemented A1254 exposed (ASeS) and selenium deficient A1254 exposed (ASeD). A1254 was applied by gavage during the last 15 days of feeding period. Heart and thyroid weights and relative weights, plasma thyroid hormone levels, as well as thyroid and heart tissue oxidative/antioxidative parameters were evaluated.*

Result and Discussion: *Results showed that A1254 exposure and selenium deficiency caused oxidative stress on both heart and thyroid. Plasma fT3 and fT4 levels markedly changed in ASeD group. In conclusion, it can be stated that A1254 exposure can cause lead to oxidative/antioxidative imbalance in both thyroid and heart and can disrupt functioning of thyroid hormones. Selenium seems to have a modifying role in A1254 toxicity in both organs, the importance of which should be evaluated with further mechanistic experiments.*

Keywords: *Aroclor 1254, cardiotoxicity, oxidative stress, selenium, thyroid disorders*

ÖZ

Amaç: *Poliklorlu bifeniller (PCB'ler) geçmişte endüstriyel ürünlerde çok yaygın olarak kullanılmıştır. Bu kimyasallar, toksik etkileri nedeniyle 1970'lerde yasaklanmıştır. PCB'ler, çevrede kalıcı oldukları için insan sağlığını hala etkileyebilir. Aroclor 1254 (A1254), elektrik transformatörlerinde, fluoresan aydınlatma armatürlerinde ve televizyon veya buzdolabı gibi eski cihazlarda kullanılan ticari bir PCB türevidir. Bu çalışmada erkek Sprague-Dawley sıçanlarında A1254'ün kalp ve tiroid üzerindeki toksik etkilerinin değerlendirmesi amaçlanmıştır. Ayrıca selenyum durumunun düzenleyici rolü de değerlendirilmiştir.*

Gereç ve Yöntem: *Çalışmada 8 haftalık erkek Sprague-Dawley (SD) ratlar kullanılmıştır. Hayvanlar, kontrol grubu, selenyum suplemente grup (SeS); selenyum eksikliği olan grup (SeD); A1254 maruziyet grubu (A); selenyum suplemente A1254'e maruziyet grubu (ASeS) ve selenyum eksikliği olan A1254'e maruziyet (ASeD) olarak rastgele 6 gruba ayrıldı. A1254, beslenme sürecinin son 15 gününde uygulanmıştır. Kalp ve tiroid ağırlıkları ve bağıl ağırlıkları, plazma tiroid hormonu seviyeleri ve ayrıca tiroid ve kalp dokusu oksidatif/antioksidatif parametreleri değerlendirilmiştir.*

Sonuç ve Tartışma: *Sonuçlar, A1254 maruziyetinin ve selenyum eksikliğinin hem kalp hem de tiroit dokusunda oksidatif strese neden olduğunu göstermiştir. Plazma fT3 ve fT4 seviyeleri ASeD grubunda belirgin şekilde değişmştir. Sonuç olarak, A1254 maruziyetinin hem tiroit hem de kalpte oksidan/antioksidan dengesizliğe yol açabileceği ve tiroit hormonlarının işleyişini bozabileceği söylenebilir. Selenyum, her iki organda da A1254 toksisitesinde değiştirici bir role sahip olduğu görünmektedir ve bunun önemi daha ileri mekanistik deneylerle değerlendirilmelidir.*

Anahtar Kelimeler: *Aroklor 1254, kardiyotoksisite, oksidatif stress, selenyum, tiroit bozuklukları*

INTRODUCTION

Polychlorinated biphenyls (PCBs) belong to the class of "manmade halogenated aromatic hydrocarbon compounds" [1]. Since they are very stable substances in terms of chemical and physical structure, they are mainly used in the production of capacitors, transformers, hydraulic pumps, printing ink, paints, pesticides, and electrical insulation liquids [2].

Although most countries have banned the commercial production of PCBs since the 1970s, the compounds can still be detected in the environment [3]. Some researchers suggest that these compounds are still being used in the industry in developing countries while others suggest that their environmental persistence cause their presence in human and animal tissues and biological fluids. As PCBs are highly

lipophilic organic pollutants, they can accumulate and bioaccumulate in the food chain. Therefore, their body load increases due to increasing biological age in humans [4].

It has been determined by various studies that high levels of PCBs are found in water sources and soil samples in developing countries [5-7]. In various studies conducted in different countries, PCB concentrations in biological samples such as serum, breast milk, and adipose tissue indicates that human exposure exceeds the tolerable daily intake (TDI, 20 ng/kg body weight), which was determined by the World Health Organization (WHO) [8-11]. The threat posed by PCB accumulation in the environment leads to low-dose PCB toxicity as well as long-term human exposure. This phenomenon has received much attention among both environmental engineers and toxicologists as these compounds, which were banned years ago, are still a significant health threat today. PCB exposure may cause pathological effects, such as reproductive, neurological, endocrinologic, cardiovascular, and immunological disorders in both humans and animals [12-18].

Various studies have demonstrated that PCBs cause pathological changes in the thyroid gland and a decrease in serum thyroid hormone levels [19-23]. It has been observed that thyroid hormones increase in response to PCB exposure and this effect is sometimes followed by a decrease. Such effects usually depend on the type of PCB [24-25]. It is thought that the effects of PCBs on thyroid functions may be due to their structural similarity to thyroid hormones and their competing for binding to the same globulins in the blood [2,16,25,26].It is known that inflammation and oxidative stress in the thyroid gland may also play a role in thyroid hormone disorders and that PCBs may lead to inflammation and oxidative/antioxidative balance in multiple organs and systems [27,28].

Exposure to PCBs has been associated with different cardiac and circulatory pathologies such as heart failure, heart disease, atherosclerotic cardiovascular diseases and hypertension. Numerous epidemiological studies have found that exposure to PCBs, particularly dioxin-like (DL)-PCBs, is associated with an increased risk of cardiovascular disease [29-31].

Aroclor 1254 (A1254) was a highly used PCB congener. A1254 contains 54% chlorine by weight and contains 5 chlorines per biphenyl molecule [7,32]. Because Aroclors are composed of dozens of chlorinated PCB components, their biodegradation takes a very long time [33]. A1254 is known to have various toxic effects, including cardiac and thyroidal toxicities [34-37].

Selenium, an essential trace element, has important roles in many cellular processes in the human body, especially in the antioxidant and immune systems. Enzymes and proteins they have selenium as a component are called “selenoproteins”. Crucial proteins and antioxidant enzymes such as iodothyronine deiodinases, glutathione peroxidases, thioredoxin reductases, and selenoprotein P are all selenoproteins. Selenium supplementation is known to have a protective effect against the oxidative stress caused by physical, chemical and biological agents and selenium supplementation may be beneficial in chronic diseases at appropriate doses [38-41].

Our study aimed to evaluate the toxic effects of A1254 exposure on thyroid and heart in adult male Sprague-Dawley rats. The toxic effects of this PCB congener was determined by measuring oxidant/antioxidant parameters as well as plasma thyroid hormone levels. In addition, the modifying role of selenium status after A1254 exposure was evaluated.

MATERIAL AND METHOD

Chemicals and Kits

A1254 (purity 99%), alcohols, Tris, diethylenetriaminepentaacetic acid (DTPA), phenylmethanesulfonyl fluoride (PMSF), and BCA Protein Assay Kit were purchased from Sigma-Aldrich (St. Louis, MO). Total antioxidant capacity (TAOC), Malondialdehyde (MDA) and protein carbonyl assay kits were purchased from Cayman (Ann Arbor, MI). Rat thyroxine, T4 ELISA Kit was from Biomatik (Kitchener, Canada) and Rat Triiodothyronine (T3) ELISA Kit was from MyBioSource (Vancouver, Canada), respectively. All other chemicals were from Sigma-Aldrich.

Experimental Groups

3-week-old male Sprague-Dawley (SD) rats were obtained from the Laboratory of Experimental Animals at Hacettepe University. Six groups were randomly created (n=6 animals for each) and each

group was housed in polypropylene cages with stainless steel grid tops. The cages were kept in regulated humidity (at 50%), temperature (at 23°C), and 12-hour light/dark cycle. Every week body weights (bw) of the animals were measured. The feeding period lasted for five weeks and the animals had unlimited access to food and water. The experimental groups were as follows:
1-Control group was fed with normal rat diet (0.15 mg/kg Se) for 7 weeks.
2-Selenium supplemented (SeS) group was fed with selenium-supplemented diet (1 mg/kg Se) for 7 weeks.
3-Selenium deficient (SeD) group was fed with selenium-deficient diet (≤0.05 mg/kg Se) for 7 weeks.
4-Aroclor 1254 (A) group was fed with normal rat diet (0.15 mg/kg Se) for 7 weeks and received 10 mg/kg A1254 by gavage during the last 15 days of feeding period.
5-Selenium supplemented Aroclor 1254 (ASeS) group was fed with selenium-supplemented diet (1 mg/kg Se) for 7 weeks and received 10 mg/kg A1254 by gavage during the last 15 days of feeding period.
6-Selenium deficient Aroclor 1254(ASeD) group was fed with selenium-deficient diet (≤0.05 mg/kg Se) for 7 weeks and received 10 mg/kg A1254 by gavage during the last 15 days of feeding period.

Thyroid Hormone Levels

After decapitation, 5 ml blood samples were taken into heparinized tubes. Tubes were centrifuged at 3500 rpm for 10 min. Plasma samples were aliquoted and stored at -80^{o}C. fT3 levels were measured with a competitive ELISA kit and fT4 levels were determined by a quantitative sandwich ELISA kit.

Preparation of Tissue Homogenates

Teflon pestle homogenizer used for heart and thyroid homogenates 10% (w/v). Total homogenate in a volume of ice-cold buffer containing Tris (10 mM), diethylenetriaminepentaacetic acid (1 mM), and phenylmethanesulphonyl fluoride (1 mM; adjusted to pH 7.4). The supernatant's total antioxidant capacity (TAOC), malondialdehyde (MDA), and carbonyl concentrations were assessed after centrifugation at 1500 x*g*, 4 °C, for 10 min. The rest of the supernatants were recentrifugated at 9500 x*g*, 4°C for 20 min, and the antioxidant enzyme activities (SOD, CAT) were determined in the supernatant. All spectrophotometric measurements were performed using a spectrophotometer SpectraMax M2 (Molecular Devices, Sunnyvale, CA).

Determination of Antioxidant Enzyme Activities and Oxidative Stress Parameters

Catalase (CAT) activity was determined with the enzymatic decomposition of H_2O_2. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 in one min was followed directly at 240 nm [42]. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 in one min.

The total superoxide dismutase (total SOD) activity was determined by monitoring the auto-oxidation of pyrogallol at 420 nm [43]. One unit of total SOD activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%.

TAOC of experimental groups was evaluated with a commercial kit based on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-Azino-di- [3-ethylben, followedulphonate]) to ABTS®•+ by metmyoglobin. The amount of ABTS®•+ produced can be monitored by reading the absorbance at 750 nm or 405 nm.

The carbonyl groups as the biomarker of protein oxidation were determined by a quantitative analysis of carbonyl groups is based on the formation of stable hydrazones after the derivatization of these groups with DNPH. Subsequently, the absorbance of the stable hydrazones formed is measured spectrophotometrically at 370 nm by "carbonyl assay kit".

MDA levels were measured as a biomarker of lipid peroxidation with a commercial kit. The basis of the Cayman kit used in the measurement is the reaction of malondialdehyde with thiobarbituric acid in acidic conditions and the color intensity of the pink compound formed is measured colorimetrically at 530 nm.

Total Protein Determination

Total protein measurement was performed using a commercial kit using the bicinchoninic acid (BCA) assay. This experiment is based on the spectrophotometric measurement of the absorbance of the purple complex formed by the reaction of BCA and Cu^{+2} ions and the Cu^{+1} ions formed as a result of the reaction of the protein in alkaline media, at 562 nm [44].

Statistical Analysis

The results were expressed as mean±standard deviation (SD). The differences among the groups were evaluated with Kruskal–Wallis one-way analysis of variance, followed by Mann–Whitney U test using a Statistical Package for Social Sciences Program (SPSS) version 17.0 (Chicago, IL). P values <0.05 were considered as statistically significant.

RESULT AND DISCUSSION

In our study, we determined the effects of A1254 exposure on heart and thyroid at different selenium status in rats. The results suggested that A1254 exposure may cause decrease in tissue weights, oxidative stress on organs, and hormonal disorders in rats. The results of this study can be discussed in five parts:

Heart Organ Weights

Heart weights in all study groups were lower than the control. In SeS and SeD groups, there were 8% and 18% decreases in heart weight while A group had 47% lower heart weight compared to control ($p<0.05$, all). ASeS group had 20% lower heart weight ($p>0.05$) while ASeD group has 21% decreased heart weight ($p<0.05$) vs. control (Figure 1A).

Relative heart weights in SeS (11%) and SeD (15%) groups were markedly lower than control while in A group there was 10% insignificant decrease vs. control group. Both ASeS (14%) and ASeD (11%) groups had significantly lower heart weights vs. control (Figure 1B).

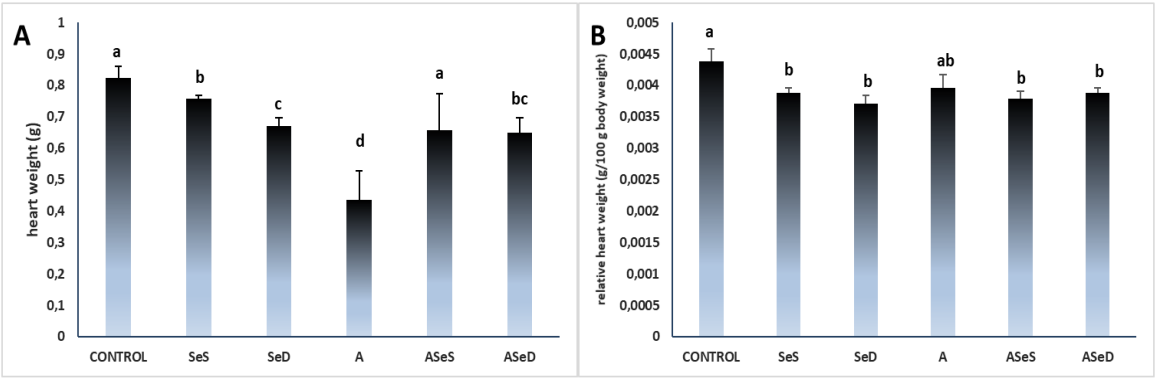

Figure 1. Heart and relative heart weights of groups.
A. Heart weights of groups; B. Relative heart weights of groups.
[a,b,c,d]Bars that do not share same letters (superscripts) are significantly different from each other ($p < .05$)

Wang et al. (2021) found that low dose (0.5 µg/kg and 50 µg/kg) PCB126 exposure caused an increase in relative heart weights. This increase is thought to be related to cardiac hypertrophy seen with low-dose exposure [45]. In our study, we can suggest that the inhibition of heart tissue development due to oxidative damage caused by high-dose A1254 exposure may be the underlying factor of lower heart and relative heart weights in A1254-exposed groups. Moreover, the decreases in heart and relative heart weights in selenium deficiency might be due to both increased cardiac oxidative stress and the lower antioxidant capacity of the heart tissue. In addition, other mechanisms yet to be identified might contribute to lower heart and relative heart weights in both selenium deficiency and/or A1254 exposure.

The contradiction between the results of our study and the study conducted by Wang et al. (2021) may be due to the difference in the PCB congeners.

Determination of Antioxidant Enzyme Activities and Oxidative Stress Parameters on Heart Tissue

Heart MDA levels in A group was higher than control (69%, $p<0.05$). There were no significant changes in MDA levels in other study groups (Fig 2A). Heart carbonyl levels in ASeD group was significantly higher than control (~2-fold). Carbonyl levels of SeD and A groups were higher vs. control group (46% and 27%, respectively; $p>0.05$, both) (Fig 2B). Heart TAOC levels in ASeD group was markedly lower than control (23%) (Fig 2C).

The results indicate that A1254 exposure leads to lipid peroxidation in heart. Heart carbonyl levels in the ASeD group were significantly higher than control (~2-fold). Although A1254 exposure alone does causes higher levels of protein oxidation and lower TAOC levels, the differences between A and control groups were not statistically significant for both of the measured parameters. These results suggest that selenium deficiency exacerbates the protein oxidation caused by A1254 exposure. All these results suggest that there is a deterioration in the oxidant/antioxidant balance in the heart tissue, particularly after A1254 exposure and selenium deficiency. The increase in CAT activity indicates the response of the organ to protect itself against the oxidative stress that occurs in the heart tissue with the application of A1254 in selenium deficiency. Several *in vivo* studies also suggest that different PCBs and PCB congeners lead to tissue damage, oxidative stress, disrupt the work of calcium and potassium channels and affect enzyme activities [46-50]. In studies conducted on humans, PCB exposure has been associated with important cardiac pathologies such as myocardial infarction, heart attack, coronary atherosclerosis and heart failure [31,51,52]. In addition, various studies show that selenium deficiency can have negative effects on cardiovascular health [40,53]. As selenium is the major component of glutathione peroxidases (GPxs), which are crucial antioxidant enzymes, the decrease in selenium levels mainly affects GPx activity. Lower GPx activity exacerbates endothelial dysfunction, a major contributing factor in the severity of chronic heart failure symptoms, in various conditions such as hyperhomocysteinemia. This suggests that homocysteine may be involved in the chronic heart failure associated endothelial dysfunction through a peroxide-dependent oxidative mechanism [54]. According to our results, it can be postulated that PCB toxicity may occur more predominantly in selenium deficiency and its consequences may be more pronounced.

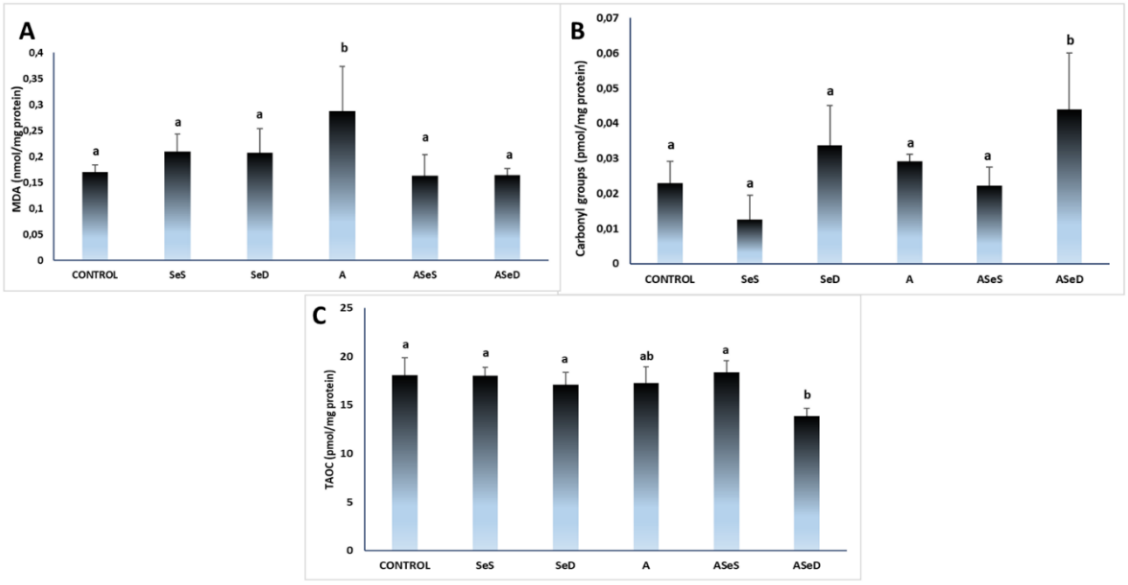

Figure 2. Heart oxidative stress parameters.
A. MDA levels in heart.; B. Carbonyl levels in heart; C. TAOC levels in heart.
[a,b,c,d]Bars that do not share same letters (superscripts) are significantly different from each other ($p < .05$)

CAT activity was significantly higher in ASeD group (33%) while there were no marked changes in SOD activities of the study groups vs. control.

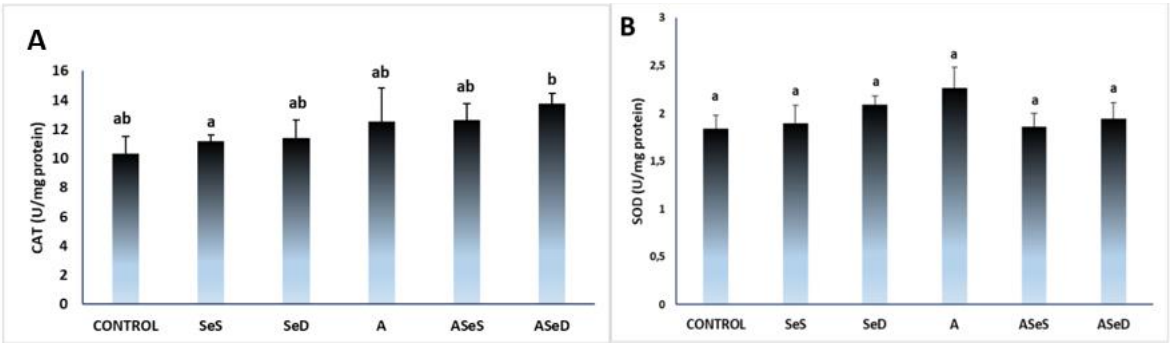

Figure 3. Heart antioxidant enzyme activities.
A. CAT activity of heart tissue; B. SOD levels of heart tissue
[a,b,c,d]Bars that do not share same letters (superscripts) are significantly different from each other ($p < .05$)

Thyroid Organ Weights

The thyroid weights in SeD (38%), A (39%), ASeS (55%) and ASeD (50%) groups were markedly lower than control (Fig. 4A). Relative thyroid weights were significantly lower in all study groups (21% in SeS, 34%in SeD, 30% in A, 52% in ASeS and 41% in ASeD groups vs. control) (Fig. 4B). These results indicate that Se deficiency and/or A1254 exposure cause more pronounced decreases in thyroid tissue weights and relative organ weights. As both A1254 alone and selenium deficiency alone lead to lower thyroid/relative thyroid weight, it is not suprising that the combination of A1254 exposure and selenium deficiency produces a more pronounced effect on thyroid/relative thyroid weights.

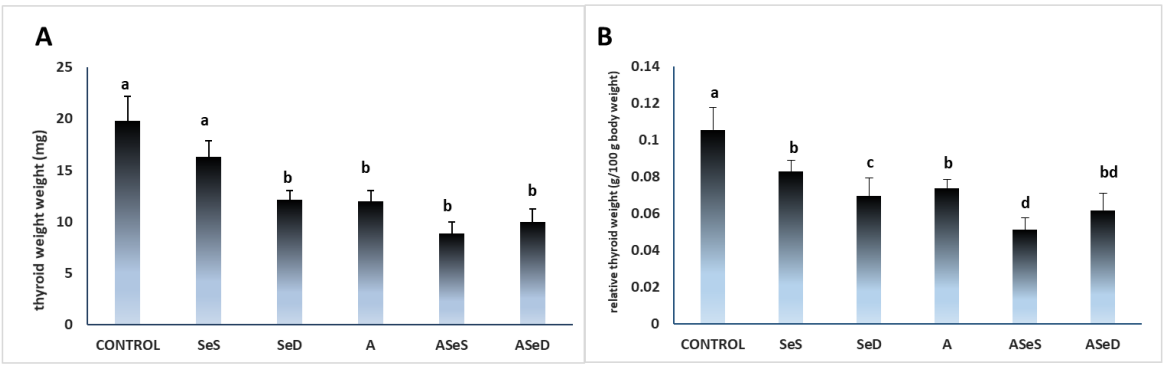

Figure 4. Thyroid and relative thyroid weights of groups.
A. Thyroid weights of groups; B. Relative thyroid weights of groups.
[a,b,c,d]Bars that do not share same letters (superscripts) are significantly different from each other ($p < .05$)

Thyroid Hormone Levels

fT4 levels in SeD group were significantly higher than control (64%). fT4 levels were markedly lower in A (51%), ASeS (22%) and ASeD (10%) groups vs. control group (Fig. 5A). fT3 levels were lower in A (37%) group vs. control (Fig. 5B). Numerous studies have shown that selenium is directly related to thyroid tissue and hormones as selenium is the integral component of iodothyronine deiodinases, which are a subfamily of deiodinase enzymes important in the activation and deactivation of thyroid hormones [38,55].

Limited number of *in vivo* and human studies indicate that exposures to A1254 and other PCBs can cause changes in thyroid hormone levels. Although the results of these studies varied depending on

the exposure period and dose, the data showed that rodents showed significant changes in thyroid hormone levels after PCB exposure. These alterations caused by A1254 in thyroid may be due to their structural similarity to thyroid hormones. In addition, as exposure to A1254 caused a significant decrease in serum fT3 levels, it can be concluded that A1254 also has an endocrine disrupting effect in the thyroid, most possible due to disruption of hormonal feedback flow and changes in enzyme levels that metabolize thyroid hormones. Moreover, thyroidal damage, oxidative stress and/or the interactions of A1254 with thyroid hormone receptors may lead to alterations in thyroid hormone levels [16, 27, 34, 56-63]. However, more mechanistic studies are needed to show the exact endocrine disrupting mechanisms of different PCB congeners in the thyroid.

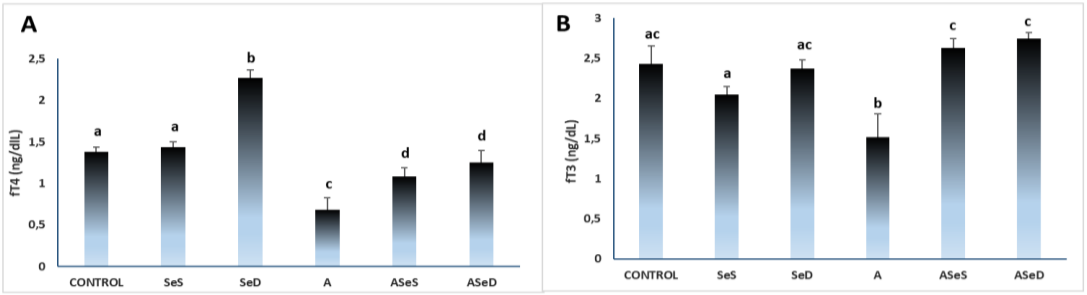

Figure 5. Plasma thyroid hormone levels
A. fT4 levels; B. fT3 levels
[a,b,c,d]Bars that do not share the same letters (superscripts) are significantly different from each other ($p < .05$)

Determination of Oxidative Stress Parameters on Thyroid Tissue

Thyroid MDA levels in SeD (58%), A (1.7-fold), ASeS (70%) and ASeD (2.5-fold) groups were markedly higher than control (Fig 6A). TAOC levels in SeD (36%), A (50%), ASeS (36%) and ASeD (33%) groups were significantly lower than control (Fig 6B). It has been determined that lipid peroxidation in thyroid was much more pronounced with the selenium deficiency with accompanying A1254 exposure. TAOC levels in SeD (36%), A (50%), ASeS (36%) and ASeD (33%) groups were significantly lower than control in thyroid. It has been observed that exposure to A1254 in the presence of selenium deficiency cause a more significant decrease in the antioxidant defense capacity of thyroid tissue. Several studies and comprehensive reviews suggested that selenium deficiency causes higher oxidative stress in thyroid in accordance with the results of the presence study [65-69].

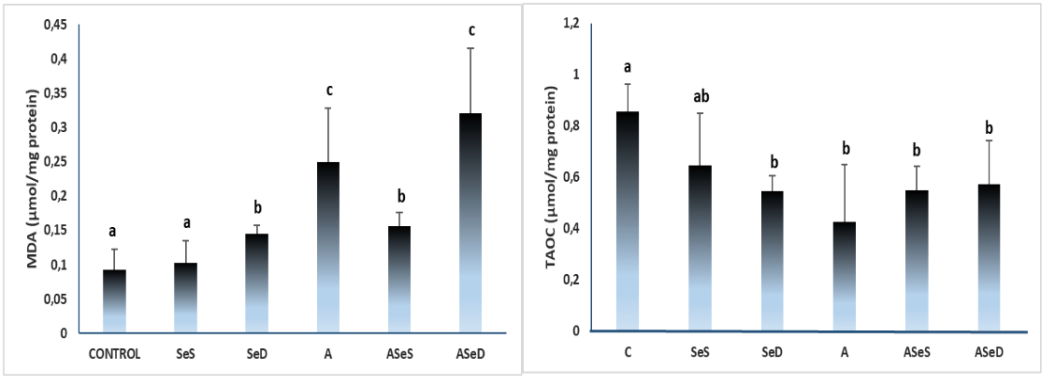

Figure 6. Thyroid oxidative stress parameters.
A. MDA levels of thyroid tissue; B. Carbonyl levels of thyroid tissue
[a,b,c,d]Bars that do not share same letters (superscripts) are significantly different from each other ($p < .05$)

In conclusion, it can be suggested that A1254 causes toxic effects in both heart and thyroid in rats. A1254 disrupts thyroid enzymes and causes oxidative/antioxidative imbalance in both thyroid and heart. Selenium, a crucial essential element, seems to have modifying role in the cardiac and thyroidal toxicity of A1254. The exact mechanism through which selenium is partially protective against toxicity of A1254 in heart and thyroid is not clear. However, as selenium is the integral component of many important antioxidants, selenium supplementation seems to augment the toxicity of A1254, at least partially. Mechanistic studies are needed to clarify the exact effects of selenium in PCB toxicity. moreover, as selenium is also an important element for the endothelial functioning through GPxs, the mechanism through which selenium shows a protective effect in heart against A1254 toxicity should be investigated in detail.

AUTHOR CONTRIBUTIONS

Concept: P.E., B.G.; Design: P.E., B.G.; Sources: A.B.Ö., G.Ö., Ü.Y., A.A., P.E., B.G.; Materials: A.B.Ö., G.Ö., Ü.Y., A.A., P.E., B.G.; Data Collection and/or Processing: A.B.Ö., Ü.Y., A.A., P.E.; Analysis and/or Interpretation: A.B.Ö., G.Ö., Ü.Y., A.A., M.K.; Literature Review: A.B.Ö., P.E.; Manuscript Writing: A.B.Ö., P.E.; Critical Review: P.E., B.G.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

The study was approved by Hacettepe University Animal Ethics Committee (02.04.2012-2012/20-7) and animals were treated humanely.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

FABRICATION AND CONTROLLING MORPHOLOGY OF POLYETHYLENE OXIDE/SODIUM ALGINATE BEADS AND/OR FIBERS: EFFECT OF VISCOSITY AND CONDUCTIVITY IN ELECTROSPINNING

POLİETİLEN OKSİT/SODYUM ALJİNAT BONCUKLARININ VE/VEYA LİFLERİNİN ÜRETİMİ VE MORFOLOJİK KONTROLÜ: ELEKTROEĞİRMEDE VİSKOZİTE VE İLETKENLİĞİN ETKİSİ

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ABSTRACT

Objective: *The aim of the study is to determine the viscosity and conductivity of PEO/NaAlg polymer solutions, to formulate PEO/NaAlg beads, bead-on-string fibers and fibers via electrospinning, and to perform advanced morphological characterization studies on them.*
Material and Method: *Effect of PEO and NaAlg concentration and ratio of them on spinnability, viscosity and conductivity of solutions, and also on the morphological properties of PEO/NaAlg electrospun fibers, and beads (length, width and aspect ratio of beads, number of beads and bead area) were investigated.*
Result and Discussion: *As a result, electrospun materials were produced using PEO/NaAlg beads and/or fibers, which are valuable for medical and biological applications such as tissue engineering, wound dressing, drug delivery system. Viscosity and conductivity of solutions, and morphological properties of the obtained materials were found to be affected by PEO and NaAlg concentration and the ratio of them. Spinnability was improved thanks to the increase in conductivity in the presence of PEO. The lower viscosity and conductivity resulted in the production of beads that were generally smaller and greater in number in the material and having higher area. The morphological properties of PEO/NaAlg electrospun materials can be modified by controlling the parameters examined in the study.*

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Keywords: *Conductivity, electrospinning, electrospun beads, polyethylene oxide (PEO)/sodium alginate (NaAlg), viscosity*

ÖZ

Amaç: *Çalışmanın amacı, PEO/NaAlg polimer çözeltilerinin viskozite ve iletkenliklerini belirlemek, PEO/NaAlg boncukları, boncuk-iplik lifleri ve lifleri elektroeğirme yöntemiyle formüle etmek ve bunlar üzerinde ileri morfolojik karakterizasyon çalışmaları yapmaktır.*
Gereç ve Yöntem: *PEO ve NaAlg derişiminin ve oranlarının çözeltilerin eğrilebilirliği, viskozitesi ve iletkenliği ve ayrıca PEO/NaAlg elektroeğrilmiş liflerin ve boncukların morfolojik özellikleri (boncukların uzunluğu, genişliği ve en boy oranı, boncuk sayısı) üzerindeki etkisi incelenmiştir.*
Sonuç ve Tartışma: *Sonuç olarak, doku mühendisliği, yara örtüsü, ilaç taşıyıcı sistem gibi tıbbi ve biyolojik uygulamalar için değerli olan PEO/NaAlg boncuklar ve/veya lifler elektroeğirme yöntemiyle üretilmiştir. Çözeltilerin viskozite ve iletkenliği ile üretilen materyalin morfolojik özelliklerinin PEO ve NaAlg derişiminden ve oranlarından etkilendiği bulunmuştur. PEO varlığında iletkenlikteki artış sayesinde eğrilebilirlik iyileştirilmiştir. Daha düşük viskozite ve iletkenlik, genellikle daha küçük, daha fazla sayıda ve daha büyük alana sahip boncukların üretilmesine neden olmuştur. PEO/NaAlg elektroeğrilmiş materyallerin morfolojik özellikleri, çalışmada incelenen parametreler kontrol edilerek değiştirilebilir.*
Anahtar Kelimeler: *Elektrik iletkenliği, elektroeğirme, elektroeğrilmiş boncuklar, polietilen oksit(PEO)/sodyum aljinat (NaAlj), viskozite*

INTRODUCTION

Electrospinning is a reproducible, one-step, inexpensive method that allows the production of beads and/or fibers with many polymers and solvents. In electrospinning, a jet is created by applying a high voltage over a polymer solution/emulsion to create an intense electric field on a droplet at the tip of a needle. The jets are then collected on a metal collector and the dried electrospun materials can be used for many purposes such as wound healing, drug delivery system and tissue engineering applications [1,2].

Sodium alginate (NaAlg) is a natural linear polysaccharide polymer of mannuronic acid and β-D-α-L-guluronic acid. It is extracted from brown seaweed or produced from the bacteria (Pseudomonas and Azotobacter). It is a hyrophilic polymer widely used in the pharmaceutical industy due to being nontoxic, biocompatible, biodegredable and has unique gel ability, swelling, film-forming and mucoahesive properties. It is also known to be antibacterial, antiinflammatory and antioxidant. In addition, NaAlg-based drug delivery systems draw attention with their advantages such as controlled release, better efficacy at lower doses and frequency of administration, and therefore reducing toxicity. Thus, NaAlg has a wide range of uses in the pharmaceutical industry such as wound dressing, tissue scaffolding, skin healing and drug delivery system [3-7]. Despite all these advantages, spinnability of pure alginate is a great challenge due to its polyelectrolyte structure, high viscosity and limited solubility. In the study, polyethylene oxide (PEO), flexible and uncharged polymer, was preferred because of its ability to improve the spinnability of NaAlg, by reducing the repulsive force between polyanionic molecules when added to NaAlg [7].

PEO is another preferred hydrophilic polymer in the pharmaceutical industry. It has a repeating structural unit of CH_2CH_2O and has a wide range of different molecular weights (100 000 to 8 000 000). It is also known as poly(ethylene glycol). PEO is preferred as a drug delivery system and biomaterial thanks to its advantages such as high biocompatibility, nontoxicity, ease of production, and insensitivity to the pH of physiological fluids [8]. Unlike NaAlg, PEO is a linear polymer and can easily spinnable.

Viscosity and conductivity of solutions are crucial parameters in the electrospinning. The viscosity plays critical role in the formation of stable taylor cone on the droplet, so it is one of the most effective parameters on the production of beads or fibers and their morphological characterization [1,9-11]. Since the polymer solution must be stretched by the charges collected on the drop at the needle tip, the solution must have sufficient electrical conductivity for electrospinning. The spinnability of solutions, the production of beads or fibers and their morphology are directly affected by the electrical conductivity of the solutions [9,12,13]. Therefore, viscosity and conductivity of the polymer solutions

were determined in this study.

Although at first bead-on-string fibers were considered to be useless in electrospinning, nowadays it is thought that beads can be used, especially for high drug loading [14]. In the future, electrospinning may be a preferred method for the production of beads, especially to be used for purposes such as scaffold or wound dressing, as it enables the production of beads in the form of scaffolds. At the same time, in order to obtain beadless fiber, the factors affecting bead formation should be clarified. The main objective of this study is to determine the viscosity and conductivity of PEO/NaAlg polymer solutions, to formulate PEO/NaAlg beads, bead-on-string fibers and fibers via electrospinning, and to perform advanced morphological characterization studies on them. Although there are many studies in the literature examining the production and morphology of the fibers in materials produced by electrospinning, there are not enough studies on the production of beads by electrospinning and the detailed characterization of the produced beads. The study is the first to investigate the effects of the use of mixtures of different polymers at different concentrations and in different ratios on the effects of viscosity and conductivity of the polymer solutions and on the advanced morphological properties of beads (average length and width of beads, aspect ratio of beads, number of beads and bead area), and as well as fibers in electrospun materials. The current study provides fabrication and morphological characterization of PEO/NaAlg beads and/or fibers which are valuable for producing electrospun materials for medical and biological applications such as tissue engineering, wound dressing, drug delivery system.

MATERIAL AND METHOD

Materials

PEO (MW 400.000) and NaAlg were obtained from Sigma-Aldrich. All chemicals used were of analytical grade.

Preparation of PEO/NaAlg Electrospun Materials via Electrospinning

3-5% w/v PEO and 1-2% w/v NaAlg were dissolved in distilled water. They were mixed in different proportions for different formulations and poured in a syringe. Then, the syringe was fixed in a syringe pump. Flow rate (1 ml/h), voltage (16 kV) and needle tip-collector distance (14 cm) were set from the electrospinning device (Ne-200, Inovenso, Turkey). The collector of the device was covered by aluminum foil and they were dried at room temperature for 48 h. PEO/NaAlg electrospun materials produced in the study were given in Table 1.

Table 1. PEO/NaAlg electrospun materials prepared in the study

	PEO (w/v%)	**NaAlg (w/v%)**	**Ratio of PEO:NaAlg (w/w)**
F1	3	1	1:1
F2	3	1	2:1
F3	3	2	1:1
F4	3	2	2:1
F5	4	1	2:1
F6	5	1	2:1
F7	4	2	1:1

Viscosity of Polymer Solutions

The viscosity of the polymer solutions were determined by Brookfield Viscometer (LVDV-I Prime, U.S.A.) at 100 rpm (spindle no: 14). All the tests were done in triplicate at room temperature. Values were expressed as mean viscosity [millipascal-second (mPa·s)] ± standard deviation.

Electrical Conductivity of Polymer Solutions

Electrical conductivity was measured by an electric conductivity meter (SevenEasy Conductivity, Mettler-Toledo, Switzerland) at room temperature (n=3). Values were expressed as mean conductivity [milisiemens (mS)] ± standard deviation.

Morphologies of Electrospun Fibers and/or Beads

Morphologies of the beads and/or fibers were examined by scanning electron microscope (SEM) (QUANTA 400 F Field Emission SEM, Holland). Electrospun mats were coated with gold before SEM analysis. The length and width of beads and mean diameters of fibers were determined by the measurement of 100 beads/fibers using SEM images via Image J. Aspect ratio of beads divided by lengths and widths for each beads was calculated (n=100). Number of beads and bead area % were also determined using SEM images via Image J.

Statistical Analysis

Statistical analysis was done using SPSS 18.0 (SPSS, Chicago, IL). The significance was determined with one-way ANOVA result with Tukey's post hoc test. The data of $p<0.05$ was considered significant. Values were expressed as mean ± SD.

RESULT AND DISCUSSION

Viscosity and Conductivity of Solutions

The viscosity and conductivity of the aqueous solutions of PEO and NaAlg at the concentrations used in the study were given in Table 2.

Table 2. Viscosity and conductivity of the aqueous solutions of PEO and NaAlg[a]

	Viscosity (mPa·s)	Conductivity (mS)
3% PEO	84.4±0	123.2±0.4
4% PEO	237.2±1.3	116.3±0.6
5% PEO	724.1±2.4	115.1±0.2
1% NaAlg	27.3±0.4	32.2±1.1
2% NaAlg	294.5±2.1	44.1±0.8

[a]Values are expressed as mean± standard deviation (n=3)

It is known that the viscosity of the polymer solution plays an important role in the electrospinning process. An ideal (neither low nor high) viscosity is desirable for the spinnability and especially fiber formation rather than beads. The viscosity of the polymer solution should be low enough not to prevent jet formation by the electric field, and high enough to allow the polymer solution to form a taylor cone without flowing at the needle tip [1,9-11]. While the viscosity of PEO solutions was 84-724 mPa·s, it varied between 27-295 mPa·s in NaAlg solutions. There was a significant difference between the viscosities of all the polymer solutions ($p<0.05$). As expected, the increase in both PEO and NaAlg concentrations resulted in an increase in viscosity ($p<0.05$) (Table 2).

Conductivity of PEO solutions ranged from 115 to 123 mS, while the conductivity of NaAlg solutions ranged from 32 to 44 mS. It was remarkable that the conductivity of NaAlg solutions was very low (approximately 3-4 times) compared to PEO solutions ($p<0.05$) (Table 2).

The viscosity and conductivity of the polymer solutions, in which the electrospun materials were produced, were given in Table 3.

The viscosity of polymer solutions producing electrospun materials ranged from 47 to 248 mPa·s and was related to the amount of PEO and NaAlg they contained. Except for F6 and F7 ($p>0.05$), there was a significant difference between the viscosities of the formulations ($p<0.05$). The viscosity increased with the increasing PEO and NaAlg concentration and the ratio of PEO to NaAlg ($p<0.05$) (Table 3).

Table 3. Viscosity and conductivity of polymer solutions which produced electrospun materials[a]

	Viscosity (mPa·s)	Conductivity (mS)
F1	46.9±0	144.3±0.4
F2	64.8±0.4	141.9±0.5
F3	120.5±1.6	134.8±0.3
F4	151.2±1.4	139.5±0.8
F5	133.2±1.4	142.1±0.3
F6	245.6±1.0	144.4±0.4
F7	247.9±0.6	137.5±0.6

[a]Values are expressed as mean± standard deviation (n=3)

The conductivity of the solutions is another parameter in the electrospinning process. It affects the spinnability of solutions, the production of beads or fibers and their morphology. Low electrical conductivity of the polymer solution leads to bead formation. It is also known that the increase in the electrical conductivity causes an increase in the stretching forces on the jet, leading to the formation of finer jets and finer fibers. As a result, high electrical conductivity leads to finer fiber production with fewer beads [9,12]. It explained the smooth fiber production with very few beads in the F6 which had the highest electrical conductivity (144 mS). While the conductivity in F1 (144.3 mS) was similar to that of F6 (144.4 mS) ($p>0.05$), the formation of beads instead of fibers in F1 was due to the insufficient viscosity of F1 compared to F6 ($p<0.05$). In addition, although the viscosity of F7 (248 mPa·s) was similar to that of F6 (246 mPa·s) ($p>0.05$), the beaded fibers were formen in F7, unlike F6, which formed smooth fibers. This was due to F7 not having high enough conductivity. In conclusion, the current study has proven that both viscosity and electrical conductivity are highly effective in producing fibers or beads, and it is not sufficient for fiber production to be ideal of only one of them.

Preparation and Morphology of PEO/ NaAlg Electrospun Materials

PEO/NaAlg beads and fibers were produced successfully by altering the formulation parameters, viscosity and conductivity. The concentration of PEO (3-5%) and NaAlg (1-2%) and the polymer ratio (1:1 and 2:1) affected the viscosity and conductivity of the polymer solutions and the advanced morphological properties of fibers (diameter and distribution) and beads (mean length and width of beads, aspect ratio of beads, number of beads, and bead area) in electrospun materials. For this purpose, various formulations given in Table 1 were prepared.

The spinnability of pure PEO and NaAlg was also determined. While pure PEO was spinnable, pure NaAlg was not suitable for electrospinning. Because the electrical conductivity of NaAlg aqueous solutions was not sufficient to stretch the charges collected on the drop at the needle tip [9,12]. As seen in Table 2, the conductivity of the NaAlg solutions was 32 and 44 mS, which was quite low compared to PEO solutions ($p<0.05$). In fact, one of the main goals of using PEO was to improve the spinnabililty of NaAlg. Hu et al. claimed that reducing the repulsive force between the polyanionic molecules of NaAlg via hydrogen bonding in the presence of PEO allows fiber production [7]. In addition, the current study also proved that NaAlg could not spin due to its very low conductivity and the increase in conductivity in the presence of PEO improved the spinnability.

The effect of NaAlg concentration and PEO/NaAlg ratio on the morphological properties of beads and fibers of PEO/NaAlg electrospun materials were given in Figure 1 and Table 4.

It was observed from the SEM images that the F1-F4 formulations had a high bead ratio (53-85%) and consisted of many beads with few fibers (Figure 1, Table 4). Increasing the PEO/ NaAlg ratio from 1:1 (F1 and F3) to 2:1 (F2 and F4) increased the viscosity, resulting in an increase in the average width and aspect ratio of the beads, while decreasing the bead area in percent ($p<0.05$). As a result, increasing PEO content and decreasing NaAlg content resulted in the formation of more elliptical beads rather than spherical beads with smaller area in the electrospun material. The main reason was that increasing viscosity reduces bead formation and increases fiber formation ($p<0.05$) (Table 3) [15]. In fact, the decrease in viscosity increased the aspect ratio of the beads ($p<0.05$), indicating a transition from beads

to fiber. It is known that increasing the viscosity of the solution causes the formation of thicker fibers by electrospinning [16,17]. On the contrary, in our study, although the viscosity always increased with the increase in PEO/NaAlg concentration, there was not always an increase in fiber diameter. There was even a reduction in fiber diameters in the F1-F2 formulations (Figure 1, Table 4).

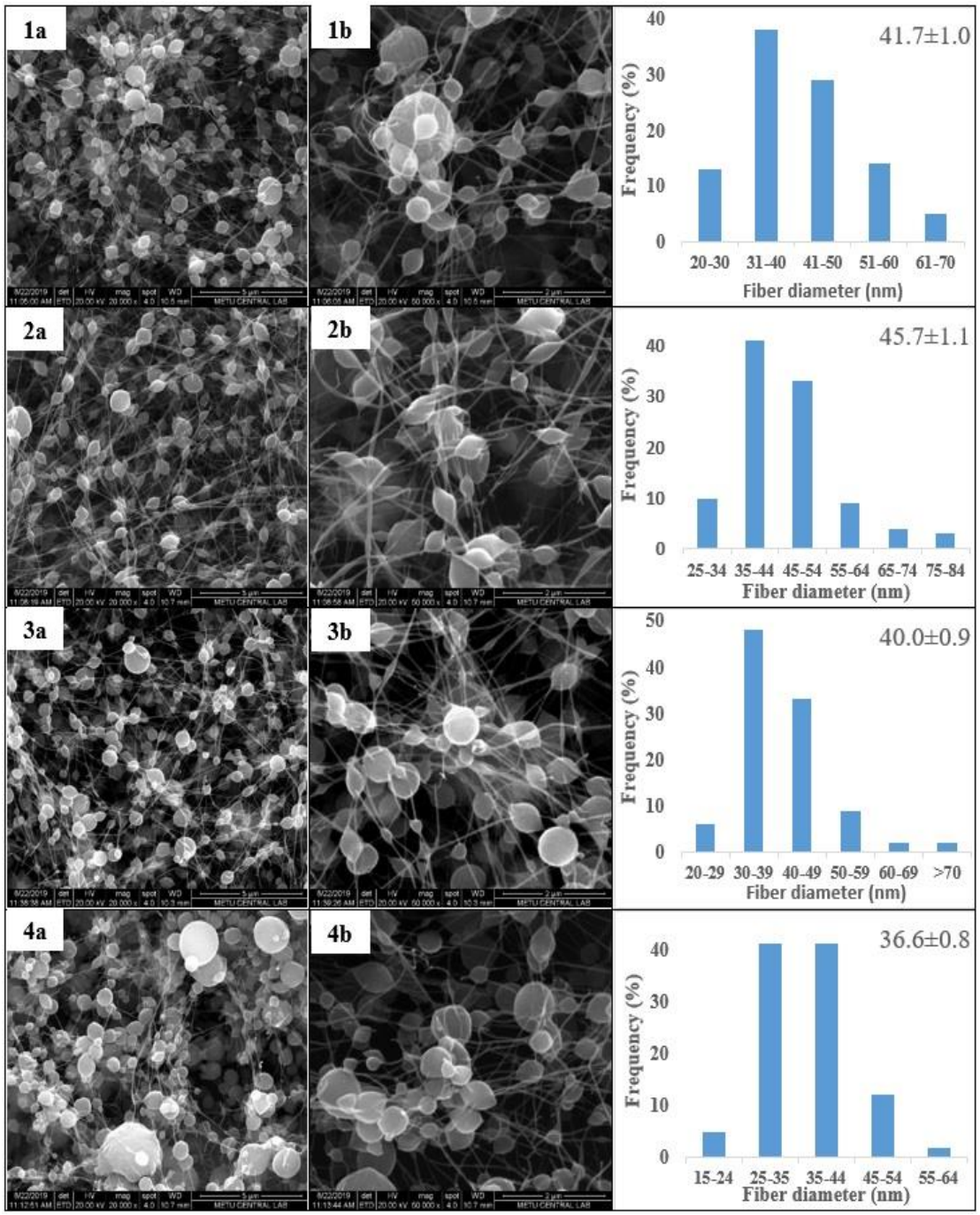

Figure 1. Effect of NaAlg concentration and the ratio of PEO/NaAlg on the morphology and fiber diameter distribution of the electrospun materials[a]

[a]Containing 3%PEO and 1%NaAlg in the ratio of PEO/NaAlg of [F1: (1-1), and F2: (2-1)] and 2%SA in the ratio of [F3: (1-1), and F4: (2-1)] (magnification = a: 20.000×, b: 50.000×)

Table 4. Effect of NaAlg concentration and the ratio of PEO/NaAlg on the morphological properties of beads of PEO/NaAlg electrospun materials[ab]

	F1	F2	F3	F4
The structure	Many beads+fibers	Beads+fibers	Many beads+fibers	Beads+fibers
Bead area %	85.4	53.1	67.1	52.77
Number (beads/μm^2)	3.11±0.03	1.98±0.02	2.63±0.03	3.35±0.32
Aspect ratio of beads	1.20±0.04	1.76±0.53	1.39±0.03	1.48±0.04
Average length of beads (nm)	632.7±32.7	756.5±22.8	660.9±21.5	562.0±35.6
Average width of beads (nm)	552.8±28.4	451.6±21.2	491.6±21.8	388.4±27.1

[a]Values are expressed as mean± standard deviation (n=3)
[b]Containing 3%PEO and 1%NaAlg in the ratio of PEO/NaAlg of [F1: (1-1), and F2: (2-1)] and 2%SA in the ratio of [F3: (1-1), and F4: (2-1)]

In the current study, increasing the PEO:NaAlg ratio from 1:1 to 2:1 resulted in both a decrease (F1 to F2) and an increase (F3 to F4) in conductivity, while increasing the viscosity ($p<0.05$). The increase in conductivity led to the formation of more beads (increase in numbers of beads/μm^2) which had greater length, and thinner fibers in both groups independent of the increase in viscosity ($p<0.05$). It clearly proved the effect of the increase in conductivity on the number of beads, length of beads and diameter of fibers ($p<0.05$). As the electric charge density in the ejected jets increases with the increase in electrical conductivity, it causes stronger elongation forces. Since the excess charges will create a self-repunsion force, it leads to the formation of finer jets and thus finer fibers [18-20]. In previous studies, it was also stated that the low electrical conductivity of the polymer solution used resulted in thinner fibers and more beads [12,21]. In conclusion, the present study proved that the conductivity of polymer solutions is more effective than the viscosity on the properties of electrospun materials.

Increasing the NaAlg concentration from 1% to 2% also led to a reduction in bead area due to reduced conductivity (Table 3). While the decrease was statistically significant for F1-F3 ($p<0.05$), it was not statistically significant for F2-F4 ($p>0.05$). This can be explained by the fact that the decrease in conductivity between F1-F3 is greater than the decrease between F2-F4 (Table 3). NaAlg concentration and PEO:NaAlg ratio had no significant effect on fiber diameter ($p>0.05$). This proved that the increase in viscosity and decrease in conductivity were more effective on bead formation and morphology rather than the fiber diameters.

Effect of PEO concentration on the morphological properties of fibers and beads of PEO/NaAlg electrospun materials were given in Figure 2 and Table 5.

While the bead area was 53.1% for 3% PEO (F2), it decreased to 13.7% when the PEO concentration was increased to 4% (F5) and to 10.7% when increased to 5% (F6) ($p<0.05$). Similarly, the number of beads also decreased (Figure 2 and Table 5) ($p<0.05$). As a result, the bead area and number of beads were directly related to the PEO concentration. The fiber diameter was also affected by the PEO concentration, and the increase in concentration resulted in thicker fiber production. These might be brought on by the increasing both viscosity and conductivity of solutions with increasing PEO concentration (Table 3) ($p<0.05$). It is known that high electrical conductivity leads to finer fiber production with fewer beads [9,12], while high viscosity leads to thicker fiber production with fewer beads [1,13]. In F6, many fibers with few bead-on-string fibers were produced due to the highest viscosity, whereas beads were dominant at lower viscosities. Therefore, fewer beads were formed with the increase in both viscosity and conductivity ($p<0.05$), the increase in viscosity was more effective on the the fiber diameter than the increase in conductivity, and an increase in fiber diameters was observed. Moreover, the increase in PEO concentration also caused an increase in length and width of beads

($p<0.05$). As a result, it was shown that the increase in concentration and viscosity resulted in the production of fewer but larger beads.

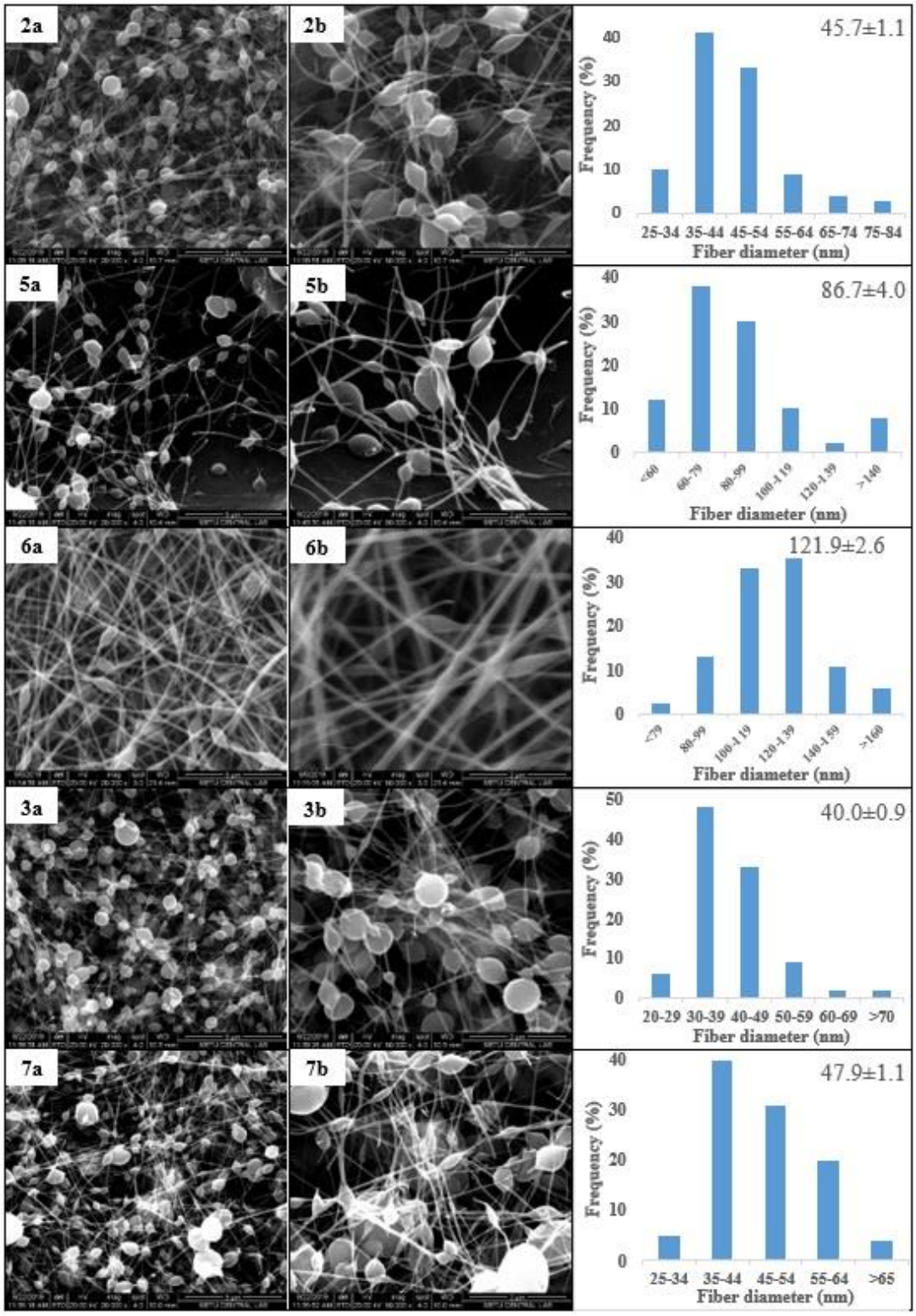

Figure 2. Effect of PEO concentration on the morphology and fiber diameter distribution of the electrospun materials (magnification = a: 20.000×, b: 50.000×)[a]

[a]F2, F5 and F6 containing 1% NaAlg in the ratio of PEO/NaAlg of 2:1 [F2: 3% PEO, F5: 4% PEO, F6: 5% PEO], F3 and F7 containing 2% NaAlg in the ratio of PEO/NaAlg of 1:1 [F3: 3% PEO, F7: 4% PEO].

Table 5. Effect of PEO concentration on the morphological properties of beads of PEO/NaAlg electrospun materials[ab]

	F2	F5	F6	F3	F7
The structure	Beads+fibers	Few beads +few fibers	Many fibers +few bead-on-string fibers	Many beads+fibers	Beads+fibers
Bead area %	53.1	13.7	10.7	67.1	45.8
Number (beads/µm^2)	1.98±0.02	0.46±0.01	0.13±0.01	2.63±0.03	1.69±0.07
Aspect ratio of beads	1.76±0.53	1.55±0.04	2.56±0.08	1.39±0.03	1.62±0.05
Average length of beads (nm)	756.5±22.8	757.3±23.7	1623.0±36.5	660.9±21.5	731.7±36.9
Average width of beads (nm)	451.6±21.2	500.9±18.2	645.0±18.3	491.6±21.8	471.4±31.4

[a]Values are expressed as mean± standard deviation (n=3)
[b]F2, F5 and F6 containing 1% NaAlg in the ratio of PEO/NaAlg of 2:1 [F2: 3% PEO, F5: 4% PEO, F6: 5% PEO], F3 and F7 containing 2% NaAlg in the ratio of PEO/NaAlg of 1:1 [F3: 3% PEO, F7: 4% PEO]

As a conclude, the study is the first to describe in detail the effects of the use of PEO and NaAlg at different concentrations and ratios on the viscosity and conductivity of the polymer solutions and hence on the advanced morphological properties of beads and fibers in electrospun materials. Viscosity and conductivity of solutions, and morphological properties of the obtained materials were found to be affected by PEO and NaAlg concentration and the ratio of them. Spinnability was improved thanks to the increase in conductivity in the presence of PEO. Morphological properties of PEO/NaAlg electrospun materials can be modified by controlling the parameters examined in the study. The lower viscosity and conductivity resulted in the production of generally smaller beads, having greater in number and in area in the electrospun material. Consequently, lower viscosity and conductivity may be preferred for bead production, while higher viscosity and conductivity are required for fiber production. However, it should be noted that sufficiently high viscosity and conductivity are required for the electrospinning process. The current study also points to the production and morphological characterization of PEO/NaAlg electrospun material, which is valuable for medical and biological applications such as tissue engineering, wound dressing, drug delivery system.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

KLİNİK *KLEBSIELLA PNEUMONIAE* VE *ACINETOBACTER BAUMANNII* İZOLATLARINDA İMİPENEM DİRENCİNİN HIZLI TESPİTİNDE AST FAST ES/NF AGAR BESİYERİNİN DEĞERLENDİRİLMESİ

EVALUATION OF AST FAST ES/NF AGAR MEDIUM FOR RAPID DETECTION OF IMIPENEM RESISTANCE IN CLINICAL KLEBSIELLA PNEUMONIAE AND ACINETOBACTER BAUMANNII ISOLATES

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ÖZ

Amaç: *Klebsiella pneumoniae ve Acinetobacter baumannii başta olmak üzere dirençli gram negatif mikroorganizmalar yaşamı tehdit eden enfeksiyonlara neden olmaktadır. Bu nedenle klinik izolatların antibiyotik duyarlılıklarının en kısa sürede ve doğru tespiti kritik öneme sahiptir. Günümüzde karbapenem direnci fenotipik ve/veya genotipik yöntemlerle belirlenmektedir. Fenotipik yöntemlerde (disk difüzyon, mikrodilüsyon) hem özgüllük hem de duyarlılık açısından farklılıklar sıklıkla ortaya çıkabilmektedir. Nispeten daha güvenilir sonuçlar veren polimeraz zincir reaksiyonu gibi moleküler metotlar ise iş gücü gerektiren ve yüksek maliyetli yöntemler olduğundan, antibiyotik direncini saptamaya yönelik hızlı, güvenilir ve ekonomik yeni yöntemlere ihtiyaç duyulmaktadır.*
Gereç ve Yöntem: *Çalışmada kromojenik AST Fast ES/NF besiyerinin EUCAST standart disk difüzyon metoduyla antibiyotik duyarlılıklarının saptanmasında kullanımının uygunluğu*

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araştırılmıştır. Karbapenem dirençli olduğu moleküler yöntem/otomatize sistemle belirlenen dört K.pneumoniae ve beş A.baumannii izolatıyla çalışılmıştır.
Sonuç ve Tartışma: *Çalışma sonucunda AST Fast ES/NF besiyerinde inhibisyon zon çaplarının 4-6 saat içerisinde ölçülebilir duruma geldiği belirlenmiş ve üretici firmanın önerilerine uygun şekilde izolatların duyarlılıkları belirlenmiştir. Mueller Hinton Agar kullanılarak gerçekleştirilen disk difüzyon test sonuçlarının, AST Fast ES/NF agar kromojenik besiyeriyle elde edilen sonuçlar ile büyük oranda uyumlu olduğu belirlenmiştir. Standart disk difüzyon test yönteminde önerilen inkübasyon süresinin 16-20 saat olduğu ve ancak bu süre sonunda duyarlılık kategorisinin belirlendiği düşünüldüğünde kromojenik besiyerinin zaman açısından önemli bir avantaj sağlayabileceği düşünülmektedir.*
Anahtar Kelimeler: *Acinetobacter baumanni, AST fast agar, disk difüzyon metodu, kromojenik besiyeri, Klebsiella pneumoniae*

ABSTRACT

Objective: *Resistant gram-negative microorganisms, especially Klebsiella pneumoniae and Acinetobacter baumannii, cause life-threatening infections. Hence, it is critical to determine the antibiotic susceptibility of clinical isolates rapidly and accurately. Nowadays, carbapenem resistance is determined by phenotypic/genotypic methods. For phenotypic methods (disk diffusion, microdilution), differences in specificity and sensitivity can often occur. Since molecular methods such as polymerase chain reaction giving relatively more reliable results, are labor-intensive and costly, new fast, reliable and economical methods are needed.*
Material and Method: *The performance of chromogenic AST Fast ES/NF agar for the EUCAST standard disk diffusion method was investigated. Four K. pneumoniae and five A. baumannii isolates, which were determined to be carbapenem resistant by molecular method and automated system results, were included in the study.*
Result and Discussion: *The inhibition zone diameters were became measurable within 4-6 hours in AST Fast ES/NF and the susceptibility categories were interpreted according to the manufacturer's recommendations. The results of disk diffusion test performed with MHA, were largely consistent with the results obtained with the AST Fast ES/NF. Considering that the incubation period recommended by EUCAST in the standard disk diffusion test method is 16-20 hours and the sensitivity category is measured at the end of this period, it is thought that the chromogenic medium may provide a significant advantage.*
Keywords: *Acinetobacter baumannii, AST fast agar, chromogenic medium, disk diffusion method, Klebsiella pneumoniae*

GİRİŞ

Enterobacterales ailesi; *Escherichia coli, Klebsiella* ve *Enterobacter* türleri başta olmak üzere insan sağlığını tehdit eden ve yüksek morbidite/mortalite oranlarıyla seyreden farklı enfeksiyonlara neden olan bakteri türlerini içermektedir [1]. Özellikle *Klebsiella pneumoniae* türü neden olduğu ciddi klinik seyirli farklı enfeksiyonlar, hastane enfeksiyonları ve yüksek antibiyotik direnç oranlarıyla öne çıkan fırsatçı patojenlerdendir [2,3]. İnsan vücudunda gastrointestinal sistem ve nazofarenkste flora elemanı olarak rastlanan *Klebsiella* türleri, üriner sistem enfeksiyonları, septisemi, pnömoni, nozokomiyal enfeksiyonlar, cerrahi ve kateter ilişkili enfeksiyonlarda sıklıkla hastalık etkeni mikroorganizma olarak izole edilmektedir [4,5]. Kapsül yapısı, sideroforların varlığı, lipopolisakkaritler ve fimbria yapısı bu türlerin önemli virulans faktörleridir [4]. Ayrıca *Klebsiella* türlerinin doğal/ kazanılmış antibiyotik direnç mekanizmaları, enfeksiyonların mevcut antibiyotiklerle etkili bir şekilde tedavisini daha da güçleştirmektedir [6].

Hastane enfeksiyonu etkeni bakteri türlerinden *Acinetobacter* türleri, yoğun bakım ünitelerinde tedavi gören hastalardan sıklıkla izole edilen ve çoklu ilaç direnci gösteren fırsatçı patojen türlerden bir diğeridir [7]. *Moraxellaceae* üyesi olan, Gram negatif, hareketsiz ve nonfermentatif *Acinetobacter baumannii* türleri, önceleri virulansı düşük bir patojen olarak düşünülmüşse de, yapılan araştırmalar sonucunda pek çok farklı virülans faktörüne ve antibiyotik direnç mekanizmasına sahip olduğu anlaşılmıştır [7,8]. *Acinetobacter* türleri, farklı risk faktörlerini bulunduran hastalarda, immünsupresif bireylerde ağır klinik tablolarla seyrebilen enfeksiyonlara yol açmaktadır [9]. Bu mikroorganizma

türünün başlıca virülans faktörleri; polisakkarit kapsül, lipit A yapısı, siderofor, polisakkarit kapsül, fimbria, sitotoksik dış membran proteini, çoğunluğu algılama sistemleri, biyofilm oluşumu ve antibiyotik direnç genleri olarak sıralanabilir [10]. Özellikle yoğun bakım ünitelerinde yatan hastalarda biyofilm ilişkili enfeksiyonlara, kateter ilişkili bakteriyemi ve ventilatör ilişkili pnomoniye neden olan *A. baumannii* türlerinde, enfeksiyonların mortalitesinin diğer türlerin etken olduğu enfeksiyonlara kıyasla dokuz kat yüksek olduğu belirtilmektedir [9]. Bu durumun en önemli nedenlerinden birisi olarak *Acinetobacter* türlerinin doğal ve kazanılmış antibiyotik direnç mekanizmaları gösterilebilir [9,10]. Günümüzde *K. pneumoniae* ve *A. baumannii* türlerinin neden olduğu enfeksiyonların tedavisinde geniş spektrumlu antibiyotikler başta olmak üzere, farklı antibiyotiklerin tek başlarına ve/veya kombinasyonları halinde kullanılmaktadır. Tedavide yaygın olarak karbapenemler, beta-laktam/beta-laktamaz inhibitör kombinasyonları, aminoglikozitler kullanımaktadır [11].

Ancak son yıllarda çoklu ilaç direnci (ÇİD) gösteren, başka bir deyişle, en az üç farklı antibiyotik sınıfından birer ilaca direnç gösteren *K. pneumoniae* ve *A. baumannii* izolatlarının sayısında dünya çapında gözlenen artış endişe verici boyutlara ulaşmıştır. Dünya Sağlık Örgütü tarafından 2020 yılında yayınlanan antimikrobiyal direnç sürveyans raporunda, ülkemizde farklı sağlık kuruluşlarından hastalardan izole *K. pneumoniae* ve *A. baumannii* izolatlarında çoklu ilaç direncinin sırasıyla %40 ve %80 oranlarında olduğu belirtilmiştir (Tablo 1) [12].

Tablo 1. Kan ve BOS örneklerinden izole edilen *Klebsiella pneumoniae* ve *Acinetobacter* spp. izolatlarında antibiyotik direnç oranları (2019, Türkiye)

	Klebsiella pneumoniae			***Acinebacter spp.***	
Antibiyotik	**n**	**% R**	**Antibiyotik**	**n**	**% R**
Piperasilin-tazobaktam	3565	60	Piperasilin-tazobaktam	-	-
Seftazidim	3742	70	Seftazidim	-	-
Amikasin	3760	27	Amikasin	2179	70
Gentamisin/tobramisin	3925	45	Gentamisin/tobramisin	2404	80
Siprofloksasin/Levofloksasin/ Ofloksasin	3933	65	Siprofloksasin/Levofloksasin/ Ofloksasin	2391	91
İmipenem/meropenem	4028	39	İmipenem/meropenem	2390	90
Çoklu İlaç Dirençli*	3689	40	Çoklu İlaç Dirençli**	2362	80

n: İzolat sayısı, %R: Dirençli izolatların yüzde oranı

* *Klebsiella pneumoniae* için çoklu ilaç direnci, üç antimikrobiyal grubun en az bir temsilcisine karşı kombine direnç olarak tanımlanır: florokinolonlar (siprofloksasin, levofloksasin ve/veya ofloksasin), üçüncü kuşak sefalosporinler (sefotaksim, seftriakson ve/veya seftazidim) ve aminoglikozitler (gentamisin ve/ veya tobramisin) ****Acinetobacter** spp. için çoklu ilaç direnci, üç antimikrobiyal grubun en az bir temsilcisine karşı kombine direnç olarak tanımlanır: florokinolonlar (siprofloksasin ve/veya levofloksasin), aminoglikozitler (gentamisin ve/veya tobramisin) ve karbapenemler (imipenem ve/veya meropenem). Bir veya daha fazla grupta eksik veri bulunan izolatlar, çoklu ilaç direnci analizinin dışında tutulur.

Tedavi seçeneklerini oldukça kısıtlayan bu durum karşısında etkili bir tedavi için klinisyenler amprik tedavi ve kombinasyon terapilerine başvurmaktadır [13]. *K. pneumoniae* ve *A. baumannii* türlerinde en sık görülen antibiyotik direnç mekanizmalarının başında betalaktamaz enzimlerin varlığı gelmektedir [14]. Karbapenemler, bakteri hücre duvar sentezini bozarak etki gösterir ve geniş etki spektrumuna sahip bakterisidal ajanlardır. Ancak farklı bakteri türleri çeşitli betalaktamaz enzimleriyle karbapenemlere direnç geliştirebilmektedir. DSÖ'nün sürveyans raporları incelendiğinde Gram negatif bakterilerde karbapenem direncinin yüksek seviyelere ulaştığı açıkça görülmektedir. Ülkemizde 2019 yılında izole edilen *Acinetobacter* izolatlarından %90'ının, *K. pneumoniae* izolatlarının ise %39'unun karbapenem dirençli olduğu belirtilmiştir (Tablo 1) [12].

Dirençli mikroorganizmaların sıklıkla menenjit, septisemi bakteriyemi gibi ciddi enfeksiyonlara yol açtığından, klinik izolatların antibiyotik duyarlılıklarının mümkün olan en kısa sürede ve doğru şekilde tespiti kritik öneme sahiptir. Bu sayede tedaviye daha doğru antibiyotik seçimi ile daha kısa sürede başlanması, morbidite/mortalite oranlarının ve ekonomik kayıpların azaltılması mümkün

olabilecektir [15,16]. Günümüzde gelişen teknolojinin yardımıyla karbapenemaz tespiti için kullanılan çok çeşitli biyokimyasal ve moleküler tabanlı yöntemler bulunmaktadır. Karbapenem direncinin belirlenmesi genellikle fenotipik (disk difüzyon, mikrodilüsyon) /genotipik yöntemler (Polimeraz zincir reaksiyonu) kullanılarak yapılmakta, sonuçlar European Committee on Antimicrobial Susceptibility Testing (EUCAST) ve Clinical and Laboratory Standards Institute (CLSI) gibi uluslararası kılavuzlara göre değerlendirilmektedir. Özellikle fenotipik yöntemler arasında hem özgüllük hem de duyarlılık açısından önemli farklılıklar olabilmektedir. Nispeten daha güvenilir sonuçlar veren moleküler metotlar ise zaman alıcı ve yüksek maliyetli olduğundan, antibiyotik direncini saptamaya yönelik daha hızlı, güvenilir ve ekonomik yöntemlere ihtiyaç duyulmaktadır [15-17]. Bu çalışmada bakterilerin metabolik faaliyetlerine bağlı olarak birkaç saat içinde renk değiştiren kromojenik bir besiyeri AST Fast ES/NF agar (Diagnotis, Türkiye) besiyeri ve Mueller Hinton agar kullanılarak disk difüzyon yöntemiyle, klinik *K. pneumoniae* ve *A. baumannii* izolatlarının imipenem duyarlılığı araştırılmıştır.

GEREÇ VE YÖNTEM

Çalışmaya Ege Üniversitesi Tıp Fakültesi Tıbbi Mikrobiyoloji Anabilim Dalı Bakteriyoloji Laboratuvarı'nda izole edilen *Klebsiella pneumoniae* (n=4) ve *Acinetobacter baumannii* (n=5) izolatı dahil edilmiştir. *K. pneumoniae* izolatları, polimeraz zincir reaksiyonu (PCR) ile blaOXA-48, blaVIM, blaNDM ve blaKPC genlerini taşıdığı bilinen izolatlardan seçilmiştir. *A. baumannii* izolatları otomatize sistemle karbapenem dirençli olduğu bildirilen ve farklı klonlardan olduğu PCR ile saptanmış izolatlardır. İzolatların imipenem duyarlılıkları disk difüzyon test yöntemi ile belirlenmiş olup bu yöntemde Mueller Hinton Agar (MHA, Merck, Germany), AST Fast ES Agar (Diagnotis, Türkiye) (*K. pneumoniae* için) ve Ast Fast NF Agar (*A. baumannii* için) kullanılmıştır. Antibiyotik duyarlılık profillerini belirlemek için disk difüzyon metodu uygulanarak, MHA üzerinde oluşan zon çapları ve izolatların duyarlılık kategorileri EUCAST kriterleri doğrultusunda yorumlanmıştır [18]. AST Fast ES/NF agar besiyerinde oluşan zon çapları üretici firma tarafından belirtilen sınır değerlere uygun şekilde değerlendirilmişti. Çalışmada antibiyotik diski olarak imipenem (IMP, 10 µg) (Bioanalyse, Türkiye), kontrol suşu olarak *Escherichia coli* ATCC 25922 ve *Pseudomonas aeruginosa* ATCC 27853 kullanılmıştır.

Disk difüzyon yöntemiyle imipenem duyarlılıklarının belirlenmesi için, MHA besiyerine ekilen bakteri izolatları bir gece boyunca 37°C'de inkübasyona bırakılmıştır. Oluşan taze kolonilerden, steril bir öze yardımıyla, serum fizyolojik çözeltisi içerisinde 0.5 McFarland bulanıklık standardına uygun inokulum süspansiyonları hazırlanmıştır. İnokulasyon öncesinde oda sıcaklığında olması sağlanan agar plaklara, hazırlanan bakteri süspansiyonlarından 15 dakika içerisinde steril eküvyon yardımıyla ekim yapılmıştır. Ardından imipenem içeren diskler agar plak üzerinde merkezi konuma steril bir pens yardımıyla yerleştirilmiştir. Diskler yerleştirildikten sonra plaklar ters çevrilerek etüvde 35°C'de 16-20 saat inkübasyona bırakılmıştır. İnkübasyon sonunda MHA besiyeri üzerinde oluşan inhibisyon zon çapları (mm), agar plak koyu renkte bir zemin üzerine konularak cetvel yardımıyla ölçülmüştür. Ölçülen zon çapları EUCAST tarafından belirtilen sınır değerlerle kıyaslanarak izolatın duyarlılık kategorisi belirlenmiştir. EUCAST tarafından disk difüzyon yönteminde imipenem sınır değerleri *K. pneumoniae* ve *A. baumannii* için aşağıda belirtilen şekildedir. *K. pneumoniae* için imipenem zon çapı sınır değerleri EUCAST rehberinde ' ≥ 22 mm duyarlı (S), < 19 mm dirençli (R)' olarak belirtilirken, *A. baumannii* için ' ≥24 mm duyarlı (S), < 21 mm dirençli (R)' olarak belirtilmektedir [18].

Çalışmada, EUCAST tarafından önerilen MHA besiyeri dışında, kromojenik bir besiyeri olan AST Fast Agar ES ve AST Fast Agar NF besiyerleri kullanılarak disk difüzyon yöntemiyle izolatların imipenem duyarlılıkları araştırılmıştır. Bu test besiyerine bakteri izolatlarının inokulasyonu, inkubasyonu ve sonuçların değerlendirilmesi üretici firmanın önerileri doğrultusunda gerçekleştirilmiştir. Bu amaçla ekimi yapılacak besiyerleri oda sıcaklığına getirilip 0.5 McFarland bulanıklık standardında bakteri süspansiyonları hazırlanmıştır. Hazırlanan inokulum süspansiyonları steril eküvyon yardımıyla AST Fast Agar ES/NF besiyeri üzerine yayılarak inokulasyon tamamlanmıştır. Bunu takiben her bir agar plakta merkezi konuma bir adet IMP diski yerleştirilmiştir. Plaklar 35-37°C'ye ayarlanmış etüvde inkübasyona bırakılmıştır. İnkübasyonun 4. ve 6. saatinde plaklarda oluşan inhibisyon zonları ölçülerek üretici firma tarafından belirtilen değerlendirme

standartlarına göre yorumlanmıştır. Üretici firma tarafından AST Fast Agar ES besiyeri disk difüzyon testinde imipenem inhibisyon zonu değerlendirme standartları, *K. pneumoniae* için ≤ 13 mm için dirençli, > 13 ve < 15 için sınırda duyarlı, ≥ 15 için duyarlı olarak belirtilmiştir. AST Fast Agar NF besiyeri imipenem inhibisyon zonu değerlendirme standartları, *A. baumannii* için ≤ 20 mm için dirençli, > 20 mm ve < 23 mm için sınırda duyarlı, ≥ 23 için duyarlı olarak belirtilmiştir**.**

SONUÇ VE TARTIŞMA

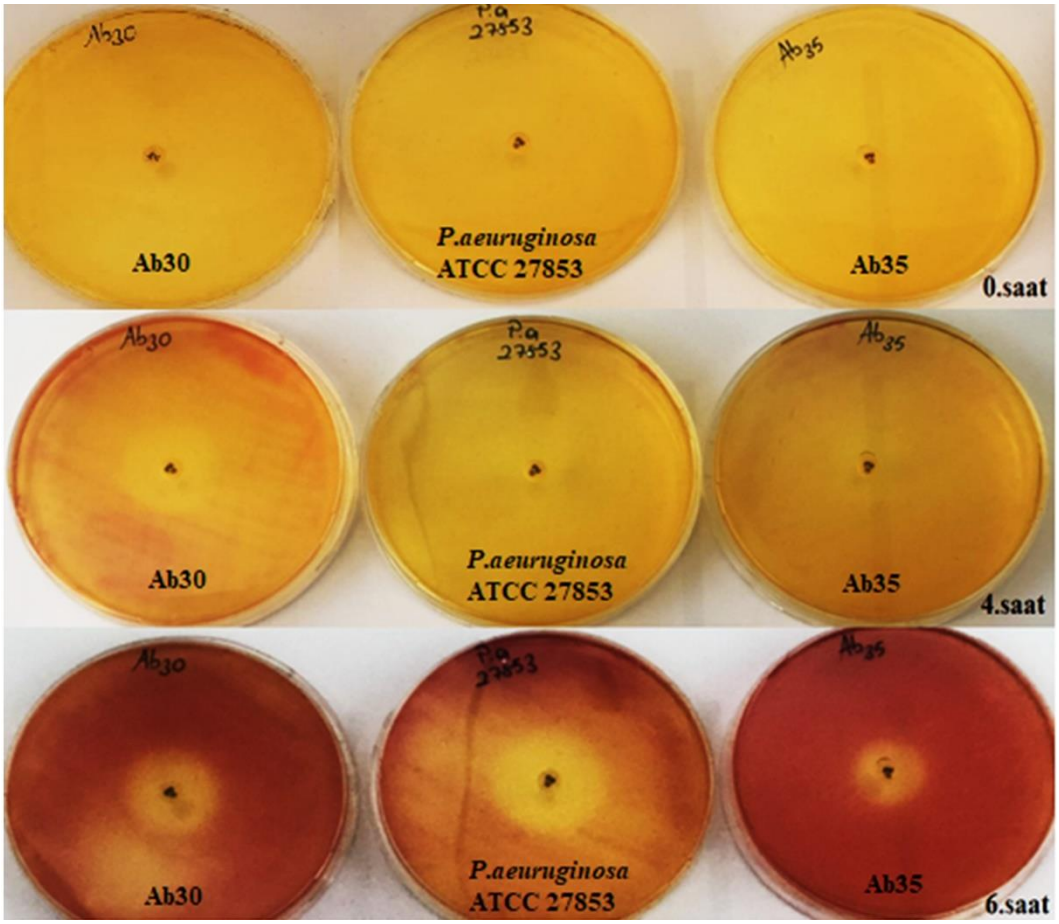
Çalışmamızda standart disk difüzyon testinde *K. pneumoniae* ve *A. baumannii* izolatlarının imipenem zon çaplarının 18-20 mm arasında değiştiği ve EUCAST kriterlerine göre dokuz izolattan ikisinin imipenem orta duyarlı, yedi izolatın ise imipenem dirençli olduğu belirlenmiştir. Aynı izolatlarla disk difüzyon testi AST Fast Agar kromojenik besiyeri kullanılarak yapıldığında imipenem inhibisyon çaplarının inkübasyonun 4. saatinde 11-24 mm arasında, 6. saatinde ise 15-24 mm aralığında değiştiği saptanmıştır. Tüm izolatlar için her iki besiyerinde ölçülen zon çapı değerleri ve yorumlama kriterlerine uygun şekilde belirlenen duyarlılık kategorileri Tablo 2'de özetlenmiştir. AST Fast Agar NF ile yapılan disk difüzyon testinde ölçüm yapılan 4. ve 6. saatlerde iki *A. baumannii* izolatına ait agar plakların görüntüsü Şekil 1'de yer almaktadır.

Tablo 2. *K. pneumoniae* ve *A. baumannii* izolatlarının imipenem duyarlılıkları ve zon çapları

	AST Fast ES/NF Agar				**MHA**	
	IMP zon Çapı (mm)				**IMP zon Çapı (mm)**	
İzolat	4.saat	Duyarlılık Kategorisi	6.saat	Duyarlılık Kategorisi	20.Saat	Duyarlılık Kategorisi
Kp14	12	R	18	R	19	I*
Kp35	11	R	24	S	20	I*
Kp111	10	R	19	S	11	R
Kp114	12	R	18	R	12	R
Ab30	24	S	20	R	20	R
Ab35	22	I*	19	R	19	R
Ab50	20	R	15	R	19	R
Ab55	20	R	15	R	18	R
Ab89	21	I*	20	R	19	R

IMP: İmipenem, MHA: Mueller Hinton Agar, Kp: *Klebsiella pneumoniae,* Ab: *Acinetobacter baumannii*,
S: Duyarlı (Susceptible), I: Orta duyarlı (Susceptible, increased exposure), R: Dirençli (Resistant)
EUCAST tarafından belirtilen imipenem duyarlılık/dirençlilik sınır değerleri:
K. pneumoniae için S ≥ 22, R < 19, 19 ≤ I < 22, *A. baumannii* için S ≥ 24 R < 21, 21 ≤ I < 24
Üretici tarafından AST Fast ES/NF Agar için belirtilen imipenem duyarlılık/dirençlilik sınır değerleri:
K. pneumoniae için S ≥ 15, R ≤ 13, 13 < I < 25, *A. baumannii* için S ≥ 23 R ≤ 20, 20 < I < 23
*: Sonuçlar yorumlanırken orta duyarlı olarak saptanan izolatlar (I) dirençli olarak değerlendirilmiştir.

Çalışmamızda kromojenik test besiyerinde yapılan disk difüzyon test sonuçlarının, EUCAST standartlarına uygun şekilde MHA kullanılarak yapılan test sonuçlarıyla büyük oranda uyumlu olduğu gözlenmiştir. Test edilen kromojenik besiyerinin fermantatif ve non fermantatif mikroorganizma türleri için iki formu bulunmaktadır. Çalışmamızda test besiyerinin nonfermentatif mikroorganizmalar (*Pseudomonaceae* ve *Acinetobacter*) için üretilen formu olan AST Fast Agar NF besiyeri beş farklı klondan *A. baumannii* izolatlarının imipenem duyarlılıklarını belirlemede kullanılmıştır. Şekil 1'de görüldüğü üzere inkübasyonun ilerleyen saatlerinde başlangıçta sarı olan test besiyerinin rengi üreyen bakterilerin metabolik aktiviteleri sonucunda turuncu-kırmızı renge dönmektedir. Kromojenik besiyerinde belirgin renk değişimlerinin 3. saat itibariyle başladığı ve söz konusu zon çaplarının 3.-6. saat aralığında ölçülebilir hale geldiği gözlenmiştir. *A. baumannii* izolatları için 6.saatte ölçülen inhibisyon zon çaplarının EUCAST standart disk difüzyon metoduyla tam uyumlu olduğu saptanmıştır.



Şekil 1. Ab ve Ab35 numaralı izolatlarının ve oluşan zon çaplarının AST Fast NF agar besiyerinde çaplarının farklı zaman aralıklarında görünümü

Çalışmamızda test besiyerinin fermentatif mikroorganizma türleri için olan formu AST Fast Agar ES, farklı betalaktamazlar taşıdığı polimeraz zincir reaksiyonu ile önceden saptanmış *Klebsiella pneumoniae* izolatlarının imipenem duyarlılıklarının belirlemede kullanılmıştır. İnkübasyonun ilerleyen saatlerinde (2.5-3.saat) AST Fast Agar ES besiyerinin başlangıçta kırmızı olan renginin bakteri üremesine bağlı olarak sarıya döndüğü gözlenmiştir. Bu besiyerinde inkübasyonun 4. Saati sonunda ölçülen inhibisyon zon çapları ile belirlenen duyarlılık profillerinin, MHA kullanılarak yapılan standart disk difüzyon metodu sonuçlarıyla benzer olduğu saptanmıştır. Standart disk difüzyon test yönteminde önerilen inkübasyon süresinin 16-20 saat olduğu ve ancak bu süre sonunda MHA üzerinde zon çapının değerlendirilerek duyarlılık kategorisinin belirlendiği düşünüldüğünde kromojenik besiyerinin zaman açısından önemli bir avantaj sağlayabileceği düşünülmektedir.

Literatürde yer alan ve kolorimetrik besiyeri kullanılan bir başka çalışmada Çoban ve ark. *Staphylococcus aureus* izolatlarında metisilin direncinin hızlı tespiti için Quicolor (QC) ES agar besiyerinin kullanımını değerlendirmiştir. Çalışmada metisilin duyarlı *S. aureus* izolatlarında oksasilin ve sefoksitin sonuçlarının QC ES'de 4-7 saat (ortalama 5,5 saat) içinde alınırken, metisilin dirençli *S. aureus* izolatlarında her iki antibiyotik için 5,5-9 saat (ortalama 6,6 saat) içinde sonuç elde edilebildiğini ve sonuçların MHA besiyerinde yapılan disk difüzyon test sonuçlarıyla uyumlu olduğu bildirilmiştir [19]. Ercis ve ark. tarafından CLSI standartları izlenerek fenotipik doğrulama testi ile 50'si GSBL pozitif ve 50'si negatif olarak önceden belirlenmiş, *Enterobacterales* üyesi 100 izolat ile yapılan çalışmada disk difüzyon ve E-test kullanılarak GSBL'nin saptanmasında MHA besiyeri ile QC agar kullanımı karşılaştırılmıştır. Çalışmamızın sonuçlarına paralel şekilde, Ercis ve ark. tarafından tüm suşlar için QC agar ile 4-6 saat içinde elde edilen GSBL sonuçlarının standart besiyeri sonuçları ile uyumlu olduğu bildirilmiştir. Aynı çalışmada E-test yöntemi ile GSBL pozitif sekiz suşun daha saptandığı belirtilmiştir

[20]. Kolorimetrik besiyerleriyle yapılan az sayıda çalışmada elde edilen sonuçlarla çalışmamızın sonuçları paralel olmakla birlikte antibiyotik direncinin hızlı tespiti açısından umut vadetmektedir. Ancak kolorimetrik besiyerinin kullanıldığı antibiyotik duyarlılık test yöntemin bazı kısıtlıkları da bulunmaktadır. Kolorimetrik besiyerinde inhibisyon zonlarının tespiti, agar üzerinde üreyen bakteri türlerinin metabolik aktivitesi sonucu besiyerinde oluşan renk değişimi esasına dayanmaktadır. *Staphylococcus epidermidis* gibi yavaş üreyen bakteri türlerinde testin sonuçlanması daha uzun sürebilmektedir. Ayrıca test besiyeri üzerinde oluşan renkli inhibisyon zon çapları nın oluştukları zaman aralığında (4-6 saat) ölçülerek kaydedilmelidir, bu süre içerisinde ölçüm yapılmadığında renkli zonlar kaybolabilmekte ve bir gecelik inkübasyonun sonuna kadar beklenmesi gerekmektedir.

Sonuç olarak çoklu ilaç direnci gösteren izolatların hızlı ve doğru tespiti; hastanın en doğru tedaviye ulaşabilmesi, hastanede kalış süresinin azalması, sağlık bakım giderlerinin azaltılması ve antimikrobiyal dirençle mücadele açısından önemli bir gerekliliktir. Günümüzde gelişen teknolojinin de yardımıyla antibiyotik direncinin saptanmasında kullanılan farklı otomatize sistemler ve moleküler yöntemler mevcutsa da bu yöntemlerin oldukça maliyetli olduğu bilinmektedir. Bu noktada daha az maliyetli disk difüzyon gibi standart yöntemler yeniden gündeme gelmekle birlikte bu klasik yöntemlerle daha hızlı sonuç alınmasını sağlayacak modifikasyonlar ve araştırmalar değer kazanmaktadır. Bu kapsamda disk difüzyon yönteminde kolorimetrik AST Fast Agar ve benzeri yeni besiyerlerinin kullanım potansiyeli ve performansının değerlendirileceği daha fazla sayıda araştırmanın alana önemli katkı sunacağı düşünülmektedir.

YAZAR KATKILARI

Kavram: A.T., Y.T.; Tasarım: A.T., Y.T.; Denetim: A.T., Y.T.; Kaynaklar: A.T., Y.T.; Malzemeler: A.T., Y.T., M.H.L., F.Ç.; Veri Toplama ve/veya İşleme: A.T., Y.T., M.H.L., F.Ç.; Analiz ve/veya Yorumlama: A.T., Y.T., M.H.L., F.Ç.; Literatür Taraması: A.T., Y.T.; Makalenin Yazılması: A.T., Y.T.; Kritik İnceleme: A.T., Y.T., M.H.L., F.Ç.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

NEPHROPROTECTIVE EFFECT OF *ALOE VERA* EXTRACT WITH REGULATION OF OXIDATIVE STRESS, APOPTOSIS AND AQUAPORIN 3 EXPRESSION LEVELS IN STREPTOZOTOCIN INDUCED DIABETIC RATS

ALOE VERA EKSTRAKTININ STREPTOZOTOSİN İNDÜKLÜ DİYABETIK RATLARDA OKSİDATİF STRESS, APOPTOZİS VE AQUAPORİN 3 EKSPRESYON DÜZEYİNİ DÜZENLEYEREK NEFROPROTEKTİF ETKİSİ

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ABSTRACT

Objective: *In this study we examined the protective activity of Aloe vera with considering anti-oxidant, anti-apoptotic properties, and the status of Aquaporin 3 (AQP3) channel protein.*
Material and Method: *Twenty-one adult female rats were divided into three groups (n=7); Control, Diabetes, Treatment. Control group did not expose to any application. Animals in Diabetes and Treatment were exposed to experimental diabetes with administration of streptozotocin. Rats in Treatment received 300 mg/kg Aloe vera extract daily for 14 days. Rats were sacrificed and kidney samples were used for analyses.*
Result and Discussion: *Analyses indicated that lowest malondialdehyde (MDA) and luminol levels in control group were increased significantly (P<0.05) in diabetic animals. Severe pathological changes observed in Diabetes group while microscopic examinations. Bax, Caspase-3 and apoptotic index (AI) were elevated significantly (P<0.05) in this group compared to Control. Oxidative stress, apoptotic protein expression levels and TUNEL Assay positive cell ratio were down-regulated in Treatment group. When AQP3 levels were measured, immunopositivity reduced significantly (P<0.05) in cortical kidney of Diabetes group which is normalized significantly in Treatment group.This study reporting anti-diabetic potency of Aloe vera extract has capability to avoid streptozotocin induced diabetic renal injury via regulating anti-apoptotic and anti-oxidant cellular signaling. Furthermore, Aloe vera consumption in diabetes might regulate AQP3 levels. Although we observed promising results, more studies are required to explore anti-diabetic, anti-hyperglycemic and nephroprotective activity of Aloe vera.*

Keywords: *Aloe vera, apoptosis, aquaporin 3, diabetes, kidney*

ÖZ

Amaç: *Bu çalışmada Aloe vera'nın koruyucu aktivitesini antioksidan, anti-apoptotik özellikleri ve Aquaporin 3 (AQP3) kanal proteininin durumu dikkate alınarak inceledik.*
Gereç ve Yöntem: *Yirmi bir yetişkin dişi sıçan Kontrol, Diyabet, Tedavi olmak üzere üç gruba (n=7) ayrıldı. Kontrol grubu herhangi bir uygulamaya maruz bırakılmadı. Diyabet ve Tedavideki hayvanlar, streptozotosin uygulanarak deneysel diyabete maruz bırakıldı. Tedavideki sıçanlara 14 gün boyunca günde 300 mg/kg Aloe vera özütü verildi. Sıçanlar sakrifiye edildi ve analizler için böbrek örnekleri kullanıldı.*
Sonuç ve Tartışma: *Analizler control grubundaki en düşük malondialdehit (MDA) ve luminal düzeylerinin diyabetik hayvanlarda anlamlı olarak arttığını (P<0.05) gösterdi. Diyabet grubunda mikroskobik incelemelerde ciddi patolojik değişiklikler gözlendi. Bax, Kaspaz-3 ve apoptotic indeks (AI) bu grupta Kontrol'e gore anlamlı olarak yüksek bulundu (P<0.05). Tedavi grubunda oksidatifstres, apoptotik protein ekspresyon seviyeleri ve TUNEL Assay pozitif hücre oranı aşağı regüle oldui. AQP3 düzeyleri ölçüldüğünde, Tedavi grubund aanlamlı olarak normalize edilen immünopozitiflik, Diyabet grubunun kortikal böbreğinde önemli ölçüde azaldı (P<0.05). Bu, Aloe veraekstraktının anti-diyabetik gücünü bildiren, anti-apoptotik ve antioksidan hücresel sinyallemeyi düzenleyerek streptozotosin kaynaklı diyabetik böbrek hasarını önleme yeteneğine sahip olduğunu göstermektedir. Ayrıca, diyabette Aloe vera tüketimi AQP3 seviyelerini düzenleyebilir umut verici sonuçlar gözlememize rağmen, Aloe vera'nın anti-diyabetik, anti-hiperglisemik ve nefroprotektif aktivitesini keşfetmek için daha fazla çalışmaya ihtiyaç vardır.*

Anahtar Kelimeler: *Aloe vera, apoptoz, aquaporin 3, böbrek, diyabet*

INTRODUCTION

Diabetes mellitus (DM) is one of the mostly encountered metabolic disorders in individuals of undeveloped and developing countries. DM affects kidneys, retina, cardiovascular system and neural system structure due to prolonged hyperglycemia [1]. In 2019 it was reported that the globally prevalence of DM will increase to 578 million people until 2030 and 700 million people until 2045 [2]. DM is divided into subgroups of insulin dependent Type 1 (T1DM) and Type 2 (T2DM) diabetes mellitus which is a result of insulin resistance [3,4]. T1DM results with diabetic nephropathy and renal dysfunction. Underlying mechanism of diabetic nephropathy is linked with increased oxidative stress due to over accumulation of reactive oxygen species (ROS) in kidney [5]. Degenerated renal corpuscles and tubular system dysfunction are the other observed pathologic problems in diabetic nephropathy

which are appear due to ROS and hyperlipidemia [6]. Although insulin therapy is still a reliable modality to control glycaemia to reduce disease complications, therapy protocol may change among the patients due to lifestyle and/or severity of the disease [7]. In literature some findings reported that over and/or long term insulin consumption may lead to other diseases such as hypoglycemia, weight gain, lipohypertrophy, local allergic reactions [8-10]. In recent years, some studies also reported possible cancer risk in insulin therapy received patients and possible contribution of insulin treatment on progress of malign diseases such as endometrial cancer [11,12]. For these reasons, numerous researchers investigated natural compounds and plant-based traditional supplements to control diabetic hyperglycemia and reduce insulin dependence of the DM patients [13-15]. Phytomedical compounds are used in treatment or control of various diseases [16]. Phenolic compound containing plant based control of hyperglycemia aims to increase deposition of blood sugar and targets antioxidant activity to protect organs which are more tendency to be affected in diabetic complications [17]. *Aloe vera* can be considered as one of these phenolic compounds containing medical plants. *Aloe vera* is a succulent that used in cosmetics, traditional medicine and drug industry. Due to containing large amount of bioactive compounds and antioxidant substances, protective activity of *Aloe vera* on various diseases examined. Phenolic compounds such as catechin, genistic acid, quercetin and much more idicates a large antioxidant spectrum of this plant [18]. Some published studies reported phenolic compounds in *Aloe vera* have capability to chelate metal ions thus down-regulate oxidation mechanisms [19]. Furthermore, hypoglycemic and hypolipidemic activities of *Aloe vera* is believed this plant to be a novel treatment modality in diabetes mellitus. Although, numerous studies reported beneficial effects of *Aloe vera* treatment in various diseases, possible effects of *Aloe vera* on streptozotocin induced diabetic nephropathy hasn't been clearly understood yet. For that reasons we aim to investigate the possible effects of *Aloe vera* against streptozotocin induced diabetic nephropathy with considering oxidative stress, apoptosis related protein expression and DNA fragmentation ratio, and water/glycerol transporter channel protein Aquaporin 3 levels in tissue.

MATERIAL AND METHOD

Study Design

All experimental procedure of this study was performed with approval of the Local Experimental Animal Ethics Committee of a University (approval date & no: 14.12.2021 & 2020/09). Twenty-one mature female rats between 169-230 gm were obtained from Experimental Animal Unit of a University and animals were randomly divided into three groups (n=7) as follows; Control, Diabetes, Treatment. Rats were kept in standard animal cages in 22 ± 2°C and 12 h light-dark cycle. Tap water and standard pellet food provided to the animals ad libitum. Animals in Control were not exposed to any application during the experiment. Rats in Diabetes and Treatment were subjected to 40 mg/kg streptozotocin for induction of Type 1 diabetes with intraperitoneal route. Following the injection all of the animals in this study received 10% glucose containing water for 48 hours to avoid hypoglycemia. Seventy-two hours post streptozotocin injection, fasting blood glucose levels in all groups were measured from tail vein and animals with glucose level >240 considered as diabetic. Rats in Treatment group received 300 mg/kg *Aloe vera* extract daily by oral gavage. The administered dose of *Aloe vera* was chosen according to a previously published article that reported anti-hyperglycemic activity [20]. All animals were sacrificed by exsanguination after 14 days of confirmation of experimental diabetes. Left kidney samples were fixed in 10% formaldehyde for microscopic examinations, right kidney of the animals were frozen in -80°C for the measurement of biochemical analyses.

Preparation of *Aloe vera* Extract

Plant materials were received and Ethanolic extract was prepared as described previously [21]. Healthy leaves were washed with sterile water and solid gel was removed from leaves. Collected gels were mixed with equal volume of 95% ethanol. Prepared suspension filtered and the extract evaporated. Extract was administered to the animals with dissolving in water prior administration. Plant samples were also deposited in Herbarium of a University Faculty of Pharmacy (shelf number: AEF 30758).

Biochemical Analyses

Tissue samples were homogenized for measurement of tissue lipid peroxidation and chemiluminescence assay that are used to measure total MDA and ROS level in samples. Measurement of these substances was performed as described previously [22]. Lipid peroxidation was evaluated as MDA equivalents using an extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$ and results are expressed as nmol MDA/gm tissue. Total ROS were measured with chemiluminescenceassay which is able to use to detection of hydrogen peroxide, hydroxyl radical and hypochlorite radicals specifically. Measurement performed in a luminometer (Junior LB 9509, EG&G Berthold, Germany) with administration of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) to the tissue samples. Results of chemiluminescence assay are expressed in relative light units per mg (rlu/mg) of tissue. All obtained biochemical results were analyzed for determination of statistically difference among the groups.

Tissue Processing Protocol

Kidneys were fixed in 10% formaldehyde solution for 24 hours. After fixation tissue samples were washed under tap water and dehydrated through increasing alcohol series and embedded into paraffin block following cleared in xylene. Five µm thick sections were obtained with a rotary microtome and sections were stored for the staining of Hematoxylin and Eosin (HE), Periodic acid-Schiff (PAS) and immunohistochemistry.

Histopathological Staining

Tissue sections were deparaffinized in Xylene and rehydrated in decreasing alcohol series. Sections from each samples then stained with HE or PAS. HE staining was performed as described previously [23]. PAS staining performed with a ready to use staining kit (Cat no: 04-130802, Bio-optica MI, IT) and all steps were performed under the recommendations of the manufacturer's instructions. Stained samples were mounted with entellan and examined under a camera attached light microscope and micrographs were captured.

Immunohistochemistry and TUNEL Assay

Tissue sections were deparaffinized, rehydrated and washed in PBS. Samples were brought to citrate buffer (Ph: 6.0) and heated on a hot plate until reaching sub-boiling temperature. The samples then cooled into room temperature and endogenous peroxidase activity was blocked with incubating the samples for 15 minutes in 3% H2O2 that dissolved in methanol. Primary antibodies of Bax (Cat no: sc-7480, Santa Cruz Biotechnology, Dallas, Texas, USA), Caspase 3 (Cat no: sc-56053, Santa Cruz Biotechnology, Dallas, Texas, USA) and AQP3 (Cat no: sc- sc-20811, Santa Cruz Biotechnology, Dallas, Texas, USA) were diluted 1:100, 1:100 and 1:200 respectively in an antibody diluent prior application. Blocking step, secondary antibody and enzyme applications were performed with a ready to use kit of Large Volume Detection System, HRP (Cat no: TP-125-HL, Thermo Fisher Scientific, MA, USA) and all procedures performed according to instructions of the manufacturer. Between all steps samples were washed in two series of PBS except primary antibody application. Primary antibody incubation performed for overnight at +4°C refrigerator. Chromogenic reaction was developed with DAB chromogen. Sections were counterstained in hematoxylin, mounted with entellan and examined under light microscope. TUNEL Assay was performed with In Situ Cell Death Detection Kit, Fluorescein (Cat no: 11684795910; Roche, Basel, CH) and all steps were performed with manufacturer's directions that also described previously [24]. TUNEL Assay sections were mounted with a ready to use mounting media that contains DAPI (Cat no: sc-24941, Santa Cruz Biotechnology, Dallas, Texas, USA). Fluorescein stained sections were examined under a fluorescein attached light microscope and micrographs were captured.

Quantification of Microscopic Examinations

Immunohistochemistry samples were quantified by threshold analysis feature of Image J software (NIH) with considering 3 randomly selected cortical areas from each animal kidney section. Immunodensity was obtained with comparing DAB positive are to the total tissue section. Measured

immunodensity levels were expressed as ratio. TUNEL Assay analyses performed under fluorescence attached microscope system. For determination of Apoptotic Index (AI), randomly selected 21 renal corpuscles from each group were used and green fluorescence positive cell count in each renal corpuscle were compared with blue and only DAPI stained total glomerular cell nuclei. All obtained datasets for immunohistochemistry and TUNEL Assay were analyzed statistically.

Statistical Analysis

Statistical analyzes were performed with SPSS Statistics Version 24.0 (IBM, NY USA) software. All obtained data were analyzed statistically to determine whether the differences among the groups are significant or not. For that purpose, all obtained datasets were analyzed with the one-way analysis of variance (ANOVA). Multiple comparisons were performed with post-hoc Tukey test. All results were shown as mean ± SD and $P<0.05$ considered as significant.

RESULT AND DISCUSSION

Our analyses indicated that tissue MDA and total ROS level in the kidney of Control group was 9,46 ± 2,44 nmol/gm and 48,93 ± 2,53 rlu/mg respectively. The tissue level of MDA and total ROS were increased significantly in streptozotocin induced Diabetes group to 21,39 ± 8,37 nmol/gm and 60,67 ± 10,96 rlu/mg. Statistical analyses indicated that the difference between Control and Diabetes were significant ($P<0.05$). When we measure the tissue MDA and total ROS levels in Treatment group, it was observed that the lipid peroxidation status alleviated to 12,29 ± 3,74 nmol/gm and 54,53 ± 8,12 rlu/mg. The statistical evaluation indicated that MDA and ROS levels in Treatment group was similar ($P>0.05$) to both of these groups. Graphical demonstrations of the statistical results are shown in Figure 1.

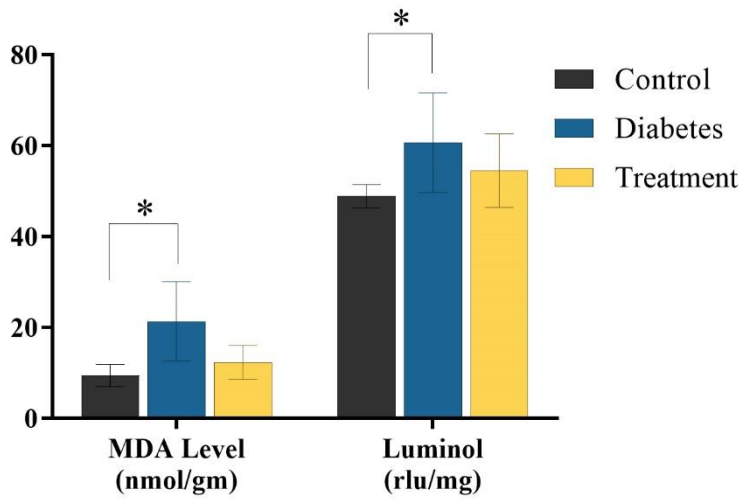

Figure 1. Tissue MDA and luminol level in the groups. The superscript between the groups indicate statistically significance (*$P<0.05$).

Morphological structure in control group was normal. Renal cortex was filled with renal glomerulus, proximal and distal tubules and upper portions of the collecting tubular system. In kidneys of Diabetes group, there were severe degenerations. Most of the glomerulus in this group was irregular. In PAS stained sections, increase in the thickness of glomerular basement membrane and depletion in microvilli structure and brush border of convoluted portion of proximal tubules were clearly visible. Vascular congestion and partially dilated vascular structure was widespread in kidney of this group. Intertubular edema was widespread. However, the mentioned kidney pathology was alleviated in *Aloe vera* exposed animals. Irregular renal glomerulus was less common. Vascular structure was normal and basement membrane thickening is not observed. Representative HE and PAS stained tissue sections are

shown in Figure 2.

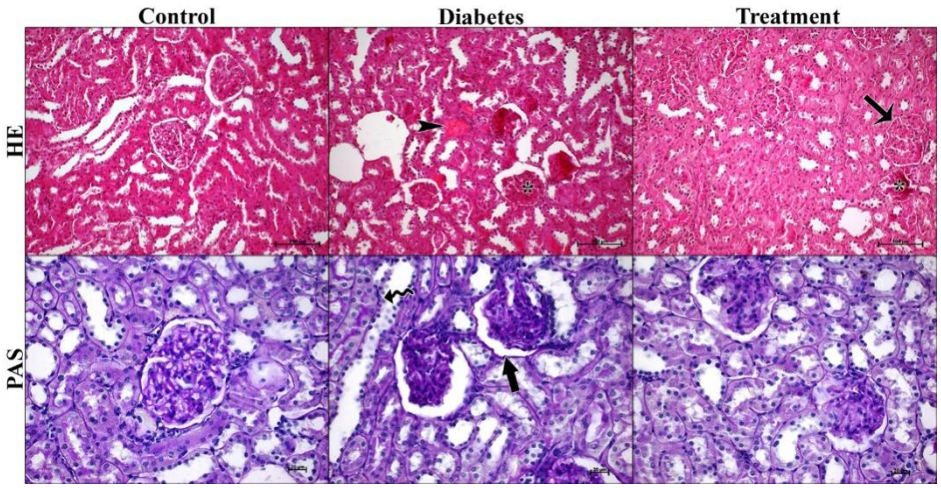

Figure 2. Light micrographs of the Control, Diabetes and Treatment groups. Irregular glomerular nephrons (*), edematous parenchyma (arrow head), decrease in filtration space (arrow), increase in thickness of glomerular basement membrane (thick arrow), depletion of brush border in proximal convoluted tubules (curved arrow). Staining: H&E, PAS, Bar: 200 µm in H&E and 20 µm in PAS sections.

Representative micrographs of the immunohistochemistry stainings are shown in Figure 3. The Baximmunodensity was lowest in Control group and it increased significantly in Diabetic animal kidney (P<0.05). Bax level in Treatment group alleviated, but it was stand between Control and Diabetes groups

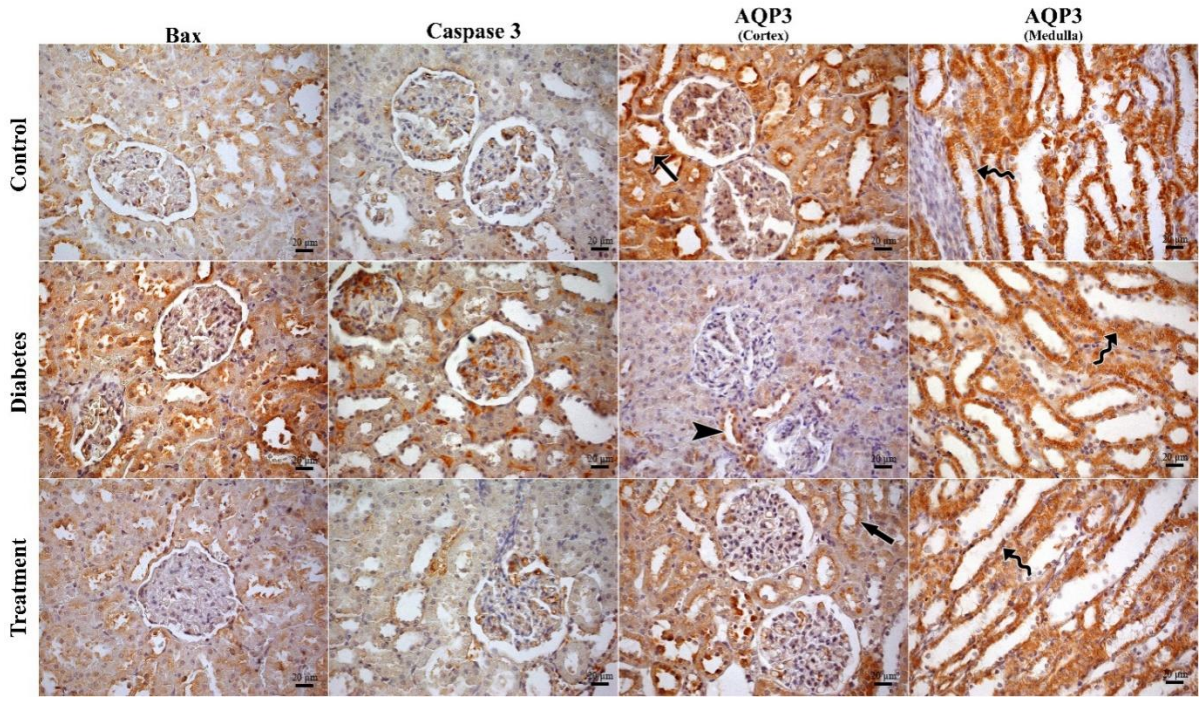

Figure 3. Representative Immunohistochemistry micrographs of Bax, Caspase 3 and Aquaporin 3 in groups. Varying AQP3 immunopositivity in proximal tubules of control (arrow), diabetes (arrow head) and treatment (thick arrow) groups, intense AQP3 immunopositivity in medullary collecting tubules (curved arrow). Bar: 20 µm.

(P>0.05). Caspase 3 immunodensity was dramatically (P<0.01) increased in diabetic animals compared to the rats in Control group. On the other hand, Caspase 3 immunodensity in Treatment group was significantly alleviated and it was similar (P>0.05) to the rest of the groups. Cortical AQP3 immunopositivity in Control group was the highest and it down-regulated in Diabetic rat kidney significantly (P<0.01). In *Aloe vera* treated animals, AQP3 immunpositivity was up-regulated but density in this group was still significantly different than Diabetes (P<0.05) and Control (P<0.01) groups. When AQP3 levels were evaluated in renal medulla, there weren't any significance (P>0.05) among the groups observed.

When TUNEL Assay sections were evaluated, TUNEL positivity was very slight in Control group, but positivity observed almost all renal cell types (Figure 4). AI in Diabetes group increased dramatically in renal glomerulus cells (P<0.01). In Treatment group, AI alleviated significantly (P<0.01) compared to the Diabetes group, but mean positive cell ration in this group was still significantly different (P<0.05) than Control group. Statistical results of immunohistochemistry threshold analyses and TUNEL Assay are shown in Table 1 and Figure 5.

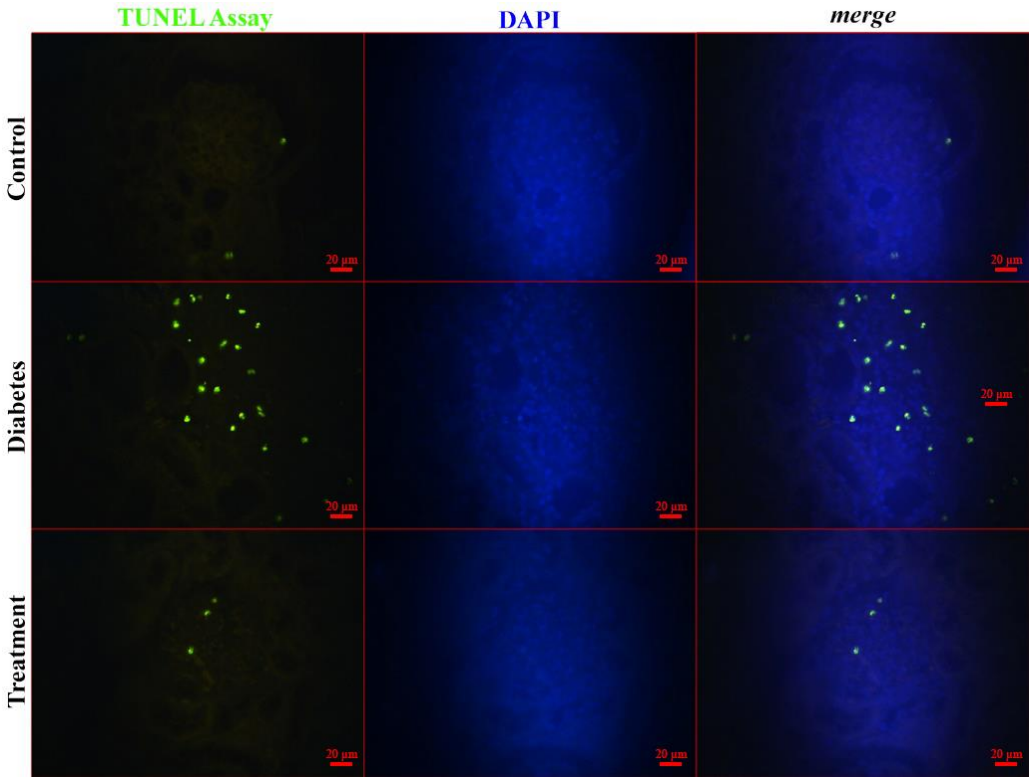

Figure 4. Representative TUNEL Assay micrographs of Control, Diabetes and Treatment groups. Green fluorescence probes indicate DNA fragmentation in kidney sections. Samples counterstained with DAPI.Bar: 20 µm.

Table 1. Statistical analyses result of immunohistochemistry and TUNEL Assay. Different superscripts among the groups indicate significantly differences between the groups ($^{a\text{-}b}P$<0.05, $^{a\text{-}c}P$<0.05, $^{b\text{-}c}P$<0.05).

	Bax	Caspase 3	AQP3 (Cortex)	AQP3 (Medulla)	AI
Control	26,68±3,63^{a}	23,48±4,31^{a}	69,00±10,94^{a}	84,29±10,92^{a}	4,22±2,36^{a}
Diabetes	30,50±5,13^{b}	32,91±8,79^{c}	49,02±12,34^{c}	80,50±9,39^{a}	10,79±3,71^{c}
Treatment	27,57±3,37^{ab}	28,24±3,93^{b}	57,64±9,22^{b}	83,87±10,31^{a}	6,40±2,27^{b}

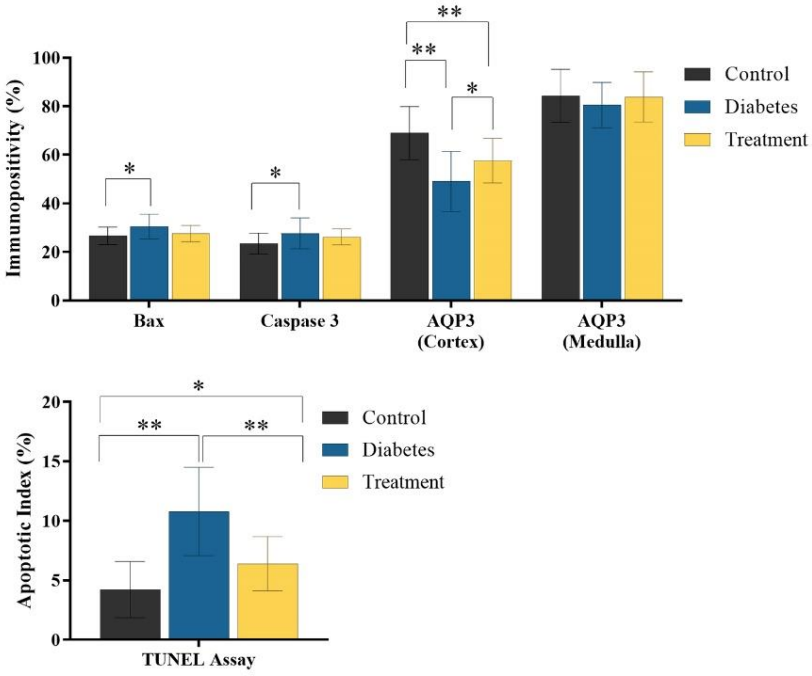

Figure 5. Graphical demonstration of immunohistochemistry and TUNEL Assay statistical analyses. Different superscripts between colums indicate significantly difference **P*<0.05, ***P*<0.01.

DM is one of the most observed metabolic disorders among the individuals. Although there are clinical applications such as insulin administration to balance blood sugar, researchers are still looking for the alternative modalities. Although many organs are affected from the hyperglycemia, the kidney are the most affected organs in DM [25]. When we review literature that is possible to reach numerous studies examined the anti-diabetic activity of herbal medicine [26]. If oxidative stress and DM evaluated as a whole, some researchers reported that DM and oxidative stress have synergistic actions over each other and each of them has ability to intensify the other one [27-30]. Although there is a limited data for the anti-diabetic activity of *Aloe vera*, in a previously published study hypoglycemic activity of Ethanolic*Aloe vera* extract investigated both in normal fasting and streptozotocin induced diabetic rats [21]. Results of this study reported that *Aloe vera* administration down-regulated blood glucose level in normal fasting animals which indicates hypoglycemic activity. Moreover, this experiment demonstrated that Ethanolic extract of *Aloe vera* increased glucose tolerance as well. Clinical experiments also reported promising results that hypoglycemic and triglyceride level regulatory activity of *Aloe vera* in T2DM patients [31]. *Aloe vera* was also found protective on pancreatic β cells besides the blood glucose and triglyceride regulatory and insulin secretion enhancing effect in streptozotocin induced diabetic rats [32]. Previously published in vitro studies also reported functional activities of *Aloe vera* in glucose metabolism. In one of these, *Aloe vera* increased Glucose transporter type 4 (GLUT4) protein gene expressions and glucose deposition [33]. In literature, most of the *Aloe vera* related experiments concluded that rich phenolic compounds and glucose depositing activity of *Aloe vera* is possible underlying hypoglycemic activity of this phenolic plant. Although kidneys are one of the most affected organs in diabetes, literature on effects of *Aloe vera* against diabetic nephropathy is limited [34]. In one of the previously published article, *Aloe vera* extract alleviated ROS level and oxidative stress in streptozotocin induced diabetic kidney [35]. Results of this study also reported diabetic nephropathy related microscopic degenerations were attenuated in treated animals. Results of this study and our current experiment are consistent. We observed severe degenerations in streptozotocin induced diabetic kidney and microscopic degenerations were reduced significantly. We also reported that *Aloe vera* oral supplementation protected kidney tissue with exerting an influence on apoptotic protein expression

levels. For that reason it's possible to say that *Aloe vera* supplementation not only has potency to down-regulate diabetes related metabolic complications with regulating blood glucose level as reported previously [21] but also affecting pro-apoptotic Bax and Caspase 3 expression levels. In another in vitro study it was reported that DNA fragmentations are increased in streptozotocin induced diabetic kidney tubular cells [36]. Furthermore, authors of this study concluded that inhibition of ferroptosis might be a potent therapeutic strategty to downregulate apoptotic cellular death in diabetic nephropathy. AI has tendency to increase dramatically in tubular system and glomerular structure within a short time as reported in two published studies [37,38]. In one of these of these studies, Sohn et al. also reported that administration of Aster koraiensis extract reduced renal AI in experimental diabetic animals. These results are consistent with our experiment if we consider relationship between AI and streptozotocin induced diabetic nephropathy. We also observed a significant decrease in AI of renal structure of *Aloe vera* treated diabetic animals.

When literature is reviewed it's possible to reach that water/glycerol transporter channel protein levels are affected in streptozotocin induced diabetic kidney [39]. Moreover, AQ3 is not only acting as glycerol and hydrogen peroxide transport channel protein, but also contributes cellular proliferation and apoptosis through regulating various cellular signaling [40].In another detail, some previously published studies and textbooks highlight that AQP3 is to be expressed in cortical basolateral membrane of collecting ducts and medullary collecting ductal systems [41,42]. However, probably as a result of upgraded sensitivity of immunodetection techniques and gene analyses, recently published studies are reporting some conflicting results that AQP3 is not only expressed in cortical collecting ducts but also proximal tubules [43, 44]. In one of these studies, Leung et al. reported down-regulated AQP3 level in streptozotocin induced diabetic kidney. However, in another study, Nejsum et al. reported streptozotocin induced diabetes up-regulated AQP3 level as a response to polyuria [45]. It is possible to reach the localization and expression levels of AQP3 in diabetic nephropathy is not defined clearly. When we compare results of our current experiment with literature, it's possible to reach partially conflicting and consistent results. First of all, we observed AQP3 is expressed in proximal convoluted cortical tubules besides the cortical collecting duct. Furthermore, results of our experiment indicated that AQP3 expression is down-regulated at renal cortex, but not affected at renal medulla in streptozotocin induced diabetic kidney. We also observed that treatment with *Aloe vera* alleviated this suppression in renal cortex. We believe that the conflicting results of AQP3 level in literature might be related with varying of experimental design of the conflicting studies and evaluation method of AQP3. The study that reported AQP3 is up-regulated in diabetic kidney is performed with intravenous administration of streptozotocin, but in the conflicting studies the induction of diabetes was performed with intraperitoneal administration route.

As a conclusion, results of our current experiment indicated that *Aloe vera* extract has potency to protect kidney in hyperglycemic animals with down regulating oxidative stress, apoptosis related protein expressions, DNA fragmentation and apoptosis. Furthermore, in our immunohistochemical analyzes we observed that AQP3 level is down-regulated in hyperglycemic diabetic animals renal cortex. We also observed that treatment with *Aloe vera* normalized AQP3 expressionin cortical structures. Although our study is expressing promising results, the anti-diabetic, anti-apoptotic and anti-hyperglycemic activity and clinically applicability of *Aloe vera* should be evaluated in more detailed studies to understand possible modality potential of the *Aloe vera*.

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AUTHOR CONTRIBUTIONS

Concept: U.S., S.S.; Design: U.S., S.S.; Control: U.S., B.C.G., M.Y., O.U.D., S.S.; Sources: U.S., B.C.G., D.S.A., S.B.B., M.Y., O.U.D., S.S.; Materials: U.S., M.Y., O.U.D., S.S.; Data Collection and/or Processing: U.S., B.C.G., D.S.A., S.B.B., M.Y., O.U.D.; Analysis and/or Interpretation: U.S., M.Y.; Literature Review: U.S., B.C.G.; Manuscript Writing: U.S., B.C.G.; Critical Review: U.S., B.C.G., D.S.A., S.B.B., M.Y., O.U.D., S.S.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

Experimental animal tissue samples of this study was obtained with approval of the Local Experimental Animal Ethics Committee of Dicle University (Approval date & no: 14.12.2021 & 2020/09).

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ASSESSMENT OF PHARMACY STUDENTS' KNOWLEDGE, ATTITUDES AND PRACTICES ABOUT COVID-19 PANDEMIC AND VACCINES

ECZACILIK ÖĞRENCİLERİNİN COVID-19 PANDEMİSİ VE AŞILARI HAKKINDA BİLGİ, TUTUM VE UYGULAMALARININ DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *The objective of this study was to determine the knowledge, attitude, and practice towards the Novel Coronavirus Disease 2019 (COVID-19) and its vaccines among pharmacy students in Turkey.*

Material and Method: *A cross-sectional study was conducted among pharmacy students aged 18 years and older. Data collected from April 27 to June 27, 2022, via an online self-reporting questionnaire developed and validated by the investigators. Descriptive statistics were performed to determine potentially associated factors with knowledge, attitudes, and practices scores of the students.*

Result and Discussion: *Among the pharmacy students, the majority was female (72.6%) and mean ± standard deviation of age was 23.17 ± 3.27 years. The mean ± standard deviation of knowledge, attitude and practice scores were 8.25 ± 1.27, 7.47 ± 2.95, and 7.20 ± 2.14, respectively. A statistically significant association was found between students who received higher knowledge scores and being a senior student or a students from governmental universities. In general, students had good knowledge and practices about COVID-19 pandemic and vaccines. However, their attitudes towards COVID-19 pandemic and vaccines was poor. These findinds were found to be similar to those health care students from other countries.*

Keywords: *Attitudes, knowldege, pharmacy students, practice, survey*

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ÖZ

Amaç: *Bu çalışmanın amacı, Türkiye'deki eczacılık öğrencilerinin Yeni Koronavirüs Hastalığı 2019 (COVID-19) ve aşılarına yönelik bilgi, tutum ve uygulamalarını değerlendirmektir.*

Gereç ve Yöntem: *Eczacılık öğrencileri arasında 18 yaş ve üzeri olan bireylerle kesitsel bir çalışma yapılmıştır. 27 Nisan - 27 Haziran 2022 tarihleri arasında, araştırmacılar tarafından geliştirilen ve valide edilen bir çevrimiçi kişisel bildirim anketi aracılığıyla veriler toplanmıştır. Öğrencilerin bilgi, tutum ve uygulama puanları ile potansiyel olarak ilişkili faktörleri belirlemek için tanımlayıcı istatistiksel analizler yapılmıştır.*

Sonuç ve Tartışma: *Ankete katılan eczacılık öğrencilerinin çoğunluğu kadındı (%72.6) ve ortalama ± standart sapma yaşı 23.17 ± 3.27 idi. Bilgi, tutum ve uygulama puanlarının ortalama ± standart sapması sırasıyla 8.25 ± 1.27, 7.47 ± 2.95 ve 7.20 ± 2.14'tür. Daha yüksek bilgi puanları alan öğrenciler ile son sınıf öğrencisi veya devlet üniversitelerinden öğrenci olmak arasında istatistiksel olarak anlamlı bir ilişki bulunmuştur. Genel olarak, öğrencilerin COVID-19 hakkında iyi bilgi ve uygulamaları vardı. Ancak, COVID-19'a karşı tutumları zayıftı. Bu bulguların diğer ülkelerden gelen sağlık öğrencilerine benzer olduğu bulunmuştur.*

Anahtar Kelimeler: *Anket, bilgi, eczacılık öğrencileri, tutum, uygulama*

INTRODUCTION

The population worldwide is currently facing a serious health problesm called as The Novel Coronavirus Disease 2019 (COVID-19) pandemic, which is caused by a novel coronavirus [1]. The coronavirus is formely known as 2019-nCoV, the new strain named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [2]. The coronavirues causes the illness named COVID-19 [2,3]. One of severe outcomes of the COVID-19 is that it can cause a fatal effect on humans after its short time of detection [2]. It mainly spread through respiratory droplets [4]. So, the people got infected when they come in contact with infected patients' saliva and nose discharges [4]. Studies demonstrated that approximately 31% of patients infected with SARS-CoV-2 was asymptomatic while 40% of symptomatic patients had mild or moderate symptoms including fever, dry cough, myalgia, tiredness [3,5]. Nearly 15% of symptomatic patients had severe symptoms including chest pain, shortness of breath or difficulty of breathing, which requires oxygen support [3,5]. The critical conditions such as respiratory failure, acute respiratory distress syndrome, sepsis and septic shock, thromboembolism and multiorgan failure were experienced by nearly 5% of the patients [5]. The COVID-19 pandemic continues to impose burdens of disease and death as it severely disrupts societies and economies worldwide [6].

While waiting the development of direct treatments of the COVID-19, vaccination approach to overcome the disease became one of the solutions. Developing a vaccine for SARS-CoV-2 generally requires months to years [7]. Different vaccines are available including live attenuated virus, viral vectors, inactivated virus, subunit vaccines, recombinant DNA, and protein vaccines [7]. Nucleic acid, live attenuated virus, protein subunit and viral vector vaccines attempts were made as a solution to the SARS-CoV-2, of which there is no active cure. More than 60 vaccine candidates have been investigated against COVID-19, but they can probably have low efficacy due to existing immunity [8]. Vaccine studies are still ongoing today and some mRNA and inactivated vector vaccines are approved. Vaccines are predicted to reduce the COVID-19 damage on the global public health [9].

Besides fighting against the pandemic with the social distance, hygiene practices; dissemination of misinformation is also an issue with COVID-19. The misinformation can cause a wide range of negative effects in society, including in the scientific community and called an infodemic [6]. The infodemic is defined as the inability of people to access reliable and accurate information due to the prevalence of false information in times of crisis such as pandemics, earthquakes, and terrorist attacks, and the rapid spread of this false information [10]. Infodemic is a public health problem when it prevents people from accessing the appropriate guidance in times of crisis such as COVID-19 pandemic [6]. Increasing digitalization, i.e., the widespread use of social media and the internet may cause infodemia to spread more rapidly [10]. Infodemia can have a significant impact on the acceptance of a COVID-19 vaccine, which was the only hope prior to treatment [6]. This can also result in increased misinformation

and vaccine opposition. Therefore, knowledge, attitude, and practice (KAP) is an important function to eliminate risk from infodemia and to improve health prevention and promotion in public [4]. KAP can be targeted for a range of health behaviours and beliefs about COVID-19 pandemic, symptoms, treatment, and outcomes.

It is very important for the students, who constitute an important part of the society, to have accurate and up-to-date information and to have the desired behaviors and practices in the fight against the pandemic. Only a few published articles evaluate the KAP of students towards COVID-19. To the best of our knowledge, this is the first study to assess KAP of pharmacy students towards COVID-19 and vaccines. Thus, this study aims to determine the knowledge, attitude, and practice towards COVID-19 and its vaccines among pharmacy students in Turkey.

MATERIAL AND METHOD

A cross-sectional, online survey in the Google Docs platform was conducted among faculties of pharmacy in Turkey during the COVID-19 period on 27 April and 27 June 2022. Students were eligible if they were 18 years or older and studying in faculty of pharmacy. Students were invited to participate in the study and after the approval from the rectorship.

The survey was used a standardized approach which included the review of the literature, group discussion among the investigators, expert panel evaluation, pilot study, and the validation of the final questionnaire. The investigators developed the initial questionnaire based on literature search and their expertise and experienes. After the initial draft of the survey, 2 clinical pharmacists, 1 sociologist, 3 pharmacists specialized in medicine and vaccine technology participated in the expert panel to review the survey questions for content validity. In the expert panel, they were asked to evaluate each question in the questionnaire in terms of content and scope, and to state their opinions and suggestions, if any. As a result of the first panel, there was no need for a second and third panel, as panel recommendations were for minor editing in the questions. A pilot study was conducted among the pharmacy students to evaluate the questions in terms of content, scope and intelligibility. During the pilot study, 55 students were included and 93% of them evaluated the questions as clear and understandable. For reliability, Cronbach's Alpha values were calculated and were found to be 0.6. It is acceptable when the Cronbach's Alpha is equal or above 0.6.

The final questionnaire included a brief introduction about this study, the declaration of anonymity and confidentiality from researchers, and the confirmation of voluntary participation from students. The first session included 7 questions about the socio-demographic characteristics of the students and their vaccination status. The session for knowledge, attitudes and practice included 10, 12, and 10 questions respectively. The attitudes and practice questions were assessed based on the 5-point Likert scale.

Beside the knowledge, attitudes and practice questions, sociodemographic characteristics such as gender, age, history of vaccination and COVID-19 disease were collected.

The continuous variables were reported as mean (standard deviation) and categorical variables were reported as number (percentage). Statistical analyses were performed using IBM SPSS Statistics 21. A Chi Square or Fisher's Exact tests were performed among the variables. Statistically significant differences were considered when the p-value < 0.05.

RESULT AND DISCUSSION

A total of 383 pharmacy students participated in this study. The majority was female (72.6%) and mean ± SD age was 23.17 ± 3.27 years. More than half of them were fourth year students (46.5%). Almost all the students had at least one dose of COVID-19 vaccine (98.4%) and one-third were infected with COVID-19 disease at least one time (33.4%). Demographic characteristics of the students are presented in Table 1.

The majority of the students responded correctly that COVID-19 is spread through the respiratory tract (92.7%), there is no effective drug treatment for COVID-19 yet (at time of the study period, 82.5%), the elderly and those with chronic diseases are more likely to experience COVID-19 (97.4%), COVID-19 mRNA vaccines contain mRNA of the antibody-forming antigenic construct of the targeted virus

(89.8%), those who have the COVID-19 vaccine can get the disease (99.0%), and the COVID-19 vaccine is never administered to people who have had the disease (97.1%) (Table 2). The mean ± SD knowledge score was 8.25 ± 1.27 (range from 4-10).

Table 1. Demographics of the pharmacy students (n=383)

Variable	n (%)
Age, mean ± SD	23.17 ± 3.27
University	
Government	366 (4.4)
Private	17 (95.6)
Education year	
First year	26 (6.8)
Second year	41 (10.7)
Third year	83 (21.7)
Fourth year	178 (46.5)
Fifth year	55 (14.4)
Gender, Female	278 (72.6)
COVID-19 vaccine	
Yes	377 (98.4)
mRNA	295 (77.0)
Inactive	37 (9.7)
Both	45 (11.7)
Influenza vaccine	29 (7.6)
History of COVID-19 diagnosis	128 (33.4)

COVID-19: The Novel Coronavirus Disease 2019, SD: standard derivation.

Table 2. Knowledge of pharmacy students about COVID-19 and vaccines

Questions	True, n (%)	False, n (%)	Do not know, n (%)
1. The COVID-19 is spread through the respiratory tract.	355 (92.7)	20 (5.2)	8 (2.1)
2. There is no effective drug treatment for COVID-19 yet.	316 (82.5)	39 (10.2)	28 (7.3)
3. The elderly and those with chronic diseases are more likely to experience COVID-19.	373 (97.4)	7 (1.8)	3 (0.8)
4. Cold chain rules should be applied to both mRNA and inactivated COVID-19 vaccines.	276 (72.1)	49 (12.8)	58 (15.1)
5. The components used alongside the virus components to increase the safety and efficacy of COVID-19 vaccines are called adjuvants.	284 (74.2)	3 (0.8)	96 (25.1)
6. COVID-19 mRNA vaccines contain mRNA of the antibody-forming antigenic construct of the targeted virus.	344 (89.8)	10 (2.6)	29 (7.6)
7. COVID-19 vaccines should be administered intramuscularly to the deltoid muscle.	267 (69.7)	27 (7.0)	89 (23.3)
8. Those who have the COVID-19 vaccine can get the disease.	379 (99.0)	3 (0.8)	1 (0.3)
9. The COVID-19 vaccine is never administered to people who have had the disease.	6 (1.6)	372 (97.1)	5 (1.3)
10. COVID-19 vaccines can be administered together with other vaccines (such as the flu vaccine).	194 (50.7)	61 (15.9)	128 (33.4)

COVID-19: The Novel Coronavirus Disease 2019.

The majority of the students were likely to accept getting vaccinated by mRNA (72.3%) or inactive vaccines (76.5%). They were likely to trust mRNA (71.3%) or inactive vaccines (56.4%). More than half of them believed that COVID-19 disease would be defeated with vaccines (62.2%) or with medications (68.7%). The majority recommended getting vaccinated (82.8%). The institutions which more than half of students trusted were the Turkish Pharmacists Association (59.3%) or World Health Organization (58.2%) (Table 3). The mean ± SD attitudes score was 7.47 ± 2.95 (range from 0-12).

In general, students had good practices about COVID-19. Based on the responses to practice

questions, the majority indicated that they used a mask even after vaccination (83.4%), followed social distancing rules (82.7%) and hygiene rules such as washing hands and using hand sanitizers (92.1%), rules set by the faculty (85.1%) and self-isolations rules such as curfew (90.3%) (Table 4). The mean ± SD practice score was 7.20 ± 2.14 (range from 0-10).

Table 3. Pharmacy students' attitudes towards COVID-19 and vaccines

Questions	Strongly agree, n (%)	Agree, n (%)	Neutral, n (%)	Disagree, n (%)	Strongly disagree, n (%)
1. I avoid getting vaccinated because of the possible side effects of mRNA COVID-19 vaccines.	14 (3.7)	35 (9.1)	57 (14.9)	105 (27.4)	172 (44.9)
2. I avoid getting vaccinated because of the possible side effects of inactivated COVID-19 vaccines.	9 (2.3)	22 (5.7)	59 (15.4)	105 (27.4)	188 (49.1)
3. I do not trust inactivated vaccines used to prevent COVID-19.	32 (8.4)	54 (14.1)	82 (21.1)	87 (22.7)	129 (33.7)
4. I do not trust mRNA vaccines used to prevent COVID-19.	21 (5.5)	26 (6.8)	63 (16.4)	108 (28.2)	165 (43.1)
5. I believe that we will defeat the COVID-19 disease with vaccines.	119 (31.1)	119 (31.1)	80 (20.9)	35 (9.1)	30 (7.8)
6. I believe that we will defeat the COVID-19 disease with drug treatments that exist or will be discovered.	129 (33.7)	134 (35.0)	85 (22.2)	24 (6.3)	11 (2.9)
7. I recommend getting vaccinated to those around me.	239 (62.4)	78 (20.4)	44 (11.5)	10 (2.6)	12 (3.1)
8. I use aspirin when I get the COVID-19 vaccine.	43 (11.2)	39 (10.2)	89 (23.2)	79 (20.6)	133 (34.7)
9. The Ministry of Health Turkey is the institution I trust most about COVID-19 and vaccines.	83 (21.7)	94 (24.5)	113 (29.5)	54 (14.1)	39 (10.2)
10. The Turkish Pharmacists Association is the institution I trust most about COVID-19 and vaccines.	104 (27.2)	123 (32.1)	102 (26.6)	44 (11.5)	10 (2.6)
11. The World Health Organization is my most trusted institution for COVID-19 and vaccines.	107 (27.9)	116 (30.3)	104 (27.2)	31 (8.1)	25 (6.5)
12. The US Food and Drug Administration is my most trusted agency for COVID-19 and vaccines.	75 (19.6)	94 (24.5)	106 (27.7)	70 (18.3)	38 (9.9)

COVID-19: The Novel Coronavirus Disease 2019.

Table 4. Pharmacy students' practices about COVID-19 pandemic measures

Questions	Always, n (%)	Mostly, n (%)	Sometimes, n (%)	Rarely, n (%)	Never, n (%)
1. I report the side effects I have experienced after vaccination through the Hayat Eve Sığar (HES) application.	103 (26.9)	59 (15.4)	70 (18.3)	63 (16.4)	88 (23.0)
2. I continue to use a face mask after vaccination.	287 (74.9)	71 (18.5)	15 (3.9)	6 (1.6)	4 (1.0)
3. I follow social distancing rules to avoid COVID-19.	215 (56.1)	102 (26.6)	46 (12.0)	12 (3.1)	8 (2.1)
4. I avoid using public transport to prevent the spread of COVID-19.	47 (12.3)	36 (9.4)	97 (25.3)	102 (26.6)	101 (26.4)
5. I use a face mask to avoid COVID-19.	218 (56.9)	75 (19.6)	43 (11.2)	26 (6.8)	21 (5.5)
6. To avoid COVID-19, I periodically wash my hands with soap and water or use hand sanitizer.	259 (67.6)	94 (24.5)	24 (6.3)	4 (1.0)	2 (0.5)
7. I follow the other rules set by our faculty to avoid COVID-19 (for example, not making crowds in and out of the classrooms, airing the classroom before and after the lesson).	214 (55.9)	112 (29.2)	36 (9.4)	14 (3.7)	7 (1.8)
8. I follow up-to-date information about COVID-19.	140 (36.6)	126 (32.9)	87 (22.7)	26 (6.8)	4 (1.0)

Table 4 (*continue*). Pharmacy students' practices about COVID-19 pandemic measures

9. I follow up-to-date information about COVID-19 vaccines.	137 (35.8)	117 (30.5)	95 (24.8)	28 (7.3)	6 (1.6)
10. I implement measures such as curfew related to COVID-19.	287 (74.9)	59 (15.4)	26 (6.8)	7 (1.8)	4 (1.0)

COVID-19: The Novel Coronavirus Disease.

According to the statistical analysis, students who received higher knowledge scores from the questionnaire were more likely senior students [odds ratio (OR) (95% confidence interval (CI)= 2.15 (1.35-3.44), p<0.001], and from governmental universities [OR (95% CI) = 3.60 (1.35-9.64), p<0.007] (Table 5).

Table 5. Associations between pharmacy students' characteristics and the KAP scores

Variables	**Knowledge**			
	Poor Score ≤ 7	**Good Score > 7**	**OR (95% CI)**	**p value**
Female **Male**	68 (24.5%) 28 (26.7%)	210 (75.5%) 77 (73.3%)	1.12 (0.67-1.87) 1	0.657
Senior students (4th and 5th year) **Junior students (3rd year or less)**	45 (19.3%) 51 (34.0%)	188 (80.7%) 99 (66.0%)	2.15 (1.35-3.44) 1	0.001
Government university students **Private university students**	87 (23.8%) 9 (52.9%)	279 (76.2%) 8 (47.1%)	3.60 (1.35-9.64) 1	0.007
Variables	**Attitudes**			
	Poor Score ≤ 8	**Good Score > 9**	**OR (95% CI)**	**p value**
Female **Male**	150 (54.0%) 67 (63.8%)	128 (46.0%) 38 (36.2%)	1.51 (0.95-2.39) 1	0.083
Senior students (4th and 5th year) **Junior students (3rd year or less)**	127 (54.5%) 90 (60.0%)	106 (45.5%) 60 (40.0%)	1.25 (0.83-1.90) 1	0.290
Government university students **Private university students**	207 (56.6%) 10 (58.8%)	159 (43.4%) 7 (41.7%)	1.10 (0.41-2.95) 1	0.854
Variables	**Practice**			
	Poor Score ≤ 7	**Good Score > 7**	**OR (95% CI)**	**p value**
Female **Male**	124 (44.6%) 53 (50.5%)	154 (55.4%) 52 (49.5%)	1.27 (0.81-1.99) 1	0.304
Senior students (4th and 5th year) **Junior students (3rd year or less)**	104 (44.6%) 73 (48.7%)	129 (55.4%) 77 (51.3%)	1.18 (0.78-1.78) 1	0.440
Government university students **Private university students**	170 (46.4) 7 (41.2%)	196 (53.6%) 10 (58.8%)	0.81 (0.30-2.17) 1	0.670

CI: Confidence Interval, KAP: knowledge, attitudes and practice, OR: Odds Ratio.

Generally, almost all students had a high level of knowledge. The scores of senior pharmacy students were higher compared to those of junior pharmacy students. Similar to our study (74.9%), the knowledge towards COVID-19 among healthcare students in Saudi Arabia and Vietnam were 65.7% [11] and 86.6% [5], respectively. The knowledge scores of university students in Vietnam were 75.61% [12], while it was 85.3% (means score out of 22 questions was 18.76) among student nurses in Philippines [2]. Similarly, the majority of nursing students in Italy had good score for knowledge about COVID-19 (94.6%) [13]. However, the knowledge score was low among undergraduate medical students in Indonesia (29.8%) and health care students in China (28.3%) [14,15]. Compared to pharmacy

students in Turkey (8.25 ± 1.27), the medical students in Turkey had the lower means of the 10 questions on the COVID-19 knowledge questionnaire, which was 7.83 ± 1.27 [16].

In our study there were no differences in knowledge scores of male and female students. However, female students were more likely to have higher scores of KAP in a study conducted among the university students in Vietnam [5,12]. Several factors including age, sex, country of residence, school type, school year, family income and perceptual awareness on COVID-19 can affect their knowledge, attitude, and/or practice toward COVID-19 [14].

Regarding attitudes towards COVID-19 and vaccines, the percentage of students who received higher scores from the attitudes questions was poor (43.3%). It was lower than those students from Vietnam (68.8% and 98.4%), China (67.8) and Italy (90.6%) [5,12,13,15]. The percentage of students who received higher scores from practice questions was moderate with 53.8% in our study. It was similar to the health care students from China (58.6%) [15]. However, health care students from Vietnam had higher percentage for positive practices about COVID-19 (94.9%) [12]. Nursing students from Italy (88.9%) had better practices about COVID-19 [13] compared to the pharmacy students in Turkey (43.3%).

Although the attitude towards COVID-19 pandemic was poor, there were some good elements of attitudes and practice. For example, students stated that they recommended getting vaccinated to those around them (83%), followed social distancing rules (83%), wore a face mask (77%), periodically washed their hands with soap and water or use hand sanitizer (92%) and followed the other rules set by their faculties to avoid COVID-19 (85%).

The most trusted agencies about COVID-19 and vaccines among the pharmacy students were the Turkish Pharmacists Association (60%), the World Health Organization (58%), and the Ministry of Health Turkey (46%), respectively. Thus, pharmacy students were likely to search for information from these agencies during the pandemic. It was also in line with the students' responses that they follow up-to-date information about COVID-19 (70%) and vaccines (66%).

There were several limitations of this study. The students participated voluntarily and not all pharmacy faculties in Turkey were invited. Therefore, the generaliazibility of the findings may not be applicable. Due to having enough sample size and participation from each selected faculties, the results of this study are still valuable. Since, the study was designed as cross sectional, we were not able to assess the impact of variables such as panic and anxiety about COVID-19 on the students' responses. There was also a risk for reporting bias as it was a self-reported questionnaire.

The results of this study showed that there is a great need for educational interventions about COVID-19 pandemic and vaccines. Although majority had a good knowledge score, the knowledge did not seem to be enough to change their attitudes and practices. The interventions must focus not only knowledge but also to improve attitudes and practice of the students. This also reflects the new curriculum need for pharmacy students where they can learn communicable diseases more and have further information about COVID-19 vaccines to have positive attitudes and practice.

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AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

The Ethics Committee for Human Research of Ankara University is approved the study (Date: January 1, 2022, Decision No: 01/08).

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

SURGICAL ORTHOPEDIC PATIENTS' PERCEPTION OF ANTIBIOTIC USE AND ANTIBIOTIC RESISTANCE

CERRAHİ ORTOPEDİ HASTALARININ ANTİBİYOTİK KULLANIMI VE ANTİBİYOTİK DİRENÇ ALGISI

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ABSTRACT

Objective: *The aim of the study was to evaluate orthopedic patients' knowledge of antibiotics and antibiotic resistance.*
Material and Method: *Patients admitted to the hospital for orthopedic surgery were given a questionnaire containing a total of 26 questions, 7 multiple choices, nine true and false questions and ten likert scale questions adapted from a survey which has been published by World Health Organization and translated to Albanian to assess their knowledge and attitude toward antibiotics and antibiotic resistance.*
Result and Discussion: *Sixty-two percent of the patients misunderstood the definition of antibiotic resistance and answered yes to the question "Antibiotic resistance occurs when your body becomes resistant to antibiotics and the antibiotic is no longer effective." 62.4% of the patient strongly agreed that 'antibiotic resistance is one of the biggest problems facing the world". Although participants are aware of antibiotic resistance and the potential threats it may pose, their knowledge of the causes of antibiotic resistance and how to prevent it is limited. In conclusion, attempts need to be made to raise antibiotic resistance awareness and knowledge among the Kosovo population.*
Keywords: *Antibiotics, antibiotic resistance, awarenes, knowledge*

ÖZ

Amaç: *Bu çalışmanın amacı ortopedi hastalarının antibiyotikler ve antibiyotik direnci üzerine bilgisini değerlendirmektir.*

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Gereç ve Yöntem: *Ortopedi ameliyatı için hastaneye yatırılan hastaların antibiyotikler ve antibiyotik direnci üzerine bilgilerini ve tutumlarını değerlendirmek amacı ile Dünya Sağlık Örgütü tarafından uygulanmış bir anketten uyarlama Arnavutça'ya çevrilen 7 adet çoktan seçmeli, 9 adet doğru yanlış ve 10 adet likert ölçeği sorusu olmak üzere toplamda 26 sorudan oluşan uyarlama anket yöneltildi.*

Sonuç ve Tartışma: *Hastaların yüzde 62'si antibiyotik direncinin tanımını yanlış anlamış ve "Antibiyotik direnci, vücudunuz antibiyotiklere dirençli hale geldiğinde ve artık işe yaramadığında ortaya çıkar." sorusuna evet cevabını vermiştir. Hastaların yüzde 62.4'ü "Antibiyotik direnci dünyanın karşı karşıya olduğu en büyük sorunlardan biridir." ifadesine kesinlikle katıldıklarını belirtmiştir. Katılımcılar antibiyotik direncinin ve sebep olması muhtemel tehlikelerin farkında olmalarına ragmen, antibiyotik direncinin sebeplerine ve antibiyotik direncinin nasıl önleneceğine dair bilgileri kısıtlıdır. Sonuç olarak, Kosova'da antibiyotik direncine dair bilinç ve farkındalığı arttırmaya yönelik çalışmalar yapılması gerekmektedir.*

Anahtar Kelimeler: *Antibiyotik, antibiyotik direnci, bilgi, farkındalık*

INTRODUCTION

Infectious diseases are a leading cause of hospitalizations, emergency department visits, and hospital-based mortality in Kosovo and around the world [1]. The easy access to antibiotics has led to antibiotic overuse, which has accelerated the emergence of antibiotic resistance globally [2]. Antibiotic resistance is brought on by both excessive and inappropriate use of antibiotics [3]. It is known that the regular administration of antibiotics combined with misuse to treat diseases such as flu, common cold, and diarrhea is seen as a major contributor of the antibiotic resistance caused by inadequate public knowledge [4]. Additionally, with the advancement in the biotechnology, broad-spectrum antibiotics became more accessible and the wide and unnecessary use of broad-spectrum antibiotics due to absence of early diagnostics, also raises antibiotic resistance [5]. In addition to direct misuse and overuse of antibiotics, people are also exposed to antibiotics through dairy products. According to the research conducted in Kosovo, dairy cattles are not receiving the prescribed treatment protocol. The uncontrolled use of antibiotics on the treatment of cattles give rise to antibiotics resiude in dairy products which causes daily intake of antibiotics for the population [6]

Planning new strategies to prevent accelerated increase in antibiotic resistance has emerged as one of the biggest obstacles that the public health sector faces [7]. World Health Organization presented the report which estimates by the year 2050, 10 million deaths can occur because of the increased antibiotic resistance [8]. Additionally, it is worthy of attention that there is a solid link between economic well-being of the countries and the antibiotic resistance [9,10]. The reason to this fact can be examined from several aspects such as the accessibility of healthcare services and the knowledge level of the public. Nevertheless, this situation should be resolved across the world because the antibiotic resistance may lead to not only a raise in infectious diseases but also biological alterations in population and bacterial evolution, and the transmission of these threats are easier than ever due globalization and increased travel rate across the world [7,9]. Surgical site infection is the term for an infection that appears in the surgical incision site following surgery. Infections at the surgical site can range from mild, affecting simply the skin, to severe, impacting organs, implanted materials, and tissues below the skin. The incidence of SSI varies greatly depending on the operative site and wound classification, ranging from 5% to 30% [11]. Infection is one of the most common complications in orthopedic surgery which may cause long-term disability, morbidity and mortality [12]. According to WHO, antibiotic-resistant bacteria cause 50% of surgical site infections [13]. Raising public awareness and knowledge about antibiotic resistance, promoting rational antibiotic use, and reducing the incidence of infections through improved hygiene and sterilization are all strategies to combat antibiotic resistance [14]. The implementation of an antibiotic stewardship program strengthens the responsible use of antibiotics while stepping up efforts to combat antibiotic resistance.

A multidisciplinary team made up of microbiologists, clinical pharmacists, and infectious diseases experts should be a part of any ideal antibiotic stewardship program [15]. Kosovo Ortomedica Orthopedic Hospital infection committee strives to accomplish a number of goals, one of which is to

improve patient understanding and awareness toward antibiotic resistance. The primary goal of this study is to determine the level of knowledge and awareness of our patients before implementing an educational program that will benefit all patients who visit the hospital.

MATERIAL AND METHOD

The antibiotic resistance knowledge and awareness of the orthopedic patients was assessed via questionnaire that translated and adapted from a survey which has been published by World Health Organization [16]. The questioner was admitted to five volunteers to ensure the readability before given to the patients. Our patients' complete the questioner and accurate demographic information as well as medical and medication histories was collected.

A questionaire containing 26 questions in total were completed by the patients who have been admitted to the Kosovo Ortomedica Orthopedic Hospital for orthopedic operation during the study period June 2018 to June 2019. Seven multiple choice questions and nine true-false questions in order to assess understanding and knowledge. Ten 5-point Likert questions to gauge the attitude of the patients towards antibiotic resistance. For the statistical analysis, cronbachs alpha was employed to assess the reliability of the questionaire; the responses for the patients were described as frequencies and percentage.

RESULT AND DISCUSSION

Within the scope of the study the questionnaires were directed to the total of 93 patients comprising 54 women (58%, n=93) and 39 men (42%, n=93). The average age in the 93 participating patients participated to the study is 54.9 ±21.9. Before directing the questionnaires about antibiotic and antibiotic resistance, the general health condition of the patients was examined. Comorbidity present among 32 of the patients (34.4%, n=93) and 27 patients (29%, n=93) had hypertension while 3 patients had Hyperlipidemia. Additionally, 1 patient (1.1%, n=93) had lower respiratory tract infection.

The reason for admission were, Coxarthrosis (5%, n=93), Gonarthrosis (9.6%, n=93), Muscle contractures (2%, n=93), Anterior cruciate ligament (6.4%, n=93), Plateau fracture (2%, n=93), Achile contractor Standing deformity (2%, n=93), femur fracture (2%, n=93), spinal stenosis (5.3%, n=93), Developmental Hip Fracture (8.6%, n=93), Meniscopathy (13.9%, n=93), Bilateral gonarthrosis (8.6%, n=93), and 1 patient each with the following conditions. tumoral mass in the sacrum tibia fracture elbow dislocation, femur and tibia deformation, bilateral developmental hip fracture etc. The cronbach alpha for the questionaire was 0.71, so we decide to rely on the patients' responses. The last time when the patients used antibiotics were in the last month for 16 (17.2%, n=93), for 29 (31.2%, n=93) it is in the last 6 months, 19 (20.4%) it is in the last year, while 19 of the patients (20.4%, n=93) had used antibiotics more than a year ago, lastly 2 patients (2.2%, n=93) never used antibiotics and 8 patients (8.6%) stated that they could not remember when they used antibiotics for the last time.

When these results are compared with an international study including participants from 12 countries, namely, Nigeria, South Africa, Barbados, Mexico, India, Indonesia, Russian Federation, Serbia, Egypt, Sudan, China, and Vietnam. It is seen that the use of antibiotic is very low in Kosovo in comparison to the average ratio of patients who took antibiotics in the last 6 months in these 12 countries, which is 65% [16]. Another comparative study subjecting pharmacy students in Australia and Sri Lanka, which is conducted to assess the use of antibiotics and awareness of microbial resistance showed very similar ratio on the last time participants used antibiotics [17]. The students in Australia who used antibiotics within the last year is 17% and in Sri Lanka it is 11% whereas it is 21% in Australia and 36% in Sri Lanka for the antibiotic consumption in the last 6 months [17]. While 50 of the patients (53.8%, n=93) answering the question if they use antibiotics as yes, 27 (29.0%, n=93) of the patients said no.

Then, the question regarding when they stop taking antibiotics during the treatment was directed to the patients, 42 (45.2%, n=93) of the patients answered as when they felt better, 33 (35.5%, n=93) of the participants declared they took all antibiotics as stated, 18 patients (19.4%, n=93) answered as they did not know. The results were compared to the pharmacy students in Australia and Sri Lanka, although the results regarding the use were very close, answers for when to stop taking antibiotics are significantly different. 4% of the students in Australia and 21% of the students in Sri Lanka answered as when they

feel better which is significantly smaller in comparison to 33% of the orthopedic patients in Kosovo [17]. The antibiotic knowledge of patients is represented in Table 1.

Table 1. Patients responses to the antibiotic knowledge questions

Multiple-Choice Questions	Choices	Number of Patients	Percentage of Patients
Age	0-20	13	13.9
	21-40	27	29
	41-60	31	33.3
	61-80	20	21.5
	81-100	2	2.1
When was the last time you used antibiotics?	Last month	16	17.2
	Last 6 months	29	31.1
	Last year	19	20.4
	More than a year ago	19	20.4
	Never	2	2.1
	Not remember	8	8.6
Did you take antibiotics from a doctor?	Yes	50	53.7
	No	30	32.2
	Not remember	13	13.9
Did you take advice from a doctor, nurse or pharmacist about how to use antibiotics?	Yes	50	53.7
	No	27	29
	Not remember	16	17.2
When do you stop taking antibiotics?	When I feel better	42	45.1
	As prescribed	33	35.4
	Not know	18	19.3
Is it okay to use antibiotics given to someone else as long as they are used to treat the same disease?	Yes	49	52.6
	No	30	32.2
	Not Know	14	15
Is it okay to buy the same antibiotics or request them from a doctor if you are sick and they have helped you fight the same symptoms in the past?	Yes	51	54.8
	No	26	27.9
	Not remember	16	17.2
Which of the diseases/conditions can be treated with antibiotics do you think?	HIV/AIDS	26	28
	Gonorrhoea	44	47.3
	Bladder infection / Urinary tract infection	37	39.8
	Diarrhea	33	35.5
	Cold/Flu	45	48.4
	Malaria	24	25.8
	Measles	25	26.9
	Skin infection	47	50.5
	Traumatic wound	38	40.9
	Sore throat	38	40.9
	Headache	4	4.3
	General body pain	12	12.9

Among the participating group, when asked ‘which of the diseases/conditions can be treated with antibiotics do you think?’ 26 patients (28.0%) answered as HIV/AIDS, 37 patients (39.8%) answered as bladder infection or urinary tract infection, 33 patients (35.5%) answered as diarrhea, 45 patients (48.4%) answered as either cold or flu, and 24 patients (25.8%) answered as malaria. Measles was chosen by 25 patients (26.9%), skin infection was chosen by 47 patients (50.5%). Also 42 patients (45.2%) answered as traumatic wound, 38 patients (40.9%) answered as sore throat and 4 patients (4.3%)

answered as headache while 12 patients (12.9%) answered general body pain. In comparison, pharmacy students from Australia answered as gonorrhoea (63%), bladder infection (92%) and skin wound infection (90%) whereas students in Sri Lanka 30%, 76% and 80% respectively [17]. These findings show that the public in many countries has relatively little general understanding about the correct consumption of antibiotics.

When asked if they get advice while using antibiotics, while 51 participants (54.8%, n=93) answered as they do, 16 participants (28%, n=93) stated that they don't get advice and lastly 16 (17.2%, n=93) did not remember.

To the question "Is it okay to use antibiotics given to someone else as long as they are used to treat the same disease?" 50 of the patients (53.8%) answered as yes, it is okay while 29 patients (31.2%) answered as no, it is not okay, and 14 of the patients (15.1%) did not remember.

When the question was asked across 12 aforementioned countries, although the answer should be no, 25% of the patients responded as yes [16]. Another study examining the extensity of self-medication of antibiotics over university students in Cairo, Egypt shows that 83.1% of the students use antibiotics relying on experience of similar symptoms [18]. When the reasons and motivation of this behaviour were questioned in another study, 47.3% of the participants from Cairo thinks that the common diseases do not require doctor consultation. Also, as already underlined, the financial aspect of the medical visits has influence on the self-medication and 33.8% of the participants stated the reason of self-medication as a result of financial issues [19]. This suggests that the public in general should be better informed about the working mechanism of antibiotics and should be educated why antibiotics cannot be shared. Also, this situation should be better examined and the relation between antibiotics sharing, and accessibility of health service should be investigated. Because it might be the situation that, if a person struggles to access to the professional health service, then they may turn to unprofessional 'help' which is a threat to public health.

Following that, it is asked "Is it okay to buy the same antibiotics or request them from a doctor if you are sick and they have helped you fight the same symptoms in the past?" The answer to the question was yes from 51 patients (54.8%) and no from 26 patients (28.0%) while 16 of the patients (17.2%) did not remember. These results from Kosovo are especially compatible with the results from other 12 countries in which the average of the 'yes' answers is 43% [16]. This situation especially should be taken under examination with the healthcare professionals who may seem to face persistence of patients to prescribe unnecessary antibiotics.

This pattern also shows itself as use of non-prescribed antibiotics when the drug tracking policies are not strength. One of the studies which is conducted to evaluate the use of non-prescription antibiotics in Nsukka Nigeria shows that more than 86% of the participants use non-prescribed antibiotics [18]. The sample size of the study is given as 400 and the age range of the participants are given between 18-60 years. According to the study, the use of non-prescribed antibiotics is very common regardless of sex, age or education level in Nigeria [20].

According to these results, it is clearly shown that the use of antibiotics is quite common. According to the data presented above, the use of antibiotics is terminated when the patient starts feeling better rather than the physician recommendation. Patients fail to see the sensitivity in the use of antibiotics and pressure physicians to prescribe the antibiotic they receive advice from. Similarly, there is a common but wrong idea that an antibiotic that is good for a family member can be used by them when they show similar symptoms.

Various questionnaires were given to the participants to evaluate their understanding of antibiotics and antibiotic resistance. The 9 questions were answered as True or False by patients and the responses with the corresponding percentage are given in the Table 2.

The results presented in Table 2 shows that the main frame of the antibiotic resistance is conceptually well-established in the vast of majority. The patients are aware of the fact that antibiotic resistance complicate the treatment and has negative effects on the process. However, most of the patients are not aware of the extensive use of antibiotics in the production sector.

Table 2. Results of the analysis of the patients' attitudes towards antibiotics.

Knowledge-Based Questions	**Answers**	**Number and percentage of patients answered**	
		N	**%**
Antibiotic resistance occurs when your body becomes resistant to antibiotics and the antibiotic is no longer effective. (false)	True False	62 31	66.7 33.3
Many infections are becoming more resistant to antibiotic therapy. (true)	True False	55 38	59.1 40.9
If bacteria are resistant to antibiotics, the infections they cause may be very difficult or impossible to treat. (true)	True False	58 35	62.4 37.6
Antibiotic resistance is a problem that can affect my family or me. (true)	True False	56 37	60.2 39.8
Antibiotic resistance is a problem in other countries, but not in Kosovo. (false)	True False	55 38	59.1 40.9
Antibiotics are widely used in agriculture and breeding farms in Kosovo. (true)	True False	26 67	28.0 72.0
Antibiotic resistance is only a problem for people who take antibiotics regularly. (false)	True False	61 32	65.6 34.4
Bacteria that are resistant to antibiotics can be transmitted from person to person. (true)	True False	55 38	59.1 40.9
Antibiotic-resistant infections can make medical procedures such as surgery, organ transplants and cancer treatment much more dangerous.(true)	True False	43 50	46.2 53.8

In the last part of the survey of the study, the fact that 62.4% of the patients stated that antibiotic resistance is one of the biggest problems facing the world, 49.5% of them are worried about the impact of antibiotic resistance on their own and their family's health are noteworthy results considering the awareness of the possible threat caused by the increased antibiotic resistance. Whereas 97% and 72% of the pharmacy students from Australia and Sri Lanka thinks that antibiotic resistance is an issue that could affect them or their family, respectively [17]. It is seen that the awaresness regarding the possible threat posed by antibiotic resistance is better acknowledged by the students compared to the patients.

On the other hand, 45.2% of the patients think that they are not at risk of acquiring antibiotic-resistant infections as long as they take antibiotics correctly. Also, when the same question is asked to the students, 89% and 70% of the students from Australia and Sri Lanka respectively stated that antibiotic resistance is only problem for people who take antibiotics regularly [17]. According to these results, it is seen that both the patients and the pharmacy students should be better trained from the aspect of the mechanism of antibiotic resistance.

Similarly, 41.9% of patients think that antibiotics left over from previous treatments should not be used to cure other diseases, and 39.8% think that doctors should prescribe antibiotics only when needed.

Following that, to evaluate their perspectives on antibiotics, 10 questions were directed to the patients and answered on 5-point likert scales as Strongly Agree, Slightly Agree, Undecided, Slightly Disagree and Strongly Disagree. The results with corresponding statistics are given in the Table 3. When the results given in Table 2. are evaluated in detail it is seen that even though the 60.2% of the patients answered the questions 'Antibiotic resistance is a problem that can affect my family or me.' as yes and 39.2% answered as no, when they answer the question 'I am concerned about the impact of antibiotic resistance on my health and the health of my family.' with the options Strongly Agree, Slightly Agree, Undecided, Slightly Disagree and Strongly Disagree statements, none of the patients chose strongly disagree and 76.4% of the patients answered as either Agree of Strongly Agree.

On the other hand, when the answers for the following questions from two survey are more competent. 65.6% of the patients think that antibiotic resistance is only a problem for people who take

antibiotics regularly. Also, 75.3% of the patients are either agree or strongly agree to the thought that if they take their antibiotics right, they are not at risk of getting antibiotic-resistant infections.

Additionally, when the questions regarding to the use of antibiotics in production sector, although only 28% of the patients think that Antibiotics are widely used in agriculture and breeding farms in Kosovo and only 73.9% of the patients agree or disagree with the statement of farmers should give less antibiotics to breeding.

Table 3. The results of the patients' knowledge regarding antibiotic resistance.

	Strongly Disagree		**Disagree**		**Indecisive**		**Agree**		**Strongly Agree**		
	f	**%**	**f**	**%**	**f**	**%**	**f**	**%**	**f**	**%**	**Mean ± sd**
Antibiotic resistance is one of the biggest problems facing the world.	1	1.1	3	3.2	3	3.2	28	30.1	58	62.4	4.495±0.803
I am concerned about the impact of antibiotic resistance on my health and the health of my family.	0	0.0	8	8.6	14	15.1	25	26.9	46	49.5	4.172±0.985
If I take my antibiotics right, I'm not at risk of getting antibiotic-resistant infections.	1	1.1	7	7.5	15	16.1	28	30.1	42	45.2	4.108±1.005
People should only use antibiotics when prescribed by a doctor.	1	1.1	6	6.5	15	16.1	33	35.5	38	40,9	4.086±0.963
People should not use leftover antibiotics from previous treatments to cure other diseases.	1	1.1	6	6.5	14	15.1	33	35.5	39	41,9	4.108±0.961
Doctors should prescribe antibiotics only when needed.	2	2.2	11	11.8	8	8.6	35	37.6	37	39,8	4.011±1.078
People like me can do little to fight against antibiotic resistance.	3	3.2	12	12.9	9	9.7	25	26.9	44	47,3	4.022±1.179
Everyone should use antibiotics responsibly.	6	6.5	6	6.5	14	15.1	23	24.7	44	47,3	4.000±1.216
People should wash their hands regularly.	6	6.5	5	5.4	8	8.6	39	41.9	35	37,6	3.989±1.128
Farmers should give less antibiotics to breeding.	12	13.0	5	5.4	7	7.6	29	31.5	39	42,4	3.848±1.374

In other words, although, the general knowledge regarding the use of antibiotics in production sector is not significantly high among the participants; more than half of the patients stated that they think farmers should use less antibiotics during production.

According to these results and statements, it is seen that there is an obvious awareness against to the danger and significance of antibiotics resistance. Majority of the group thinks that this global problem must be taken seriously, and antibiotics should be only prescribed by physicians and the patients must use in accordance with the given instructions.

However, there is also significant lack of information regarding the ways of contributing to the antibiotic resistance and the correct way of using antibiotics across not only the patients in Kosovo but also in pharmacy students in Australia, Sri Lanka, and university student in Egypt. The comparison suggests that the knowledge regarding antibiotics and antibiotics resistance needsto be improved. Although there are several ways to train public on these aspects, according to the study employing pharmacies showed that education significantly improves the adherence to the prescribed use of antibiotics [21].

In conclusion, the answers of the patients show that general knowledge regarding the danger of antibiotic resistance is internalized by the population. However, these results explicitly indicate that the reasons and the impacts of antibiotic resistance should be explained more to the public. Population with increased awareness will not only avoid direct overuse and misuse of antibiotics but also prevent the

unintentional consumption through dairy products and have significant impact on the expanse of antibiotic resistance.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki. Additionally, informed consent has been obtained from the participants involved prior to the data collection. All procedures performed were in accordance with the ethical guidelines of the Chamber of Pharmacists of Kosovo Non-invasive Ethical Committee (Decision Number: 12.11.2021/378).

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

ERİŞKİN KEMİK İLİĞİ TRANSPLANTASYON ÜNİTESİNDE POTANSİYEL İLAÇ ETKİLEŞİMLERİNİN BELİRLENMESİ - PROSPEKTİF ÇALIŞMA

EVALUATION OF POTENTIAL DRUG INTERACTIONS IN THE ADULT BONE MARROW TRANSPLANTATION UNIT - A PROSPECTIVE STUDY

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ÖZ

Amaç: *Kemik iliği nakli öncesi ve sonrasında yaşanan komplikasyonlar, uzun ve zorlu tedavi sürecinin ile çoklu ilaç kullanımı, ilaç-ilaç etkileşimlerine yol açabilmektedir. Çalışmamızın amacı, erişkin kemik iliği transplantasyon ünitesinde yatan hastalarda potansiyel ilaç etkileşimlerini değerlendirmektir.*

Gereç ve Yöntem: *Bu çalışma, Türkiye'de özel bir hastanenin erişkin kemik BMT ünitesinde Ocak-Haziran 2021 tarihleri arasında yapılmış prospektif ve tanımlayıcı bir çalışmadır. Hastaların sosyodemografik özellikleri kaydedilmiştir. Potansiyel ilaç etkileşimleri, Medscape ve Lexicomp ilaç etkileşimi veritabanları kullanılarak analiz edilmiştir. Analiz için SPSS 15 kullanılmıştır.*

Sonuç ve Tartışma: *Çalışmaya dahil edilen 40 hastanın 22'sinin (%55) erkek hastalar olduğu ve medyan yaşın 48 olduğu tespit edilmiştir. Hastaların çoğuna multipl miyelom (%35) tanısının konulduğu ve %58'inde en az bir komorbid hastalık olduğu tespit edilmiştir. Yüksek doz melfalan rejimi en çok uygulanan rejim (%25) olarak bulunmuştur. Lexicomp veri tabanında tespit edilen etkileşim sayısı, Medscape veri tabanından istatistiksel olarak daha fazla olduğu belirlenmiştir (299 vs 244; $p<0.05$). Eşlik eden hastalığı olan hastalarda, olmayan hastalara göre daha fazla etkileşim saptanmıştır ($p<0.05$). Çalışmamızda kemik iliği nakli hastalarında komorbid hastalık sayısı daha fazla olduğu gözlenmiştir. Bu hastaların akılcı tedavi ve izlem gerektiren ilaç etkileşimleri sayısı*

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yüksek olabilmektedir. Bu ünitelerde hasta takibi, ilaç etkileşimlerinin yönetiminde klinik eczacıların önemli bir rolü olduğunu düşünüyoruz.
Anahtar Kelimeler: *İlaç etkileşimi, kemik iliği transplantasyonu, klinik eczacı*

ABSTRACT

Objective: *Bone marrow treatment (BMT) is a long and complicated process with multiple drug use can lead to drug-drug interactions. The aim of our study is to evaluate potential drug interactions (PDIs) in patients hospitalized in the adult bone marrow transplantation unit.*
Material and Method: *This was a prospective and descriptive study conducted in an adult BMT unit in a private hospital in Turkey between January-June 2021. Sociodemogphrapics of the patients were recorded. PDIs were analyzed via Medscape and Lexicomp drug interaction checker. SPSS 15 was used for analysis.*
Result and Discussion: *Of 40 patients 22 (55%) were male and the mean age was 46. Most of the patients were diagnosed with multiple myeloma (35%) and 58% of the patients had at least one comorbid disease. The number of PDIs detected in the Lexicomp database was statistically higher than in the Medscape database (299 vs 244; $p<0.05$). More PDIS were found in patients with concomitant disease than in patients without ($p<0.05$). The number of comorbid diseases was found to be higher in this population. Additionally, the number of PDIs was found to be high. We think that clinical pharmacists have an important role in patient monitoring and management of PDIs in BMT units.*
Keywords: *Bone marrow transplantation, clinical pharmacist, drug interactions*

GİRİŞ

Kanser hastalarının tedavi rejimlerinde ilaç etkileşimlerine sıklıkla rastlanılmaktadır. Hastaneye yatış gerektiren durumlar incelendiğinde tahminen %2'sinin ilaç etkileşimlerinin neden olduğu yan etkilerden kaynaklandığını belirten çalışmalar mevcuttur [1]. Hastaların tedavi rejimlerinin karmaşık oluşu, eşlik eden hastalık varlığı, çok sayıda ilaç kullanımı, hastaların klinisyenin bilgisi dahilinde olmadan terapötik bitki veya reçetesiz ilaç kullanma girişimi potansiyel ilaç etkileşimini artıran faktörlerdendir [2,3]. Kanser hastalarının tedavi sürecini ilerleten multidisipliner ekibin önemli bir parçası olarak eczacının yer alması, tedaviyi sistematik olarak gözden geçirmesi ve analiz etmesi hayati önem taşımaktadır. Potansiyel etkileşimlerin, istenmeyen sonuçlarını önlemek veya en aza indirgemek için gerekli müdahaleler klinik eczacı tarafından yapılmalıdır. İlaçla ilgili sorunların önceden tespit edilerek azaltılması ve tedavinin optimize edilmesi terapötik başarıyı artırmaktadır [4-6].

Klinik eczacılar, hematopoetik hücre nakli hastalarının tedavi rejiminde multidisipliner ekibin önemli bir üyesi olarak yerini almaktadır. Özellikle antineoplastik ilaçların ve infeksiyon hastalıklarının ilaç tedavisinin yönetiminde, kemoterapi ilaçlarının danışmanlığında, terapötik ilaç düzeyinin izlenmesinde, potansiyel ilaç toksisitelerinin öngörülmesinde ve engellenmesinde, diğer ekip üyelerinin, aile bireylerinin ve hastanın ilaç tedavi rejimi eğitiminde aktif olarak rol oynamaktadır [7].

Hematopoetik kök hücre transplantasyonu, bir donörden (kişinin kendisi veya verici) kök hücrelerin toplanmasını ve bir hazırlık rejiminin ardından bu kök hücrelerin alıcıya nakledilmesini içeren bir prosedürdür. Kök hücre kaynağı olarak; kemik iliği, periferik kan kök hücre ve kordon kanı kullanılmaktadır [8]. Kemik iliği nakilleri en yaygın olarak lösemi, lenfoma, hodgkin lenfoma, multipl miyelom gibi doğrudan kemik iliğini etkileyen hastalıkların tedavisinde kullanılmaktadır. Ayrıca aplastik anemi, talasemi ve bağışıklık sisteminin konjenital eksiklikleri gibi durumlarda da bu prosedür uygulanmaktadır [9].

Farklı ülkelerdeki hematoloji kliniğinde yürütülen çalışmalarda, potansiyel ilaç etkileşimlerinin oranının %40-78 arasında olduğu belirtilmektedir [1,9-12]. Hematoloji kliniğinde ayakta tedavi alan hastaların tedavisinde kullanılan ve ilaç etkileşimine sık karşılaşılan ilaçlar arasında, flukonazol, sülfametoksazol-trimetoprim, doksorubisin, siklofosfamid ve metotreksat yer almaktadır [1,12,13]. Kemik iliği transplantasyonu sürecinde tedavide sıklıkla kullanılan busulfan, siklofosfamid, etoposit gibi birçok etken madde CYP450 izoenzimleriyle metabolize ve eliminize edilmektedir. Bu sebeple potansiyel ilaç etkileşimleri sıklıkla görülmekte ve terapötik başarıyı etkilemektedir [2].

Çalışmamızın amacı, erişkin kemik iliği transplantasyon ünitesinde tedavi gören hastalarda

potansiyel ilaç-ilaç etkileşimlerinin belirlenmesidir.

GEREÇ VE YÖNTEM

Çalışmamız prospektif kesitsel nitelikte olup, gerekli etik kurul onayı Marmara Üniversitesi Tıp Fakültesi Klinik Araştırmalar Etik Kurulu tarafından 09.2020.1374 nolu protokol ile onay almıştır. Çalışmamız Helsinki Deklerasyonu Prensipleri'ne uygun olarak yapılmıştır.

Çalışmamız, 1 Ocak 2021-1 Ağustos 2021 tarihleri arasında İstanbul'daki özel bir hastanenin "Erişkin Kemik İliği Transplantasyon Ünitesi"nde yürütülmüştür. Çalışmamıza kliniğe kemik iliği pre-veya post- transplantasyon tedavisi nedeniyle yatışı yapılmış olan 18 yaş ve üstü hastalar dahil edilmiştir.

Çalışmaya katılan hastaların sosyodemografik özellikleri kaydedilmiştir. Hastaların çalışmaya dahil edildikleri güne ait doktor isteminde yer alan ilaçlar arasındaki potansiyel etkileşimler "Medscape" ve "Lexicomp" veritabanları kullanılarak analiz edilmiştir. Lexicomp veri tabanı, ilaç etkileşimlerini 5 düzeyde rapor eder: X (kombinasyondan kaçının), D (tedavi değişikliğini düşünün), C (tedaviyi izleyin), B (eylem gerekmez) ve A (bilinen etkileşim yok). Medscape veri tabanı ise, ilaç etkileşimlerini 4 düzeyde bildirmektedir: kontrendike, ciddi kullanım alternatifi, yakından izleme ve minör.

İstatistiksel Analiz

Analizler için SPSS 15.0 istatistik programı kullanılmıştır. Hastaların sosyodemografik özellikleri ve ilaç etkileşimlerine ait ile ilgili veriler "%" olarak ifade edilmiştir. Kategorik veriler Ki-Kare testleri (Pearson ve Fisher exact testi) ile analiz edilmiştir. Sonuçlar %95 güven aralığında $p<0.05$ olarak anlamlı değerlendirilmiştir.

SONUÇ VE TARTIŞMA

Çalışmaya dahil edilen 40 hastanın medyan yaşı 48 (19-76) olup, %55'i erkek hastalardan oluşmaktadır. Çalışmaya katılan hastaların 14'ünde akut myeloid lösemi tanısı ile hastaneye yatış yapıldığı ve 12'sinde de hipertansiyon tanısının eşlik ettiği belirlenmiştir. Hastaların sosyodemografik özellikleri Tablo 1'de gösterilmiştir.

Çalışma süresince hazırlık rejimi ve/veya kemoterapi protokolünün uygulandığı hasta mevcut değildi. Bu nedenle hasta dosyalarında yer alan tedavi protokolleri değerlendirilmiştir. İlk üç sırada yer alan tedavi protokolleri sırasıyla, yüksek doz melfalan, Be-EAM (Bendamustin, etoposit, sitozin arabinozid, melfalan) ve Bu/Cy/ATG (Busulfan, siklofosfamid, anti t hücre globulini) protokollerinin sırasıyla %25, %17.5 ve %15 oranında uygulandığı tespit edilmiştir (Şekil 1).

Çalışmaya katılan hastaların, çalışmaya dahil edildikleri ilk güne ait olan doktor istemlerindeki ilaçlar ve potansiyel ilaç etkileşimleri değerlendirilmiştir. 40 adet doktor istemindeki toplam ilaç sayısı 391'dir. Çalışmaya dahil edilen hastalarda, hazırlık rejimi ve/veya kemoterapi protokolü uygulanmadığı için, ilaç etkileşimlerinin değerlendirildiği doktor istemlerinde kemoterapi ilaçları bulunmamaktadır. Toplam potansiyel ilaç-ilaç etkileşim sayısı Lexicomp veritabanına göre 299 [ortalama 7.5±0.7 (0-17)] ve Medscape veritabanına göre ise 214 [5.4±0.7 (0-22)] adet olarak tespit edilmiş olup (Tablo 2), istatistiksel olarak anlamlı fark tespit edilmiştir ($p<0,0001$). Eşlik eden hastalığı olan hastalarda, olmayan hastalara göre daha fazla etkileşim saptanmıştır (Ortalama 9 vs 6; $p<0.05$). Tablo 2'de etkileşim sayılarına ait ortalamalar gösterilmiştir.

Medscape veri tabanına göre kontrendike kategorisinde yer alan potansiyel ilaç etkileşimine giren etken maddeler amfoterisin b deoksilat, siklosporin, sidofovir, amikasin, indapamid ve flukonazol olarak; Lexicomp veritabanında ise X kategorisindeki potansiyel ilaç etkileşimine giren etken maddeler risperidone, metoklopramid, alprazolam, vorikonazol ve linezolid olarak tespit edilmiştir (Tablo 3). Tablo 4'te ise kemoterapi ilaçları dışında etkileşime en çok giren ilaçlar gösterilmiştir.

Kemik iliği transplantasyonu süresince farklı rejimlerde çoklu ilaç kullanımları mevcuttur. Bununla beraber eşlik eden diğer hastalıkların olma durumu, tedaviyi daha karmaşık hale getirebilmektedir. Klinik eczacılık hizmetleri kapsamında, transplantasyon tedavisi sürecinde, ilaç etkileşimlerinin değerlendirilmesi önemlidir. Çalışmamızda, özel bir hastanede yetişkin kemik iliği transplantasyonu ünitesinde yatan hastaların doktor istemleri değerlendirilmiş ve potansiyel ilaç-ilaç

etkileşimleri tespit edilmiştir.

Tablo 1. Hastalara ait sosyodemografik özellikler

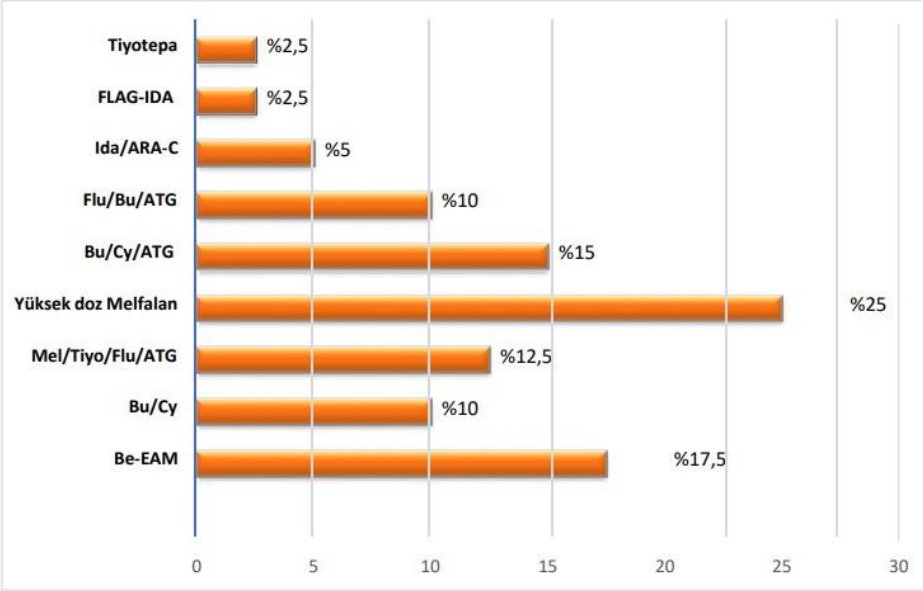
	n	%
Meslek		
Çalışmıyor	24	60
Çalışmıyor	16	40
Sigara Kullanımı		
Yok	24	60
Var	16	40
Eğitim Durumu		
İlköğretim	8	20
Lise	18	45
Üniversite	14	35
Eşlik Eden Hastalık		
Yok	17	42.5
Var	23	57.5
Eşlik Eden Hastalıklar		
Hipertansiyon	12	27
Diyabet	6	14
Obezite	4	9
KKY	3	7
KBY	3	7
Psikiyatrik Problemler	2	4.5
Hipotiroidi	2	4.5
Hepatit-B	2	4.5
Osteoporoz	1	2.3
KAH	1	2.3
Prostat Kanseri	1	2.3
Gut	1	2.3
SPH	1	2.3
STGS	1	2.3
BPH	1	2.3
KML	1	2.3
İnsülin Direnci	1	2.3
Hepatit-C	1	2.3
Kanser Türleri		
Akut Myleoid Lösemi	14	35
Multiple myeloma	10	25
Non-Hodgkin Lenfoma	7	17.5
Hodgkin Lenfoma	4	10
Akut Lenfoblastik Lösemi	3	7.5
KML	1	2.5
Anjioimmünoblastik T Hücreli Lenfoma	1	2.5

n: Hasta Sayısı; KKY: Konjestif Kalp Yetmezliği; KBY: Kronik Böbrek Yetmezliği; KAH: Koroner Arter Hastalığı; SPH: Sekonder Pulmoner Hipertansiyon; STGS: Sağ Testis Granülositik Sarkom; BPH: Benign Prostat Hiperplazisi; KML: Kronik Myeloid Lösemi

Çalışmamıza dahil edilen 18 yaş üstü 40 hastanın medyan yaşı 48 olup, %45'i kadın ve %55'i erkek hastadan oluşmaktadır. Sanchez ve ark. kemik iliği transplantasyonu ünitesinde tedavi görmekte olan 31 hasta üzerinde retrospektif bir çalışma yapmış ve potansiyel ilaç-ilaç etkileşimlerini incelemişlerdir [14]. Bu çalışmaya dahil edilen hastaların %51.6'sı kadın ve %48.4'ü ise erkek olup, medyan yaş 52 (19-66) bulunmuştur. Çalışmamızın popülasyonunun yaşı ve cinsiyet oranları farklı çalışmalarla da benzerlik göstermektedir [15-17].

Trevisan ve ark. hematopoietik kök hücre nakli için hastane yatışı yapılan 40 hasta üzerinde yaptıkları çalışmada, hastaların %52.5'inde eşlik eden hastalık olduğunu bildirmişlerdir [13]. En çok karşılaşılan hastalıkların; hipertansiyon (%94.4), dislipidemi (%33.3), hipotiroidizm (%16.7), diyalize bağımlı olmayan kronik böbrek yetmezliği (%16.7), kalp yetmezliği (%11.1) ve tip 2 diyabet (%11.1) olduğunu belirtmişlerdir [18]. Çalışmamızda ise hastaların %57.5'inde eşlik eden hastalık varlığı olduğu

tespit edilmiştir. Bu eşlik eden hastalıkların arasında en yüksek yüzdeye sahip hastalığın %52.2 ile hipertansiyon olduğunu ve sırasıyla diyabet (%26), obezite (%17.4), konjestif kalp yetmezliği (%13), kronik böbrek yetmezliği (%13) ve hipotiroidizm (%8.7) hastalıklarının olduğu tespit edilmiştir. Bu veriler doğrultusunda kemik iliği nakli yapılacak hastalarda eşlik eden kronik hastalıkların sayısının fazla olabileceği görülmektedir.



Şekil 1. Çalışmaya dahil edilen hastalara uygulanan kanser tedavi protokollerinin dağılımı (*FLAG-IDA: Fludarabin, sitozin arabinozid, idarubisin, granülosit koloni uyarıcı faktör; Ida/ARA-C: İdarubisin, sitarabin; Flu/Bu/ATG: Fludarabin, busulfan,anti t hücre globulini; Bu/Cy/ATG: Busulfan, siklofosfamid, anti T hücre globulini; Mel/Tiyo/Flu/ATG: Melfalan, tiyotepa, fludarabin, anti t hücre globulini; Bu/Cy: Busulfan, siklofosfamid; Be-EAM: Bendamustin, etoposid, sitarabin, melfalan)*

Tablo 2. Medscape ve Lexicomp veri tabanlarındaki potansiyel ilaç etkileşim sayılarının ortalamaları

Veri Tabanı	Etkileşim Derecesi	Ortalama±SH
Medscape (n=214)	Kontrendikasyon (n=4)	0.1±0.05
	Ciddi (n=15)	0.38±0.12
	Major (n=1)	0.02±0.03
	Yakından Takip Ediniz (n=125)	3.13±0.53
	Minör (n=69)	1.72±0.25
Lexicomp (n=299)	A (n=22)	0.6±0.13
	B (n=119)	2.98±0.25
	C (n=127)	3.18±0.45
	D (n=27)	0.68±0.19
	X (n=4)	0.1±0.05

SH: Standart Hata; n=etkileşim sayısı; X: kombinasyondan kaçının; D: tedavi değişikliğini düşünün; C: tedaviyi izleyin; B: eylem gerekmez; A: bilinen etkileşim yok

Tablo 3. Medscape veri tabanındaki (kontrendike ve ciddi) ve Lexicomp veritabanındaki (X ve D kategori) potansiyel ilaç etkileşimleri

		Potansiyel İlaç-İlaç Etkileşimleri	**Etkileşimin Sonucu**
Medscape	Kontrendike Etkileşimler	Amfoterisin b deoksilat-Siklosporin (n=1)	Her iki ilaç da nefrotoksisite ve/veya ototoksisite riskini artırmaktadır.
		Sidofovir-Siklosporin (n=1)	Her iki ilaç da nefrotoksisite ve/veya ototoksisiteriskini artırmaktadır.
		Amfoterisin b deoksilat- Amikasin (n=1)	Her iki ilaç da nefrotoksisite ve/veya ototoksisite riskini artırmaktadır.
		Flukonazol- İndapamid (n=1)	Her iki ilaç da QT aralığını uzatmaktadır.
	Ciddi Etkileşimler	Asiklovir-Amfoterisin b deoksilat (n=1)	Her iki ilaç da nefrotoksisite ve/veya ototoksisite riskini artırmaktadır.
		Klomipramin-Siprofloksasin (n=1)	Siprofloksasin CYP1A2 enzim grubunu etkileyerek klomipramin düzeyini artırmaktadır. Additif etki oluşturarak QT aralığını uzatıp ventriküler aritmi riskini artırmaktadır.
		Siklosporin-Teikoplanin (n=2)	Her iki ilaç da nefrotoksisite ve/veya ototoksisite riskini artırmaktadır.
		Pantoprazol-Digoksin (n=1)	Pantoprazol, gastrik pH'ı artırarak digoksinin etkisini artırmaktadır.
		Klomipramin-Flukonazol (n=2)	Her iki ilaç da QT aralığını uzatmaktadır. Flukonazol, CYP3A4 hepatik enzim ailesini etkileyerek klomipraminin etkisini artırmaktadır.
		Klomipramin-Granisetron (n=1)	Her iki ilaç da QT aralığını uzatmaktadır. Her iki ilaç da birbirinin serotonin seviyesini artırarak toksisiteye sebep olabilir.
		Aprepitant-Kolşisin (n=1)	Aprepitant, CYP3A4 hepatik enzim ailesini etkileyerek kolşisinin etkisini artırmaktadır.
		Siklosporin-Tenofovir (n=1)	Her iki ilaç da nefrotoksisite ve/veya ototoksisite riskini artırmaktadır.
		Amfoterisin b deoksilat-Kolistin (n=1)	Her iki ilaç da nefrotoksisite ve/veya ototoksisite riskini artırmaktadır.
		Metoklopramid-Essitalopram (n=1)	Her iki ilaç da serotonin seviyesini artırarak serotonin sendromu riskini artırmaktadır.
		Essitalopram-Granisetron (n=2)	Her iki ilaç da birbirinin serotonin seviyesini artırarak toksisiteye sebep olabilir.
		Flukonazol-Moksifloksasin (n=1)	Her iki ilaç da QT aralığını uzatmaktadır.
Lexicomp	X Category	Risperidon-Metoklopramid (n=1)	Metoklopramid, antipsikotik ajanların yan/toksik etkilerini artırabilir; extrapyramidal reaksiyonlara veya nöroleptik malign sendroma sebep olabilir.
		Alprazolam-Vorikonazol (n=1)	Vorikonazol gibi güçlü CYP3A4 inhibitörleri alprazolamın serum konsantrasyonunu artırabilir.
		Linezolid-Metoklopramid (n=2)	Metoklopramid, linezolid gibi monoamine oksidazinhibitörlerinin hipertansif etkilerini artırabilir.
	D Kategorisi	Allopurinol- Gaviscon (alüminyum hidroksit) (n=1)	Alüminyum hidroksit, allopurinolün serum konsantrasyonunu artırabilir.
		Deksametazon-Gaviscon (antasitler) (n=1)	Antiasitler, deksametazon gibi korktikosteroidlerinbiyoyararlanımını düşürebilir.
		Siprofloksasin-Kalsiyum 600/d vitamini(antasitler) (n=2)	Antiasitler, oral formdaki siprofloksasin gibi kinolonların absorbsiyonunu azaltabilirler.
		Siprofloksasin- Kalsiyum tuzları (n=2)	Kalsiyum tuzları, oral formdaki siprofloksasin gibi kinolonların absorbsiyonunu azaltabilirler.
		Siprofloksasin- Magnezyum hidroksit (magnezyum tuzları) (n=4)	Magnezyum tuzları, oral formdaki siprofloksasin gibikinolonların absorbsiyonunu azaltabilirler.

Tablo 3 (*devamı*). Medscape veri tabanındaki (kontrendike ve ciddi) ve Lexicomp veritabanındaki (X ve D kategori) potansiyel ilaç etkileşimleri

		Potansiyel İlaç-İlaç Etkileşimleri	**Etkileşimin Sonucu**
Lexicomp	D Kategorisi	Siprofloksasin- Çinko sülfat (n=2)	Çinko tuzları, oral formdaki siprofloksasin gibi kinolonların absorbsiyonunu azaltabilirler.
		Siklosporin-Vorikonazol (n=1)	Vorikonazol gibi antifungal ajanlar, siklosporin metabolizmasını azaltabilirler.
		Gaviscon (alüminyum hidroksit)- Kolekalsiferol (n=1)	Kolekalsiferol, alüminyum hidroksitin serum konsantrasyonunu artırabilir.
		Metotreksat-Pantoprazol (n=2)	Proton pompası inhibitörleri, metotreksatın serum konsantrasyonunuazaltabilir.
		Deksametazon-Aprepitant (n=1)	Aprepitant, deksametazonun serum konsantrasyonunu artırabilir.
		Klomipramin-Tramadol (n=1)	Trisiklik antidepresanlar, serotonerjik opioidlerin merkezi sinir sistemiüzerindeki depresan etkisini artırabilir. Serotonerjik opioidler, trisiklik antidepresanların serotonerjik etkisini artırabilir.
		Hidrokortizon-Aprepitant (n=1)	Aprepitant, hidrokortizonun serum konsantrasyonunu artırabilir.
		Aprepitant-Kolşisin (n=1)	Aprepitant gibi CYP3A4 inhibitörleri, kolşisinin serum konsantrasyonunuartırabilir.
		Alprazolam-Flukonazol (n=4)	CYP3A4 inhibitörleri, alprazolamın serum konsantrasyonunu artırabilir.
		Kolistin-Amikasin (n=1)	Aminoglikozidler, kolistinin nefrotoksisite ve nöromüsküler blokaj etkisiniartırabilir.
		Kolistin-Amfoterisin b deoksilat (n=1)	Amfoterisin b deoksilat, kolistinin nefrotoksik etkisini artırabilir.
		Enoksaparin-Essitalopram (n=1)	Antiplatelet özellikli ajanlar, enoksaparinin antikoagülan etkisini artırabilir.

n: hasta sayısı; X: kombinasyondan kaçının; D: tedavi değişikliğini düşünün

Tablo 4. Medscape ve Lexicomp veri tabanında tespit edilen potansiyel ilaç-ilaç etkileşimlerinde en çok karşılaşılan kemoterapötik olmayan ilaçların listesi

	Etken Madde	**Sayı**	**Yüzde**
Medscape	Amfoterisin b deoksilat	4	10.5
	Siklosporin	5	13.2
	Flukonazol	4	10.5
	Teikoplanin	2	5.3
	Klomipramin	4	10.5
	Granisetron	3	7.9
	Essitalopram	3	7.9
Lexicomp	Metoklopramid	3	4.8
	Alprazolam	5	8.1
	Vorikonazol	2	3.2
	Linezolid	2	3.2
	Gaviscon (alüminyum hidroksit)	2	3.2
	Deksametazon	2	3.2
	Siprofloksasin	12	19.4
	Kalsiyum 600/vitamin d	2	3.2
	Kalsiyum tuzları	2	3.2
	Mg hidroksit (magnezyum tuzları)	4	6.5
	Çinko sülfat	2	3.2
	Metotreksat	2	3.2
	Pantoprazol	2	3.2
	Aprepitant	3	4.8
	Flukonazol	6	9.7
	Kolistin	2	3.2

Çalışmamıza dahil edilen hastaların %35'i akut miyeloid lösemi, %25'i multipl miyelom, %17.5'i ise non-hodgkin lenfoma tanısı olduğu belirlenmiştir. Yetişkin kemik iliği transplantasyonu yapılan hastalar üzerinde yapılmış diğer çalışmalarda da en sık karşılaşılan hematolojik malignite akut myeloid lösemi olarak karşımıza çıkmaktadır [14,16,19,20].

Guastaldi ve ark. yaptıkları kesitsel çalışmada, kemik iliği transplantasyonu ünitesinde yatmakta olan hastaların tedavi rejimlerinde görülebilecek potansiyel ilaç- ilaç etkileşimlerinin sıklığını ve derecesini belirlemişlerdir [19]. Çalışmaya 70 hasta dahil edilmiş ve kemik iliği transplantasyonundan önceki günün reçeteleri incelenmiştir. Potansiyel ilaç-ilaç etkileşimlerini belirlemek için Micromedex veri tabanı kullanılmış ve toplam 128 adet etkileşim tespit edilmiştir [19]. Bu etkileşimlerin %85.9'u orta derece ve %14.1'i majör etkileşim olarak bulunmuştur. Elde edilen verilere göre hastaların %60'ında en az 1 potansiyel ilaç-ilaç etkileşimi olduğu ve %21.4'ünde en az bir major potansiyel ilaç-ilaç etkileşimi olduğu görülmüştür. En çok karşılaşılan ilaç etken maddeleri; siklosporin (%28.1), fenitoin (%25), flukonazol (%15.6) olarak bulunmuştur. Çalışmamızda hastaların doktor istemlerindeki ilaç-ilaç etkileşim sayısı Lexicomp veritabanına göre toplam 299 ve Medscape veritabanına göre ise 214 adet etkileşim tespit edilmiştir. Lexicomp veri tabanında tespit edilen 299 ilaç-ilaç etkileşiminin %43'ü C kategorisi, olarak bulunmuştur. Lexicomp veri tabanında ilaç- ilaç etkileşiminde en çok tespit edilen ilaç etken maddeleri ise sırasıyla siprofloksasin (%19.4), flukonazol (%9.7), alprazolam (%8.1) olarak tespit edilmiştir. Çalışmamızda, Medscape veri tabanında tespit edilen toplam 214 adet potansiyel ilaç-ilaç etkileşiminin %58'i yakından takip ediniz kategorisinde bulunmuştur. Medscape veri tabanında tespit edilen ilaç- ilaç etkileşimlerinde en çok karşılaşılan ilaç etken maddeleri ise siklosporin (%13.2), flukonazol (%10.5), klomipramin (%10.5), amfoterisin-b deoksilat (%10.5) olarak bulunmuştur. Elde edilen bu veriler doğrultusunda, çalışmamız bahsedilen çalışmanın verileriyle benzerlik göstermektedir. Kemik iliği transplantasyonu ünitesinde yatan hastaların çeşitli tedavi rejimlerinin çoklu ilaç içermesi sebebiyle potansiyel ilaç-ilaç etkileşimlerinin görülme sıklığı ve önemi artmaktadır. Tedavi süreci boyunca, özellikle ciddi etkileşimlere yol açabilecek ilaçlar klinik eczacı tarafından yakından izlenmeli ve terapötik başarısızlığa yol açabilecek faktörler tespit edilerek eliminize edilmelidir.

Çalışmamızın popülasyonu, uygulanan nakil çeşitleri ve tedaviler, ulusal ve uluslararası çalışmalarla paralellik göstermektedir. Kemik iliği transplantasyon hastalarının yarısından fazlasında, genç popülasyon olmasına rağmen, eşlik eden hastalık sayısı oldukça fazladır. Bu nedenle hem kronik olarak kullanılan ilaç sayısı hem de nakil süresince uygulanan tedavilerdeki ilaç sayısının fazla olması, polifarmasi ve ilaç kaynaklı olası sorunlardan biri olan ilaç etkileşimlerinin ortaya çıkmasına yol açmaktadır. Çalışmamızda, siklosporin, granisetron, klomipramin, flukonazol, siklosporin ve amfoterisin-b deoksilat ilaç etkileşimlerine sık rastlanılan ilaçlar olarak karşımıza çıkmıştır. Lexicomp veritabanında, klinik uygulamayla ilgili daha ayrıntılı bilgilere ulaşılmış ve daha fazla ilaç etkileşimi tespit edilmiştir. Medscape veri tabanında ise daha az ilaç etkileşimine rastlanmasına rağmen, özellikle polifarmasiye eğilimli hastalar için en az iki ilaç veri tabanının kullanılmasının faydalı olacağını düşünmekteyiz. Elde edilen bu veriler doğrultusunda, çalışmamız bahsedilen çalışmanın verileriyle İlaç etkileşimlerinin kanıta dayalı ve multidisipliner ekiple birlikte yönetilmesi için, ekipte klinik eczacının olması gerektiği ulusal ve uluslararası çalışmalarda vurgulanmaktadır. Çalışmamızın sonuçlarına göre ilaç etkileşim sayısı oldukça fazladır. Klinik eczacı eğitimi almış olan bir eczacının yer aldığı ekipte bu etkileşimlerin tespitinin ve yönetiminin akılcı ve güvenilir bir şekilde sağlanacağını ve klinik eczacının olumlu katkıları olacağını düşünmekteyiz. İlaç etkileşimlerinin farklı hematolojik maligniteleri olan hastalar üzerindeki gerçek klinik ve ekonomik etkilerini değerlendirmek için büyük, çok merkezli, prospektif, standart çalışmalara ihtiyaç vardır.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

APPLICATION OF PLACKETT-BURMAN DESIGN FOR DEVELOPMENT AND EVALUATION OF A BETAMETHASONE SUSPENSION FOR INJECTION FORMULATION

BİR BETAMETAZON ENJEKSİYONLUK SÜSPANSİYON FORMÜLASYONUNUN GELİŞTİRİLMESİ VE DEĞERLENDİRİLMESİ İÇİN PLACKETT-BURMAN TASARIMININ UYGULANMASI

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ABSTRACT

Objective: *In this study, a quality-by-design (QbD) approach was used to develop a betamethasone suspension for injection formulation and to investigate the possible effects of formulation and process variables on the critical quality attributes (CQAs) of the formulation.*

Material and Method: *It was determined that the CQAs of the formulation were particle size distribution, viscosity, sedimentation time, density and assay of active substances and preservatives, considering the quality target product profile (QTPP). Potential risk factors that may affect the CQAs of the formulation were identified using an Ishikawa diagram, and a six-factor, two-level Plackett-Burman experimental design was used to statistically investigate the effects of selected formulation and process variables. The prepared formulations were tested, and variance and multiple linear regression analyses were performed with the acquired data.*

Result and Discussion: *As a result of the one-way analyses of variance (ANOVA) and multiple linear regression analyses, the established statistical models for the assay of methyl parahydroxybenzoate and propyl parahydroxybenzoate, and viscosity were found to be significant, the established models for other independent variables were not significant. The concentration of carmellose calcium and filter type was found to be the most significant formulation and process variables. In conclusion, this study showed that understanding the formulation and process*

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variables that may affect the CQAs of injectable suspension formulations with a QbD approach could be useful for formulation development and optimization.
Keywords: *Betamethasone, design of experiments, Plackett-Burman, quality-by-design, suspension*

ÖZ

Amaç: *Bu çalışmada, bir betametazon enjeksiyonluk süspansiyon formülasyonu geliştirmek, formülasyon ve üretim değişkenlerinin formülasyonun kritik kalite özellikleri (CQAs) üzerindeki olası etkilerini araştırmak amacıyla kalite tasarımı yaklaşımı kullanılmıştır.*
Gereç ve Yöntem: *İlk olarak hedef ürün kalite profili (QTPP) dikkate alınarak formülasyonun kritik kalite özelliklerinin partikül büyüklüğü dağılımı, viskozite, sedimentasyon süresi, yoğunluk ve etkin maddeler ile koruyucuların miktar tayini olduğu belirlenmiştir. Formülasyonun kritik kalite özelliklerini etkileyebilecek potansiyel risk faktörleri Ishikawa diagram ile tanımlanmış, seçilen formülasyon ve proses değişkenlerinin etkilerini istatistiksel olarak araştırmak için altı faktörlü, iki seviyeli bir Plackett-Burman deney tasarımı kullanılmıştır. Hazırlanan formülasyonlar test edilmiş, elde edilen veriler ile varyans ve çoklu lineer regresyon analizleri yapılmıştır.*
Sonuç ve Tartışma: *Tek yönlü varyans analizi (ANOVA) ve çoklu lineer regresyon analizleri sonucunda, metil parahidroksibenzoat miktar tayini, propil parahidroksibenzoat miktar tayini ve viskozite için kurulan istatistiksel modeller anlamlı bulunurken, diğer bağımsız değişkenler için kurulan modeller anlamlı bulunmamıştır. Karmelloz kalsiyum konsantrasyonu ve filtre tipinin en kritik formülasyon ve proses değişkenleri olduğu görülmüştür. Sonuç olarak, bu çalışma enjeksiyonluk süspansiyon formülasyonlarının kritik kalite özelliklerini etkileyebilecek formülasyon ve proses değişkenlerinin QbD yaklaşımı ile anlaşılmasının formülasyon geliştirilmesi ve optimizasyonu için fayda sağlayabileceğini göstermiştir.*
Anahtar Kelimeler: *Betametazon, deney tasarımı, kalite tasarımı, Plackett-Burman, süspansiyon*

INTRODUCTION

Betamethasone is a synthetic glucocorticoid that has anti-inflammatory, immunosuppressive and antiallergic effects in disorders of many organ systems [1-4]. Betamethasone is available in several ester forms such as dipropionate, acetate, sodium phosphate, valerate, and benzoate, and in various dosage forms such as ointment, lotion, cream, injectable suspension/solution, tablet, syrup and aerosol [5,6].

The approved products in injectable suspension dosage forms contain a combination of betamethasone dipropionate and betamethasone sodium phosphate, as well as a combination of betamethasone acetate and betamethasone sodium phosphate. These two combination products are indicated for the treatment of acute and chronic corticosteroid-response disorders such as rheumatoid arthritis, osteoarthritis, bursitis, ankylosing spondylitis, chronic bronchial asthma, atopic dermatitis, discoid lupus erythematosus, psoriasis, keloids, and that are administered via intramuscular, intraarticular, periarticular, intrabursal, intradermal, and intralesional injection [7,8]

Betamethasone sodium phosphate is a soluble ester of betamethasone, responsible for the immediate activity, while betamethasone dipropionate is practically insoluble in water and provides sustained activity to control symptoms over a longer period [3,7,8]. Betamethasone dipropionate is suspended in water by using a suspending agent, a viscosity increaser agent, and a surface-active agent. In addition, it is necessary to use a filter for the sterilization of the product. Therefore, several formulations and process parameters can have potential effects on the critical quality attributes (CQAs) of formulations.

As defined by the International Conference on Harmonisation (ICH), Quality-by-Design (QbD) is a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and control, based on sound science and quality risk management [7]. Design of experiments (DoE) within the scope of QbD enhances formulation development capability and speed by allowing changing of more than one factor at the same time compared to conventional experimental approaches [7,8]. Many experimental designs are used for the optimization of products and processes. Screening designs are comprised of factorial designs and Plackett-Burman design (PBD), which are used to identify the most crucial independent variables that influence the predetermined responses [9-13].

The purpose of this study is to develop a betamethasone suspension for injection formulation using a QbD approach to investigate the potential risk factors that may affect the CQAs and to understand the influences of formulation and process parameters on the CQAs of injectable suspension formulations.

MATERIAL AND METHOD

Materials

Betamethasone dipropionate and betamethasone sodium phosphate were purchased from Symbiotica (Butterworth, Pulau Pinang, Malaysia). Additionally, the following excipients were used throughout the formulation: Polysorbate 80 (Merck KGaA, Darmstadt, Germany), Macrogol (BASF, Ludwigshafen, Germany), Carmellose Sodium (Ashland, Alizay, France), Sodium Phosphate Dibasic Anhydrate (Merck KGaA, Darmstadt, Germany), Sodium Chloride (Merck KGaA, Darmstadt, Germany), Benzyl Alcohol (Merck KGaA, Darmstadt, Germany), Methyl Parahydroxybenzoate (Lanxess Distribution GmbH, Leverkusen, Germany), Propyl Parahydroxybenzoate (Lanxess Distribution GmbH, Leverkusen, Germany), Disodium Edetate (Merck KGaA, Darmstadt, Germany), Hydrochloric Acid (Concentrated) (Merck KGaA, Darmstadt, Germany). 0.2 µm pore size cellulose acetate (CA) and polytetrafluoroethylene (PTFE) filters were purchased from Sartorius GmbH (Göttingen, Germany). All other chemicals were of analytical reagent grade.

Preparation of Betamethasone Suspension for Injection Formulation

Suspension for injection formulation was prepared using an IKA RCT Basic Magnetic Stirrer (Staufen, Germany), an IKA RW20 Digital Mechanical Overhead Stirrer (Staufen, Germany), and an IKA T25 Digital Ultra-Turrax (Staufen, Germany). Briefly, methyl parahydroxybenzoate and propyl parahydroxybenzoate were dissolved in water for injections at 80-85°C. After these two excipients are completely dissolved, the solution was then cooled down to 20-25°C. Benzyl alcohol, sodium chloride, disodium edetate, macrogol, carmellose sodium, betamethasone sodium phosphate and polysorbate 80 were added to the solution, respectively after each is completely dissolved. The pH of the solution was adjusted to pH 7.0 ± 0.1 using the hydrochloric acid solution. The weight of the solution was completed to the quantity that was stated in the manufacturing batch record using water for injections. The solution was filtered using the filter specified in the experimental design. Sterile betamethasone dipropionate was added to the filtered solution and stirred for 10 min. Then, the obtained suspension was homogenised using an ultra-turrax at different speeds. The prepared suspensions were stored in glass bottles for subsequent analyses.

Risk Identification: Ishikawa Diagram

An Ishikawa diagram was established for the risk identification of the formulation and the process parameters given in the manufacturing method in section of Preparation of Betamethasone Suspension for Injection Formulation, and to understand their potential effects on the CQAs of the formulation [14].

Table 1. Quality Target Product Profile (QTPP) for betamethasone suspension for injection formulation

QTPP Elements	Target
Dosage form	Injectable Suspension
Route of administration	Intramuscular, intraarticular, periarticular, intrabursal, intradermal, and intralesional injection
Dosage strength	6.43 mg/ml of Betamethasone Dipropionate, and 2.63 mg/ml of Betamethasone Sodium Phosphate
Drug product quality attributes	Physical Attributes (particle size distribution, viscosity, sedimentation time, density)
	Identification
	Assay (active substances and preservatives)
	Dissolution
	Impurities
	Microbiological quality

Based on the physicochemical characteristics as well as the in vitro dissolution characteristics of the reference product, a quality target product profile (QTPP) was defined for the betamethasone suspension for injection formulation (Table 1). According to the QTPP and prior scientific knowledge about injectable suspension formulations, particle size distribution, viscosity, sedimentation time, density, and an assay of active substances and preservatives were considered as the CQAs of the betamethasone suspense on for injection formulation (Table 2) [15-17].

Table 2. Critical Quality Attributes (CQAs) of betamethasone suspension for injection formulation

CQA	**Target**
Particle size distribution (d50)	≤ 20 µm
Viscosity	7.0 – 13.0 cP (20°C)
Sedimentation time	≤ 10 min
Density	0.9000 – 1.1000 g/ml
Assay of benzyl alcohol (BA)	90.0 – 110.0%
Assay of methyl parahydroxybenzoate (MP)	90.0 – 110.0%
Assay of propyl parahydroxybenzoate (PP)	90.0 – 110.0%
Assay of betamethasone sodium phosphate (BSP)	90.0 – 110.0%
Assay of betamethasone dipropionate (BDP)	90.0 – 110.0%

Experimental Design

The formulation and process parameters shown in the Ishikawa diagram were examined within the scope of a failure modes and effects analysis (FMEA) [18]. Macrogol type, concentration of polysorbate 80 and carmellose sodium were selected as critical formulation variables; while filter type, homogenization time and homogenization rate were selected as critical process variables. A Plackett-Burman statistical experimental design was performed to understand the effects of independent variables on the CQAs. The variable levels were chosen considering the previous experiments and prior scientific knowledge. As shown in Table 3, six independent variables were examined at two levels.

Minitab 19 (Minitab Inc.; State College, PA, USA) software was used to randomize the design matrix and for statistical analyses, and twelve experiments were prepared for six independent variables (Table 4). Multilinear regression analysis and one-way analyses of variance (ANOVA) were performed to test the significance of the model and the factor coefficients [19].

The response variables (CQAs) were particle size distribution (Y_1), viscosity (Y_2), sedimentation time (Y_3), density (Y_4), the assay of benzyl alcohol (Y_5), the assay of methyl parahydroxybenzoate (Y_6), the assay of propyl parahydroxybenzoate (Y_7), the assay of betamethasone sodium phosphate (Y_8), and the assay of betamethasone dipropionate (Y_9).

Table 3. The independent variables and their levels used in the Plackett-Burman Design

Independent Variables	**Levels**	
	Low	**High**
X_1: Macrogol Type	3350	4000
X_2: Concentration of Polysorbate 80 (mg/ml)	0.25	0.50
X_3: Concentration of Carmellose Sodium (mg/ml)	5.00	6.00
X_4: Filter Type	CA	PTFE
X_5: Homogenization Time (min)	5	10
X_6: Homogenization Speed (rpm)	3000	5000

Table 4. Plackett-Burman Design experimental matrix

Formulation Code	X_1 Macrogol Type	X_2 Concentration of Polysorbate 80 (mg/ml)	X_3 Concentration of Carmellose Sodium (mg/ml)	X_4 Filter Type	X_5 Homogenization Time (min)	X_6 Homogenization Speed (rpm)
F01	4000	0.25	6	CA	5	5000
F02	3350	0.50	5	PTFE	5	5000
F03	3350	0.50	6	PTFE	10	3000
F04	3350	0.25	6	CA	10	3000
F05	3350	0.50	6	CA	5	5000
F06	4000	0.50	5	CA	10	3000
F07	4000	0.25	6	PTFE	5	3000
F08	4000	0.50	6	PTFE	10	5000
F09	3350	0.25	5	PTFE	5	3000
F10	4000	0.25	5	PTFE	10	5000
F11	3350	0.25	5	CA	10	5000
F12	4000	0.50	5	CA	5	3000

Characterization of the Formulations

Particle Size Distribution

The particle size distribution of the suspension was measured by the laser diffraction method using a Mastersizer 3000E (Malvern Instruments Ltd., Malvern, UK) at 2000 rpm of stirring rate, 50% of ultrasound, and 10-20% of obscuration level. For this, approximately 10 ml of suspension was added directly to 600 ml of purified water as the dispersant. Measurements were performed in triplicate.

Viscosity

The viscosity of the suspension was measured using a rotating viscometer (DV3T LV, Brookfield, Middleborough, United States) equipped with an enhanced UL adapter, a 0 spindle at 60-80 rotation speed at 20°C. For this, 16 ml of suspension was transferred into a sample container and the sample container temperature is adjusted to 20°C. Then, the spindle was immersed into the sample, the sample was stirred, and the apparent viscosity of the sample was then measured. Measurements were performed in triplicate.

Sedimentation Time

The sedimentation time of the suspension was measured by using a 30 ml volumetric cylinder at 20°C. For this, 20 ml of suspension was transferred into the volumetric cylinder, and the time until complete sedimentation (until forming a clear solution on the top and sediment on the bottom) was measured. Measurements were performed in triplicate.

Density

The density of the suspension was measured by using a Mettler Toledo DM-40 Density Meter (Mettler Toledo, Columbus, Ohio, United States) at 20°C. For this, approximately 10 ml of suspension was injected into the sampling unit of the density meter and the results were recorded. Measurements were performed in triplicate.

Assay of the Active Substances and Preservatives

A high-pressure liquid chromatography (HPLC) method was used for the assay of benzyl alcohol, methyl parahydroxybenzoate, propyl parahydroxybenzoate, betamethasone sodium phosphate and betamethasone dipropionate (Agilent 1260 Infinity II HPLC, Agilent, Santa Clara, California, United States). The column was a C18; 150 x 4.6 mm, 4 µm (Agilent, Zorbax Poroshell EC-120), and the detector was a DAD/UV set at 254 nm. The flow rate of the mobile phase was 1.2 ml/min at gradient

conditions. The injection volume was 10 µl, the autosampler temperature was set at 10°C and the column thermostat temperature was maintained at 45°C.

Storage Stability Study

The accelerated (40°C ± 2°C/75% ± 5% RH) and long-term (25°C ± 2°C/60% ± 5% RH) stability studies were carried out to investigate the physicochemical stability of betamethasone suspension for injection formulation for 6 months. The suspension samples were analyzed at the initial time point, 3rd and 6th months for both conditions. Particle size distribution, density, assay of BA, assay of MP, assay of PP, assay of BSP, assay of BDP and impurity analyses were conducted to evaluate the physicochemical stability.

RESULT AND DISCUSSION

Assay of the Active Substances and Preservatives

A rapid, precise, and accurate HPLC method was developed and validated for robustness, selectivity, specificity, linearity, precision, solution stability and accuracy as per the ICH Q2 (R1) guideline [20]. The system suitability parameters of the developed HPLC method are given in Table 5. The run time of the analysis was 25 min, while the retention time of benzyl alcohol, methyl parahydroxybenzoate, propyl parahydroxybenzoate, betamethasone sodium phosphate and betamethasone dipropionate were 2.6 min, 3.2 min, 8.9 min, 10 min and 16.8 min, respectively. The sample chromatogram belonging to specificity study is given in Figure 1, and the sample chromatogram obtained from the standard solution is given in Figure 2.

Figure 1. Results of specificity study: Blank solution chromatogram, placebo solution chromatogram, standard solution chromatogram, test solution chromatogram

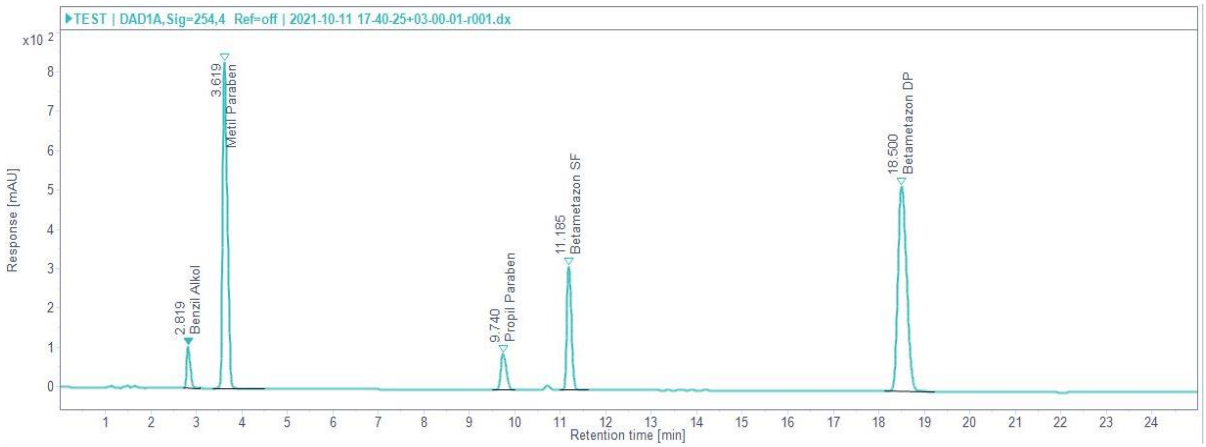

Figure 2. The sample chromatogram obtained from the standard solution and the retention time active substance and preservatives

Table 5. The system suitability parameters of the developed HPLC method

System Suitability Parameters	BA	MP	PP	BSP	BDP
Retention time (min) [a]	2.7 (0.1%)	3.4 (0.1%)	9.5 (0.2%)	11.0 (0.2%)	18.3 (0.1%)
Capacity factor [b]	2.3	3.2	10.9	12.8	21.9
Resolution [c]	-	5.1	32.8	7.6	28.11
Theoretical plate numbers	45643	48676	205983	409555	306674
Peak asymmetry (%10)	1.1	1.2	1.2	1.2	1.1

BA: Benzyl Alcohol, MP: Methyl Parahydroxybenzoate, PP: Propyl Parahydroxybenzoate, BSP: Betamethasone Sodium Phosphate, BDP: Betamethasone Dipropionate.
[a]The values given in blankets were RSD % of retention times (n=10), [b]Dead retention time was found with the injection of uracil at the same conditions: t_0 = 0.8 min. [c]Values are resolution between adjacent peaks.

Risk Identification: Ishikawa Diagram

An Ishikawa diagram was used for identifying the risks and to examine their potential effects on the CQAs of the formulation. Three formulation variables and three process variables that may have an impact on the CQAs of the formulation were identified using the Ishikawa diagram, which are the concentration of carmellose sodium, macrogol molecular weight, concentration of polysorbate 80, filter type, homogenization time, homogenization speed, and are given in red in Figure 3. The influence of these variables was investigated within the context of a follow-up Plackett-Burman experimental design.

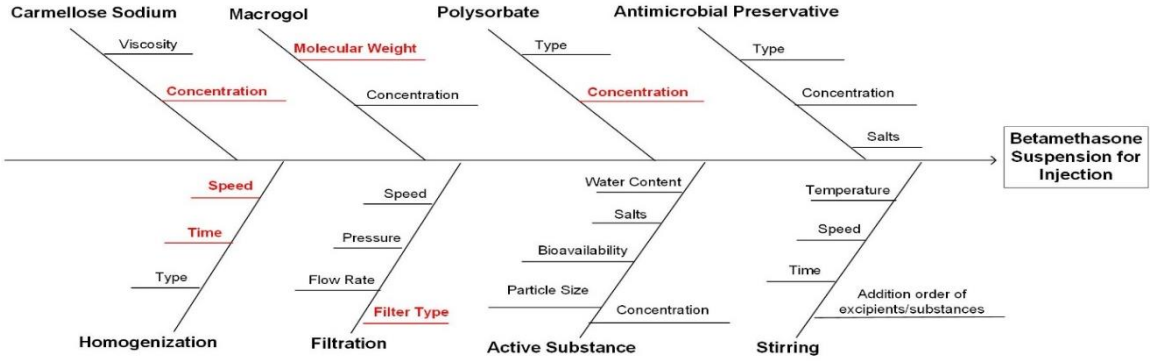

Figure 3. An Ishikawa diagram showing formulation and process variables which may have an impact on the CQAs of the formulation

Experimental Design

A Plackett-Burman design was used to establish an appropriate screening strategy for the CQAs of betamethasone suspension for injection formulation. Twelve experiments were run to screen the effects of the formulation (X_1: Macrogol Type, X_2: Concentration of Polysorbate 80 (mg/ml), X_3: Concentration of Carmellose Sodium (mg/ml)), and process variables (X_4: Filter Type, X_5: Homogenization Time (min), X_6: Homogenization Speed (rpm)) that were identified using the Ishikawa diagram. The twelve formulations that were tested and observed response variables are given in Table 6.

Table 6. Observed response variables through the Plackett-Burman Design

Formulation Code	Y_1: Particle size distribution (d50) (µm)	Y_2: Viscosity (cP)	Y_3: Sedimentation Time (min)	Y_4: Density (g/ml)	Y_5: Assay (%) of BA	Y_6: Assay (%) of MP	Y_7: Assay (%) of PP	Y_8: Assay (%) of BSP	Y_9: Assay (%) of BDP
F01	8.1	12.5	12.4	1.0118	96.50	93.70	82.80	101.40	99.45
F02	8.2	10.7	9.2	1.0111	96.30	96.50	93.20	100.20	101.20
F03	8.7	11.5	12.6	1.0106	97.10	97.60	94.80	100.40	98.80
F04	8.7	12.3	9.5	1.0110	96.90	91.60	75.00	99.70	100.20
F05	8.7	12	10.4	1.0111	95.40	90.00	76.40	99.20	99.20
F06	8.6	10.9	11.2	1.0111	97.80	91.20	79.80	98.90	98.00
F07	8.3	11.9	12.2	1.0110	98.90	97.70	92.80	98.80	100.50
F08	8.2	12	13.5	1.0116	98.60	98.60	97.10	98.50	98.40
F09	8.3	10.5	12.7	1.0110	98.80	99.50	96.00	100.30	100.60
F10	8.3	10.9	11.2	1.0110	96.80	98.00	95.00	98.50	100.00
F11	8	10.7	9.5	1.0098	98.10	93.60	83.70	97.40	96.90
F12	8.3	11.7	12.8	1.0110	97.20	93.80	86.00	96.70	98.80

BA: Benzyl Alcohol, MP: Methyl Parahydroxybenzoate, PP: Propyl Parahydroxybenzoate, BSP: Betamethasone Sodium Phosphate, BDP: Betamethasone Dipropionate.

As a result of the ANOVA and multiple linear regression analyses, the established statistical models for viscosity (Y_2), the assay of MP (Y_6) and the assay of PP (Y_7) as response variables were found to be significant ($p < 0.05$) while the established models for other independent variables, particle size distribution (Y_1), sedimentation time (Y_3), density (Y_4), the assay of BA (Y_5), the assay of BSP (Y_8) and the assay of BDP (Y_9), were not statistically significant ($p > 0.05$) (Table 7). After a multiple linear regression analysis of the data, the following polynomial equations were constructed to describe the quantitative impact of the independent variables on the responses (Equations (1-9)).

$$\text{Particle size distribution (µm)}(Y_1) = 7.517 - 0.0667\,X_1 + 0.667\,X_2 + 0.167\,X_3 + 0.0333\,X_4 + 0.0200\,X_5 - 0.000117\,X_6 \quad (1)$$

$$\text{Viscosity (cP)}(Y_2) = 5.48 + 0.1833\,X_1 - 0.000\,X_2 + 1.133\,X_3 + 0.2167\,X_4 - 0.0333\,X_5 - 0.000000\,X_6 \quad (2)$$

$$\text{Sedimentation Time (min)}(Y_3) = 9.37 + 0.783\,X_1 + 1.47\,X_2 + 0.667\,X_3 - 0.467\,X_4 - 0.073\,X_5 - 0.000400\,X_6 \quad (3)$$

Density (g/ml) (Y_4)= 1.000910 + 0.000242 X_1 + 0.00060 X_2 + 0.000350 X_3 - 0.000042 X_4 - 0.000063 X_5 + 0.000000 X_6 (4)

Assay of BA (%) (Y_5)= 99.38 + 0.333 X_1 - 1.87 X_2 - 0.133 X_3 - 0.317 X_4 + 0.100 X_5 - 0.000350 X_6 (5)

Assay of MP (%) (Y_6)= 100.35 + 0.350 X_1 - 4.27 X_2 - 0.567 X_3 - 2.833 X_4 - 0.020 X_5 - 0.000083 X_6 (6)

Assay of PP (%) (Y_7)= 100 + 1.20 X_1 + 1.33 X_2 - 2.47 X_3 - 7.10 X_4 - 0.060 X_5+0.00032 X_6 (7)

Assay of BSP (%) (Y_8)= 94.88 - 0.367 X_1 - 1.47 X_2 + 1.000X_3 - 0.283 X_4 - 0.107 X_5 + 0.000033 X_6 (8)

Assay of BDP (%) (Y_9)= 101.63 - 0.146 X_1 - 2.17 X_2 + 0.175 X_3 - 0.579 X_4 - 0.248 X_5 - 0.000146 X_6 (9)

Table 7. Statistical analysis of response variables of Plackett-Burman Design

Independent Variables	Y_1: **Particle size distribution (d50) (µm)**	Y_2: **Viscosity (cP)**	Y_3: **Sedimentation Time (min)**	Y_4: **Density (g/ml)**	Y_5: **Assay (%) of BA**	Y_6: **Assay (%) of MP**	Y_7: **Assay (%) of PP**	Y_8: **Assay (%) of BSP**	Y_9: **Assay (%) of BDP**
	p Value	*p Value*	*p Value*	*p Value*	*p Value*	*p Value*	*p Value*	*p Value*	*p Value*
β_0: Constant	0.342	0.012	0.451	0.467	0.622	0.024	0.020	0.819	0.408
X_1: Macrogol Type	0.340	0.081	0.116	0.139	0.336	0.470	0.315	0.460	0.680
X_2: Concentration of Polysorbate 80 (mg/ml)	0.245	1.000	0.676	0.608	0.489	0.287	0.883	0.706	0.454
X_3: Concentration of Carmellose Sodium (mg/ml)	0.245	0.001	0.456	0.258	0.840	0.555	0.303	0.325	0.804
X_4: Filter Type	0.621	0.049	0.310	0.774	0.358	0.001	0.001	0.563	0.143
X_5: Homogenization Time (min)	0.465	0.367	0.676	0.301	0.461	0.915	0.894	0.586	0.122
X_6: Homogenization Speed (rpm)	0.124	1.000	0.377	0.689	0.314	0.860	0.780	0.945	0.680
Model (ANOVA)	0.342	0.012	0.451	0.467	0.622	0.024	0.020	0.819	0.408
R^2	0.6400	0.9205	0.5784	0.5699	0.4818	0.8947	0.9027	0.3521	0.6026

For viscosity (Y_2), the two most significant variables were the amount of carmellose sodium ($p < 0.05$) and filter type ($p < 0.05$), respectively (Figure 4). The R^2 was 0.9205 indicating a good fit for the model being tested (Table 7). The individual value plot was used to detect any outliers and compare distributions, as shown in Figure 5. A visual evaluation shows that the use of PTFE filter has resulted in a slightly lower viscosity compared to CA filter with both 5% and 6% carmellose sodium concentration.

For the assay of MP (Y_6) and the assay of PP (Y_7), the significant variable was the filter type ($p < 0.05$) (Figure 6). The R^2 values were 0.8947 and 0.9027, respectively, indicating a good fit for the model being tested. The *p* values of the main effects of filter type obtained from ANOVA were both 0.001

(Table 7).

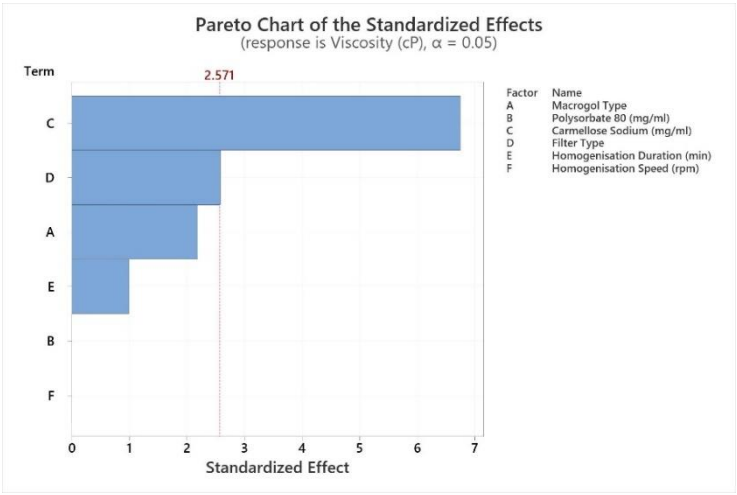

Figure 4. The Pareto chart of the independent variables showing the statistical significance of each variable on the viscosity

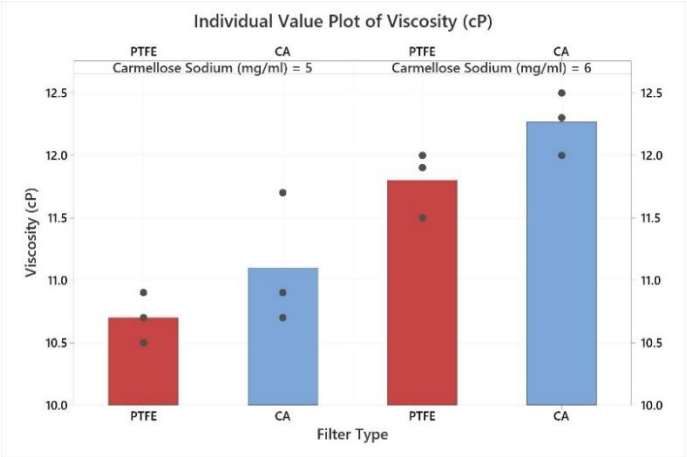

Figure 5. The individual value plots showing the effect of carmellose sodium and filter type on the viscosity

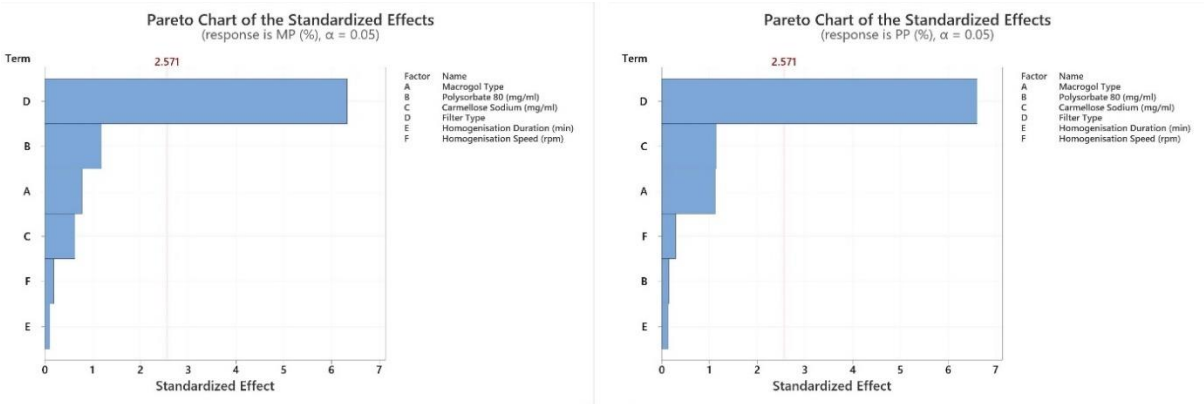

Figure 6. The Pareto chart of the independent variables showing the statistical significance of each variable on the assay of MP (left) and the assay of PP (right)

Storage Stability Study

The results of stability studies are given in Table 8. The particle size distribution, density, assay of BA, assay of BSP, assay of BDP of the formulation were found to be stable with no significant change. It can be concluded that excipient composition and manufacturing method of the optimized formulation was accurately justified. However, assay of MP and PP were decreased, when compared to the initial time point values. The decrease of the assay at the 40°C, 75% RH condition was higher than the decrease at the 25°C, 60% RH. This is thought to be related to increased reaction rates at higher temperatures and relative humidity levels. In order to understand the impact of lowered levels of antimicrobial preservatives, an Antimicrobial Effectiveness Testing (AET) was conducted according to the European Pharmacopeia. AET results showed that the lowest concentration of MP and PP obtained from stability studies provided an adequate level of antimicrobial preservation. The impurity data revealed decomposition with increased temperature and relative humidity levels, which were consistent with the stress testing carried out during analytical method development studies. These impurity results were found to be in compliance with the shelf-life specifications. All results suggested that betamethasone suspension for injection formulation had acceptable stability at accelerated and long-term conditions for at least 6 months.

Table 8. Stability results of betamethasone suspension for injection formulation at 40°C, 75% RH and 25°C, 60% RH conditions

Test	Storage Condition and Time Period				
		25°C ± 2°C/60% ± 5% RH		40°C ± 2°C/75% ± 5% RH	
	Initial	**3rd month**	**6th month**	**3rd month**	**6th month**
Density (g/ml)	1.0111	1.0075	1.0151	1.0080	1.0165
Particle size distribution (μm) (d90)	17.9	21.4	16.2	20.4	18.7
Assay of BA (%)	97.5	96.1	96.5	95.6	96.0
Assay of MP (%)	97.7	94.2	89.3	79.2	66.8
Assay of PP (%)	99.9	96.3	93.9	91.5	85.8
Assay of BSP (%)	97.8	99.8	99.1	98.7	95.1
Assay of BDP (%)	97.9	95.7	97.7	96.5	99.7
BSP Impurity (%)	0.40	0.68	0.72	1.57	2.90
BDP Impurity (%)	0.08	0.11	0.13	0.36	0.86
Total Impurity (%)	1.0	1.4	1.43	3.1	5.4

In conclusion, we investigated the effects of formulation and process variables on the CQAs of the betamethasone suspension for injection formulation using a Plackett-Burman experimental design. This study demonstrated that the filter type was the most critical process parameter for the assay of methyl parahydroxybenzoate and propyl parahydroxybenzoate in the current study. The results showed that these substances adsorbed on the PTFE filter less than the CA filter. The PTFE filter will not only reduce the extent of adsorption but also reduce the process risks. Furthermore, the concentration of carmellose sodium was the most significant formulation variable on the viscosity of the suspension. As expected, the higher concentration of carmellose sodium resulted in increased viscosity. Understanding the formulation and process parameters effects on the CQAs of suspension for injection can significantly reduce the costs of research and development due to fewer formulation trials.

AUTHOR CONTRIBUTIONS

Concept: F.Y., A.A., B.A., P.G.; Design: F.Y., A.A., B.A., P.G.; Control: F.Y., A.A., B.A., P.G., E.N.; Sources: F.Y., A.A., B.A., P.G., E.N.; Materials: F.Y., A.A., B.A., P.G.; Data Collection and/or Processing: F.Y., A.A., B.A., P.G.; Analysis and/or Interpretation: F.Y., A.A., B.A., P.G.; Literature

Review: F.Y., A.A., B.A., P.G.; Manuscript Writing: F.Y., A.A., B.A.; Critical Review: F.Y., E.N.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

SYNTHESIS AND STRUCTURE ELUCIDATION OF NEW METHYL 1*H*-BENZIMIDAZOLE-5-CARBOXYLATE DERIVATIVES

YENİ METİL 1H-BENZİMİDAZOL-5-KARBOKSİLAT TÜREVLERİNİN SENTEZİ VE YAPILARININ AYDINLATILMASI

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ABSTRACT

Objective: *In this study, in connection with previous works in our department, some methyl 1H-benzimidazole-5-carboxylate derivatives were synthesized for the first time. Compounds were modified by substituting the second position of the benzimidazole ring with 4-methylpiperidinyl groups for increasing antibacterial activity.*
Material and Method: *The targeted methyl 1H-benzimidazole-5-carboxylates were synthesized by the reaction of o-phenylendiamine derivatives with urea. Oxygen at the 2nd position was converted to chlorine in the presence of $POCl_3$. Finally, the resulting products were obtained by the nucleophylic substitution with 4-methylpiperidine. Structures of synthesized compounds were elucidated with ^1H- ^{13}C-NMR and LC-MS techniques.*
Result and Discussion: *Methyl 1H-benzimidazole-5-carboxylate derivatives bearing 4-methyl piperidinyl groups at the 2nd position were synthesized to improve better in vitro antibacterial activity profiles. In vitro antibacterial activity of the synthesized compounds in this study is under investigation.*
Keywords: *^1H-^{13}C-NMR, 1H-benzimidazole, 4-methylpiperidine, methyl 1H-benzimidazole-5-carboxylates*

ÖZ

Amaç: *Bu çalışma kapsamında daha önce bölümümüzde sentezlenen bileşiklere ek olarak bazı yeni metil 1H-benzimidazol-5-karboksilat türevlerinin sentezi gerçekleştirilmiştir. Antibakteriyel aktiviteyi artırmak için benzimidazol halkasının 2. konumu 4-metilpiperidinil grubuyla modifiye edilmiştir.*

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Gereç ve Yöntem: *Tasarlanan metil 1H-benzimidazol-5-karboksilat bileşikleri, o-fenilendiamin türevlerinin üre ile reaksiyonu sonucu sentezlenmiştir. 2. konumda bulunan oksijen atomu $POCl_3$ varlığında klor ile yer değiştirmiştir. Son olarak, 4-metil piperidin ile nükleofilik sübstitüsyonla hedeflenen bileşiklere ulaşılmıştır. Elde edilen bileşiklerin yapıları ^1H-^{13}C-NMR ve LC-MS teknikleriyle aydınlatılmıştır.*

Sonuç ve Tartışma: *Bu çalışmada daha iyi in vitro antibakteriyel aktivite elde etmek için 2. konumda 4-metilpiperidinil grubu taşıyan metil 1H-benzimidazol-5-karboksilat türevleri sentezlenmiştir. Bu çalışmada sentezlenen bileşiklerin in vitro antibakteriyel etkileri araştırılmaktadır.*

Anahtar Kelimeler: *^1H-^{13}C-NMR, 1H-benzimidazol, 4-metilpiperidin, metil 1H-benzimidazol-5-karboksilat*

INTRODUCTION

Benzimidazole is an important pharmacophore and a privileged structure in medicinal chemistry [1]. Many benzimidazole derivatives containing ester groups on the benzene ring have been synthesized for their antimicrobial [2,3], anti-inflammatory [4], antihypertensive [5] and anticancer [6] activities. In addition, antibacterial [7-9] and antifungal [8,9] effects of the compounds that bear 4-methyl piperidine moiety have been reported in various references. Furthermore, our previous work [10] showed that benzimidazoles containing methyl or ethyl ester groups display good antibacterial and antimycotic activity. **13f** and **13h** were found the most active compounds against *Staphylococcus aureus* with MIC values of 0.78 and 1.56 µg/ml and against MRSA with MIC values of 0.78 and 0.39 µg/ml, respectively (Figure 1). Taking into consideration these structural features and the expectation of much better antibacterial activity with 4-methylpiperidine groups, it has been planned to prepare benzimidazoles carrying the ester groups on the benzene ring and with additional substitution at position C-2. And their *in vitro* antibacterial activity studies are planned to test in further analysis.

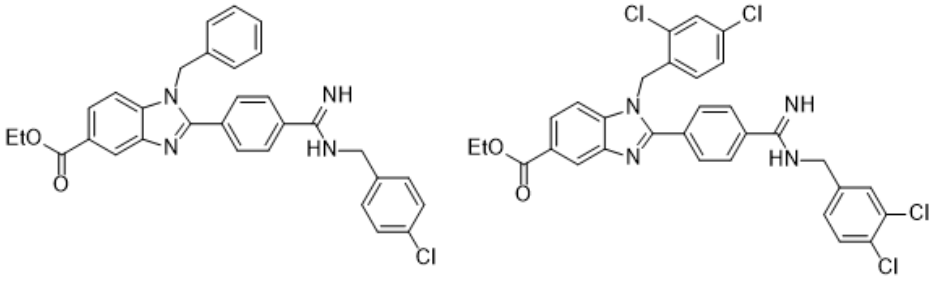


Figure 1. Previously synthesized potent benzimidazoles containing ethyl ester groups **13f** and **13h**, respectively

MATERIAL AND METHOD

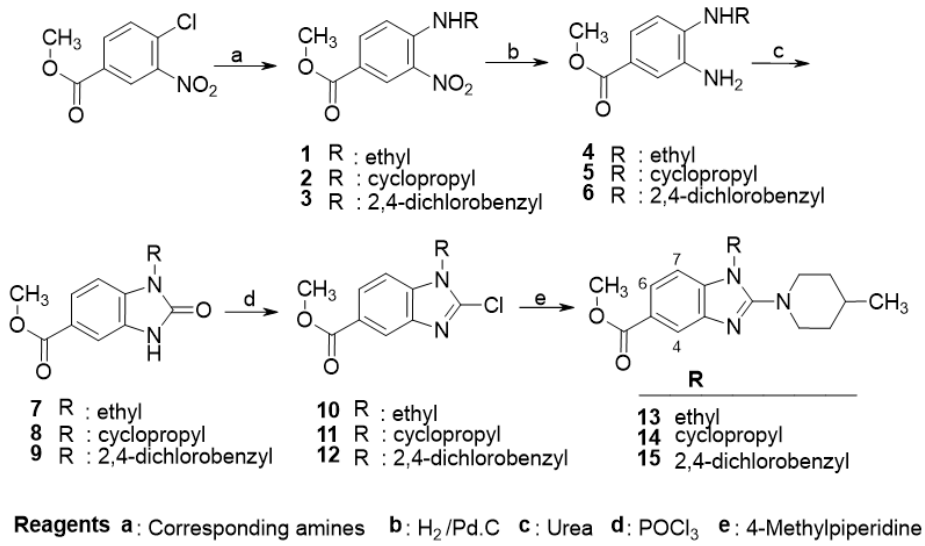
Experimental

Uncorrected melting points were measured on an Büchi B-540 capillary melting point apparatus. ^1H (400 and 500 MHz) and ^{13}C (100 and 125 MHz) NMR spectra were recorded employing a Varian Mercury (AGILENT) 400 MHz and BRUKER AVANCE NEO 500 MHz FT spectrometers, chemical shifts (δ) are in ppm relative to TMS. The samples (5-15 mg) were prepared in 0.75 ml of $CDCl_3$. TMS was used as an internal standard. The liquid chromatography mass spectrometry (LC-MS) spectra were taken on a Waters Micromass ZQ connected with Waters Alliance HPLC (Waters Corporation, Milford, MA, USA), using the ESI (+) method with a C-18 column (XTerra®, 4.6 X 250 mm, 5 µm).

Chemistry

The synthetic pathways for the preparation of targeted compounds are outlined in Scheme 1.

Compounds **1,2,4,5** [10,11], **7,8** [11], and **11** [12] were synthesized according to the lit. method. Reduction of the nitro groups of **1-3** afforded **4-6**. These compounds were fused with urea for obtaining **7-9.** Treatment of **7-9** with $POCl_3$ gave **10-12**. Final compounds **13-15** were prepared by the nucleophilic substitution reaction of **10-12** with 4-methylpiperidine.



Scheme 1. Synthesis of targeted benzimidazoles

General Procedure for Synthesis of 1-3

To a solution of methyl 4-chloro-3-nitrobenzoate (4.6 mmol) in DMF (1.5 ml), the corresponding amines (9.3 mmol) were added and the mixture was heated for 3-8 h at 80°C. The mixture was allowed to cool, and water was added. The resultant precipitate was filtered off and crystallized from the mixture of ethanol-water.

General Procedure for Synthesis of 4-6

Compounds **1-3** (3.4 mmol) in ethanol (75 ml) were reduced by hydrogenation using 40 psi of H_2 and 10% Pd-C (40 mg) until the cessation of H_2 uptake. The catalyst was filtered on a bed of Celite, washed with ethanol and the filtrate was concentrated in vacuo. The crude amines were used for further steps without crystallization [13].

General Procedure for Synthesis of 7-9

A mixture of **4-6** (2 mmol) and urea (11.9 mmol) was heated at 150°C for 5 h. Water was added, the precipitate was collected and recrystallized from toluene [13].

General Procedure for Synthesis of 10-12

A mixture of **10-12** (1.92 mmol) and $POCl_3$ (80 mmol) was refluxed with stirring for 7 h and dry HCl gas was passed through the refluxing liquid during the first 4 h, then $POCl_3$ was evaporated, the reaction mixture was poured into ice-cold water, 4N NaOH was added and the mixture was extracted with EtOAc. The extract was washed with water, dried over Na_2SO_4 and evaporated. Recrystallization of the mixture from EtOAc: *n*-hexane gave **10-12** [13].

General Procedure for Synthesis of 13-15

A mixture of **10-12** (1.7 mmol) and 4-methylpiperidine (2 mmol) in DMF (0.5 ml) was heated for 8 h at 110°C, water was added and the mixture was extracted with EtOAc. The extract was washed with water, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography ($CHCl_3$:isopropanol 10:1) to give **13-15** [13].

Methyl 4-((2,4-dichlorobenzyl)amino)-3-nitrobenzoate (3)

Yield: 65%. m.p. 132-133°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 3.89 (s, 3H, OC*H*$_3$), 4.65 (d, 2H, J=6Hz, C*H*$_2$), 6.73, (d, 1H, J=8.8Hz), 7.225-7.228 (m, 2H), 7.45 (s, 1H) , 8.01 (dd, 1H, J=8.8 & 2Hz), 8.72 (br.s, 1H, N*H*), 8.90 (d, 1H, J=2.4Hz) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 165.4, 147.1, 136.5, 134.5, 133.9, 132.5, 131.95, 129.85, 129.4, 129.2, 127.6, 118.3, 113.7, 52.2, 44.4. $C_{15}H_{12}Cl_2N_2O_4$.

Methyl 1-ethyl-2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazole-5-carboxylate (7)

Yield: 65%. m.p. 198-200°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 1.37 (t, 3H, J=7.6Hz, C*H*$_3$), 3.90 (s, 3H, OC*H*$_3$), 3.98 (q, 2H, J=7.6Hz, C*H*$_2$), 7.02 (d, 1H, J=8Hz), 7.83-7.87 (m, 2H), 10.61 (br.s, 1H, N*H*) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 167.1, 155.8, 133.7, 127.9, 123.8, 123.5, 111.0, 107.1, 52.0, 35.9, 13.6. **MS** m/z (ESI+) : 221 [M+H] (68%) for $C_{11}H_{12}N_2O_3$.

Methyl 1-cyclopropyl-2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazole-5-carboxylate (8)

Yield: 70%. m.p. 225-227°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 1.04-1.07 (m, 2H, C*H*$_2$), 1.15-1.18 (m, 2H, C*H*$_2$), 2.91-2.94 (m, 1H, C*H*), 3.90 (s, 3H, OC*H*$_3$), 7.22 (d, 1H, J=8.4Hz, H-7), 7.81 (d, 1H, J=1.6Hz, H-4), 7.85 (dd, 1H, J=8 & 1.6Hz, H-6), 10.32 (br.s, 1H, N*H*) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 167.1, 156.4, 135.0, 127.4, 123.8, 123.7, 110.8, 108.2, 52.05, 22.5, 6.1. **MS** m/z (ESI+) : 233 [M+H] (100%) for $C_{12}H_{12}N_2O_3$.

Methyl 1-(2,4-dichlorobenzyl)-2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazole-5-carboxylate (9)

Yield: 60%. m.p. 219-221°C. **^{1}H-NMR** (500 MHz, $CDCl_3$) δ ppm : 3.92 (s, 3H, C*H*$_3$), 5.21 (s, 2H, C*H*$_2$), 6.91 (d, 1H, J=8.3Hz, H-7), 7.05 (d, 1H, J=8.35Hz, H-6'), 7.19 (dd, 1H, J=8.35 & 2.05Hz, H-5'), 7.47 (d, 1H, J=2.05Hz, H-3'), 7.82 (dd, 1H, J=8.3 & 1.55Hz, H-6), 7.85 (d, 1H, J=1.2Hz, H-4), 10.04 (s, 1H, N*H*) ; **COSY** ($CDCl_3$) δ ppm: [H-6 : H-7], [H-5' : H-6'] ; **^{13}C-NMR** (125 MHz, $CDCl_3$) δ ppm : 166.8, 155.8, 134.4, 133.5, 131.6, 129.65, 129.2, 127.7, 127.6, 124.33, 124.26, 111.1, 107.9, 52.2, 41.7. **MS** m/z (ESI+) : 351 [M+H] (68%), 353 [M+H+2] (31%), 355 [M+H+4] (8%) for $C_{16}H_{12}Cl_2N_2O_3$.

Methyl 2-chloro-1-ethyl-1*H*-benzo[*d*]imidazole-5-carboxylate (10)

Yield: 40%. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 1.46 (t, 3H, J=7.2Hz, C*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 4.28 (q, 2H, J=7.6Hz, C*H*$_2$), 7.35 (d, 1H, J=8.4Hz, H-7), 8.04 (dd, 1H, J=8.4 & 1.2Hz, H-6), 8.40 (d, 1H, J=1.2Hz, H-4) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 167.2, 141.9, 141.1, 137.6, 125.05, 124.8, 121.6, 109.05, 52.2, 39.75, 14.6. **MS** m/z (ESI+) : 239 [M+H] (100%), 241 [M+H+2] (31%), for $C_{11}H_{11}ClN_2O_2$.

Methyl 2-chloro-1-cyclopropyl-1*H*-benzo[*d*]imidazole-5-carboxylate (11)

Yield: 45%. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 1.14-1.32 (m, 4H, C*H*$_2$-C*H*$_2$), 3.23-3.25 (m, 1H, C*H*), 3.93 (s, 3H, OC*H*$_3$), 7.52 (d, 1H, J=8.4Hz, H-7), 8.01 (dd, 1H, J=8.4 & 1.6Hz, H-6), 8.34 (d, 1H, J=1.6Hz, H-4) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 167.5, 144.4, 141.05, 139.6, 125.2, 124.95, 121.8, 110.2, 52.4, 25.6, 7.5. **MS** m/z (ESI+) : 251 [M+H] (100%), 253 [M+H+2] (31%) for $C_{12}H_{11}ClN_2O_2$.

Methyl 2-chloro-1-(2,4-dichlorobenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (12)

Yield: 44%. m.p. 132-134°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 3.94 (s, 3H, OC*H*$_3$), 5.47 (s, 2H, C*H*$_2$), 6.54 (d, 1H, J=8.8Hz, H-7), 7.12 (dd, 1H, J=8.4 & 2Hz, H-5'), 7.18 (d, 1H, J=8.4Hz, H-6'), 7.48 (d, 1H, J=2Hz, H-3'), 7.98 (dd, 1H, J=8.8 & 1.6Hz, H-6), 8.43 (d, 1H, J=0.8Hz, H-4). **MS** m/z (ESI+) : 369 [M+H] (68%), 371 [M+H+2] (67%), 373 [M+H+4] (23%), 375 [M+H+6] (3%) for $C_{16}H_{11}Cl_3N_2O_2$.

Methyl 1-ethyl-2-(4-methylpiperidin-1-yl)-1*H*-benzo[*d*]imidazole-5-carboxylate (13)

Yield: 30%. m.p. 113-115°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 0.99 (d, 3H, J=6.8Hz, C*H*$_3$), 1.33-1.45 (m, 5H), 1.58-1.6 (m, 1H), 1.75-1.78 (m, 2H), 2.99-3.06 (m, 2H), 3.52-3.55 (m, 2H), 3.88 (s, 3H, OC*H*$_3$), 4.02 (q, 2H, J=7.6Hz, C*H*$_2$), 7.19 (d, 1H, J=8.8Hz, H-7), 7.87 (dd, 1H, J=8.8 & 1.6Hz, H-6), 8.28 (d, 1H, J=1.2Hz, H-4) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 167.9, 159.2, 140.6, 138.2, 124.0,

123.4, 119.8, 108.6, 52.2, 51.5, 39.6, 34.2, 30.9, 22.05, 14.5. **MS** m/z (ESI+) : 302 [M+H] (100%) for $C_{17}H_{23}N_3O_2$.

Methyl 1-cyclopropyl-2-(4-methylpiperidin-1-yl)-1*H*-benzo[*d*]imidazole-5-carboxylate (14)

Yield: 35%. m.p. 132-133°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 0.99 (d, 3H, J=6.8Hz, C*H*$_3$), 1.02-1.04 (m, 2H), 1.08-1.16 (m, 2H), 1.32-1.41 (m, 2H), 1.57-1.62 (m, 1H), 1.75-1.78 (m, 2H), 2.94-3.00 (m, 2H), 3.13-3.16 (m, 1H), 3.87 (s, 3H, OC*H*$_3$), 3.93-3.96 (m, 2H), 7.27 (d, 1H, J=8.4Hz, H-7), 7.82 (dd, 1H, J=8.4 & 1.2Hz, H-6), 8.17 (d, 1H, J=1.2Hz, H-4) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 168.2, 159.15, 140.7, 139.9, 123.8, 122.7, 119.1, 109.2, 52.1, 49.9, 34.15, 30.9, 26.2, 22.1, 7.9. **MS** m/z (ESI+) : 314 [M+H] (100%), for $C_{18}H_{23}N_3O_2$.

Methyl 1-(2,4-dichlorobenzyl)-2-(4-methylpiperidin-1-yl)-1*H*-benzo[d]imidazole-5-carboxylate (15)

Yield: 33%. m.p. 181-183°C. **^{1}H-NMR** (400 MHz, $CDCl_3$) δ ppm : 0.96 (d, 3H, J=6.4Hz, C*H*$_3$), 1.31-137 (m, 2H), 1.55 (m, 1H), 1.67-1.70 (m, 2H), 2.92-2.99 (m, 2H), 3.39-3.42 (m, 2H), 3.89 (s, 3H, OC*H*$_3$), 5.17 (s, 2H, benzylic C*H*$_2$), 6.74 (d, 1H, J=8.8Hz, H-7), 6.90 (d, 1H, J=8Hz, H-6'), 7.13 (dd, 1H, J=8.4 & 2Hz, H-5'), 7.47 (d, 1H, J=1.6Hz, H-3'), 7.80 (dd, 1H, J=8 & 1.6Hz, H-6), 8.30 (d, 1H, J=1.2Hz, H-4) ; **^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm : 167.65, 159.9, 141.5, 138.8, 134.3, 133.1, 132.1, 129.7, 127.84, 127.76, 124.4, 123.4, 119.9, 108.3, 51.9, 50.9, 45.7, 33.8, 30.6, 21.8. **MS** m/z (ESI+) : 432 [M+H] (100%), 334 [M+H+2] (61%), 436 [M+H+4] (12%) for $C_{22}H_{23}Cl_2N_3O_2$.

RESULT AND DISCUSSION

As shown in Scheme 1, nitro group of **1-3** was reduced to **4-6**. Cyclization of these compounds with urea under heat afforded **7-9.** Treatment of **7-9** with $POCl_3$ gave **10-12**. By the nucleophilic substitution reaction of **10-12** with 4-methylpiperidine gave the targeted compounds **13-15**. The structures of novel compounds were determined by ^{1}H-^{13}C-NMR and LC-MS. To clarify aromatic protons of **9**, the COSY (Correlated Spectroscopy-2D-NMR technique) spectrum was recorded. Analysis of the COSY spectrum, shown in Figure 2, confirms the assignment of the resonances H-6/H-7 and H-5'/H-6' as neighboring hydrogens.

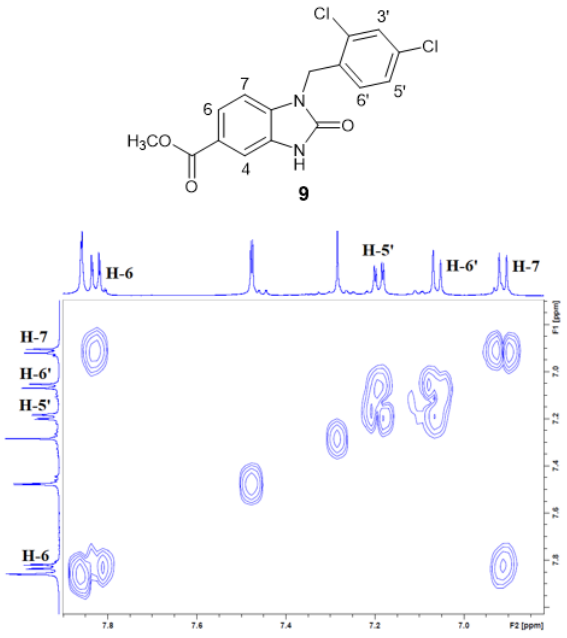


Figure 2. COSY spectrum of compound **9**

In this study, five new intermediate compounds and three new methyl 1*H*-benzimidazole-5-carboxylate derivatives were designed and synthesized, their structures were elucidated with NMR techniques. The ADME parameters of compounds **13-15** in Table 1 are presented. According to theoretical calculations, compound **15** complies with Lipinski's rules by causing one violation. Other ADME parameters of the compounds are within suitable limits. Antibacterial activity studies are under investigation.

Table 1. Calculated ADME parameters of **13-15**.

Compounds	LogP	TPSA	nON	nOHNH	MV	Vio
13	3.59	47.37	5	0	289.68	0
14	3.58	47.37	5	0	295.90	0
15	6.09	47.37	5	0	371.60	1

Log P: log octanol/water partition coefficient; TPSA: Total Polar Surface Area; nON: number of Hydrogen acceptors; nOHNH: number of Hydrogen donors and MV: Molecular Volume were calculated using Molinspiration Calculation of Molecular Properties toolkit. Vio: Violation number of Lipinski's rule.

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AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

FORMULATION OF GEL CONTAINING *PHASEOLUS VULGARIS* L. EXTRACT AND TO EVALUATE ITS EFFICACY IN THE MANAGEMENT OF INFLAMMATION - A SINGLE ARM OPEN LABELLED CLINICAL STUDY

PHASEOLUS VULGARIS L. ÖZÜ İÇEREN JELİN FORMÜLASYONU VE İNFLAMASYON TEDAVİSİNDEKİ ETKİNLİĞİNİN DEĞERLENDİRİLMESİ - TEK KOL AÇIK ETİKETLİ KLİNİK ÇALIŞMA

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ABSTRACT

Objective: *The research works objective was to develop and test a gel that contained anthocyanin of Phaseolus vulgaris L. extract and to study it's anti-inflammatory activity.*
Material and Method: *By macerating Phaseolus vulgaris L. with 1% HCl (v/v) in methanol as the solvent, anthocyanin was extracted from the seed coat. Anthocyanin gels were produced utilizing a variety of polymers, including carbopol-940, sodium-CMC, chitosan, and other ingredients, including ethanol, lavender oil, propylene glycol, methyl paraben, and propyl paraben. All of the formulations of anthocyanin gel were studied for FTIR, pH, viscosity, spreadability, extrudability, drug content, and in-vitro drug release studies. The optimal formulation was then carried through a clinical investigation.*
Result and Discussion: *Formulations for anthocyanin gels can be made utilizing various gelling agents, such as carbopol-940, sodium-CMC, chitosan at different concentrations. Gel formulated using Chitosan (F3) showed better results of composition with decreased viscosity, enhanced*

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extrudability, and a respectable amount of drug release. Formulation F3 by 2% of chitosan was best choice for anti-inflammatory activity in clinical study. Hence, Anthocyanin proved it's potential for inflammation that implies moderate to marked improvement with significant results in subjects.
Keywords: *Anthocyanin, anti-inflammatory, Phaseolus vulgaris L., topical gel*

ÖZ

Amaç: *Bu çalışmanın amacı, Phaseolus vulgaris L. ekstraktından antosiyanin içeren bir jel geliştirmek, test etmek ve bunun anti-enflamatuar aktivitesini incelemektir.*
Gereç ve Yöntem: *Phaseolus vulgaris L., çözücü olarak metanol içinde %1 HCl (v/v) ile maserasyonu yapılarak tohum kabuğundan antosiyanin ekstrakte edilmiştir. Antosiyanin jelleri, karbopol-940, sodyum-CMC, kitosan ve etanol, lavanta yağı, propilen glikol, metil paraben ve propil paraben gibi diğer bileşenler dahil olmak üzere çeşitli polimerler kullanılarak üretilmiştir. Antosiyanin jelin tüm formülasyonları FTIR, pH, viskozite, yayılabilirlik, ekstrüde edilebilirlik, ilaç içeriği ve in vitro ilaç salınım çalışmaları için incelenmiştir. Optimal formülasyon daha sonra klinik bir araştırma ile tespit edilmiştir.*
Sonuç ve Tartışma: *Antosiyanin jelleri için formülasyonlar, farklı konsantrasyonlarda karbopol-940, sodyum-CMC, kitosan gibi çeşitli jelleştirici ajanlar kullanılarak yapılabilmektedir. Kitosan (F3) kullanılarak formüle edilen jel, azalmış viskozite, gelişmiş ekstrüdasyon ve yeterli miktarda ilaç salınımı ile bileşimin daha iyi sonuçlar göstermiştir. Kitosan'ın % 2'si ile F3 formülasyonu, klinik çalışmada anti-enflamatuar aktivite için en iyi seçim olarak bulunmuştur. Bu nedenle, antosiyanin ile deneklerde orta ila belirgin iyileşme sağlayanmıştır ve antosiyaninin anti-enflamatuar potansiyeli olduğu kanıtlanmıştır.*
Anahtar Kelimeler: *Anti-enflamatuar, antosiyanin, Phaseolus vulgaris L., topikal jel*

INTRODUCTION

The immune system's biological response, inflammation, can be carried on by a variety of factors including pathogens, harmed cells, and toxic substances. The heart, pancreas, liver, kidney, lung, brain, digestive tract, and reproductive system may all experience acute or chronic inflammatory reactions, which may result in tissue damage or disease. Inflammatory cells are activated by both viral and non-infectious stimuli, as well as by cell injury, which also opens up inflammatory signalling pathways, most frequently the NF-B pathways. Organ-specific inflammatory responses, with an emphasis on the causes of inflammation, mechanisms of inflammatory response, and resolution of inflammation.

Redness, swelling, heat, pain, and loss of tissue function are signs of inflammation at the tissue level and are brought on by local immunological, vascular, and inflammatory cell reactions to infection or damage. Vascular permeability alterations, leukocyte recruitment and accumulation, and the release of inflammatory mediators are all significant microcirculatory events that take place throughout the inflammatory phase. A chemical signalling cascade that drives actions aimed at mending damaged tissues is started by the organism in response to tissue injury. These signals cause leukocytes to migrate to damaged areas from the overall circulation. The cytokines that are produced by these activated leukocytes cause inflammatory reactions [1].

Gel is a word used to describe semi-rigid systems in which the dispersing medium's ability to move is constrained by solvated macromolecules in the dispersed phase. Both "gel" and "jelly" can be traced back to the Latin word gelu, which meant "frost." Gel, which means "freeze" or "congeal," is derived from "gelatin." According to the USP, gels are semisolid systems made up of lattices of small, distinct particles or suspensions of large, liquid-pierced organic molecules or small, inorganic particles. Due to the presence of more covalent crosslinks, a higher density of physical bonds, or just less liquid, gels are typically thought to be more stiff than jellies. While some gel systems are transparent like water, and some others are turbid due to the ingredients' incomplete molecular dispersion or the possibility that they will form clumps that scatter light [2].

Phaseolus vulgaris L. belonging to the family Fabaceae or Leguminosae is one of the most widely used food crops and medicinal plants in the world and is well-known for its seed. *P. vulgaris* contains lysine, phenylalanine, and tyrosine, as well as carbs, protein, and other amino acids. In addition to

nutritional value, it contains bioactive substances such anthocyanin, phenolic acid, flavonoids, flavan-3-ol, condensed tannins [3].

MATERIAL AND METHOD

List of Chemicals

Anthocyanin (Black bean [*Phaseolus vulgaris* L.]), Methanol, Hydro Chloric acid Carbopol-940, Sodium Carboxymethyl Cellulose, Chitosan, Methyl paraben, Propyl paraben, Propylene glycol, Ethanol, Lavender oil. All ingredients used are analytical Grade.

Methods

Procedure for anthocyanin extraction: The seed coat powder of *Phaseolus vulgaris* L. taken in 1:10 ratio was soaked in 1% v/v solution of con. HCl in methanol, cover the beaker with aluminium foil and allow it stand in dark place for 24 hrs at room temperature. Later, muslin cloth was first used for filtering, then whatman filter paper. Anthocyanin was obtained by further filterate evaporation over a water bath at 40°C. The obtained dried anthocyanin was collected and preserved [4].

Identification Tests for Anthocyanin

IR Spectroscopy

FT-IR spectra of the drug sample that was obtained and the standard FT-IR spectra of the pure drug were compared using infrared spectroscopy [5].

Solubility Analysis

The drug's solubility in the intended dissolving medium was tested as aspect of the preformulation solubility analysis, which also involved choosing a suitable solvent to dissolve the drug and for anthocyanin release studies.

Initially we performed solubility by dissolving 0.5 g of Anthocyanin with 10 ml of methanol with continue stirring for 5-10 minutes. Then other solvents such as ethanol, phosphate buffer of pH 6.8 were used, as ethanol during formulation for dissolving Anthocyanin & phosphate buffer of pH 6.8 in drug release studies.

Confirmatory Test for Anthocyanin

- Using methanol as a blank, a UV-Visible Spectrophotometer was used to confrim the presence of anthocyanin in the extract. A spectra in the 200-800 nm UV-visible range was recorded.
- 2M HCl was dissolved in 1 ml of *Phaseolus vulgaris* L. extract, and the mixture was heated for 5 minutes at 100°C. The observation that the extract maintains its stable colour indicates that anthocyanin is present.
- When 1 ml of *Phaseolus vulgaris* L. extract is mixed with 2M NaOH, the extract's colour changes to show that anthocyanin is present [6,7].

Preparation of Anthocyanin Gel

The formulation of different gels, using concentrated *Phaseolus vulgaris L.* as gelling agents, Carbopol-940, Sodium CMC, and Chitosan were utilized along with extract (Anthocyanin) as the active ingredient as mentioned in Table 1 [8].

Table 1. Composition of gel formulations with different polymers such as Carbopol- 940, Sodium Carboxymethyl Cellulose and Chitosan

Weight taken in g (For 100g)	F_1	F_2	F_3
Extract	1	1	1
Carbopol-940	2	-	-
Sodium CMC	-	3	-
Chitosan	-	-	2

Table 1 (*continue*). Composition of gel formulations with different polymers such as Carbopol-940, Sodium Carboxymethyl Cellulose and Chitosan

Methyl paraben	0.2	0.2	0.2
Propyl paraben	0.02	0.02	0.02
Propylene glycol	5	5	5
Ethanol	5	5	5
Lavender oil	q.s	q.s	q.s
Water upto	100	100	100

Preparation of Carbopol-940/Sodium Carboxymethyl Cellulose/ Chitosan

Weigh accurately gelling agents, mix it with water/ glacial acetic acid and keep it aside for 24h for complete swelling. Methyl and propyl parabens (used as preservatives) were dissolved in half of the formulation water and heated further to 40°C. Gelling agents were then added and stirred with the mixture for 30 minutes at 1200 rpm using a magnetic stirrer. Anthocyanin extract in a predetermined quantity was weighed and thoroughly combined with propylene glycol and ethanol. A few drops of lavender oil were then added to the gel after this mixture had been gradually added and well combined into a gel [9-11].

By observing the consistency of gel water containing 1% glacial acetic acid was used, the weight of ingredients was taken in grams, for liquids volume was converted in mass during formulation.

Characterization of Anthocyanin Gels

pH Determination

The pH of the extract was measured using a digital pH metre after extraction for 48 hours, one week, two weeks, one month, and three months. Three repetitions of 1g of extract in 10 ml of distilled water were carried out [12].

Homogeneity

All prepared gels were placed in containers and inspected visually to determine their homogeneity. They were examined to check for aggregates and appearance [13].

Centrifuge Test

After 48 hours of preparation, formulations were placed into tubes that were 10 cm long and 1 cm wide, and they were centrifuged for 60 minutes at 2000 rpm using a centrifugal equipment (PR-24) At 5, 15, 30, and 60 minutes, the formulation's stability and sedimentation were determined [14].

Temperature Change Test

Tubes containing the formulation were placed at temperatures of 2-8°C, 25°C, and 40–45°C, and their appearance quality was checked 48 hours, 1 week, 2 weeks, 1 month, and 3 months afterwards. This was done to check the formulation's stability in various seasons and temperature conditions [15].

Evaluation of Anthocyanin Gels

Viscosity

A DV-E Brookfield viscometer was used to measure the formed gel's viscosity. Using spindle no. 64, the gels were rotated at 50 rpm, and the appropriate dial reading was recorded [16].

Spreadability

Utilizing a modified apparatus made of a wooden block with a pulley at one end, spreadability was evaluated. By using this method, spreadability was characterized based on the gels' properties of slip and drag. On this ground slide, an excess of the gel (approximately 2 g) under investigation was applied. The gel was then placed in a sandwich between this glass slide and another glass slide with a hook and a fixed ground slide dimension. For five minutes, a weight of 20 g was placed on the slide’s

top to push out air and create a consistent gel film between the slides. The edges of the gel were scraped clean of extra. Next, a 20 gramme pull was applied to the top plate. With the use of a thread fastened to the hook, record the amount of time (in seconds) needed for the top slide to travel 6.5 cm. Better spreadability is indicated by a shorter interval [17].

Extrudability

A clamp was used to stop any rollback and a closed collapsible tube containing about 20 gm of gel was squeezed firmly at the crimped end. The gel was extruded after the cap was removed. The extruded gel's volume was collected and weighed [18,19].

Anthocyanin Content

By accurately dissolving 2 g of gel in 100 milliliters of pH 6.8 phosphate buffer, the drug content of the gel formulation was determined. To fully solubilize the drug, the volumetric flask holding the gel solution was shaken for two hours. With the aid of filter paper, the solution was purified. Utilizing a UV-visible spectrophotometer (UV Shimadzu, Japan) with a maximum 536 nm reading and phosphate buffer as a blank, drug absorbance was measured using the method with appropriate dilution [20].

In-vitro Drug Release Studies

Using a modified diffusion testing apparatus, the *in-vitro* drug release study of anthocyanin from the prepared formulations was investigated. As a diffusion medium, freshly made phosphate buffer (pH 6.8) was employed. Gelatin sheet was used as a semi-permeable membrane that had been pre-soaked in the diffusion medium over the previous night. It was then attached to one end of a specially made glass cylinder with an inner diameter of 3.4 cm that was open on both sides. A glass cylinder known as the donor chamber was gently pipetted with 2 gm of the gel formulation within donar chamber. The cylinder was suspended in a 50 ml diffusion medium-filled beaker (acceptor chamber) so that the membrane just touched the surface. Using a magnetic stirrer, the acceptor chamber was kept at a temperature of 37 ± 2ºC. while being rotated at a rate of 50 rpm. At hourly intervals, 4 ml of the sample were taken out and replaced with an equal volume of diffusion media. The aliquots were examined using a UV spectrophotometer at 536 nm [21].

Clinical Studies

A Single Arm Open Label Clinical Study

Anti-inflammatory activity study for formulated Anthocyanins rich extract gel was done in prospective observational study over time period of 1 month at outpatient of BVVS Ayurved medical college & Hospital, Bagalkot. Prior approval from Institutional Ethics Committee (Reference. No: BVVS/IEC/AMVB-2020-21/767) on Human Subject Research was obtained and documented. The 10 subjects who was having Osteoarthritis of age 25-70 years were included of either sex in the study after obtaining the written consent from the subjects were treated. The data was extracted according to predefined study criteria. The subjects were instructed for the procedure of application of gel thrice a day. On each visit of study centre the containers of medication containing Anthocyanin gel was given for treatment. Subjects analysis of inflammation was made by the investigator for parameters like pain, swelling, tenderness, pain during flexion, pain during extension, crepitus. Each of these parameters was graded as absent (0), mild (1), moderate (2), severe (3) and the four scores added together to give clinical score. The data for present study was collected from subjects case report and progress chart. The obtained final results were statistically processed using the student paired 't' test [22].

RESULT AND DISCUSSION

Phaseolus vulgaris L. anthocyanin herbal gel formulations were developed and evaluated for their ability to reduce inflammation. Gel was prepared through dispersion process using a variety of polymers, including chitosan, carbopol-940, and sodium-CMC. They evaluated the pH, viscosity, spreadability, extrudability, drug content, and *in-vitro* drug release tests of the extracted drug and prepared formulation. The formulation was subjected for a clinical investigation.

Identification Tests for Anthocyanin

FTIR Studies

By using an IR spectrophotometer, compatibility studies were carried out. The peaks found in the spectra of each sample correlate to the peaks found in the spectrum of anthocyanin. The physical mixture of Anthocyanin and polymers indicates that the drug was compatible with formulation components, hence their is no interaction between them.

Solubility Analysis

The anthocyanin extract was completely soluble in methanol and other organic solvents, it was only partially soluble in water.

Confirmatory Test for Anthocyanin:

- A UV-visible spectrophotometer was used to analyse a *Phaseolus vulgaris* L. extract, and an absorbance at 536 nm confirmed the presence of anthocyanin.
- *Phaseolus vulgaris* L. extract was mixed with 2M HCl, the colour of the mixture remains stable red colour, which confirms the presence of Anthocyanin.
- *Phaseolus vulgaris* L. extract was mixed with 2M NaOH, the colour of extract red turns to stable brown colour, which confirms the presence of Anthocyanin.

Development of UV Spectroscopic method: The anthocyanin extract λ_{max} was found to be 536 nm and result of obtained peak is represented in Figure 1.

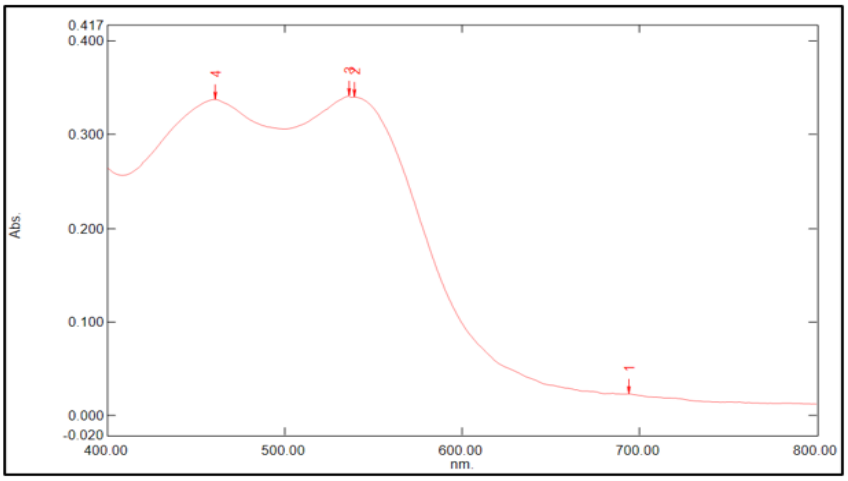

Figure 1. UV-Visible λ_{max} of Anthocyanin

Characterization of Anthocyanin Gels

pH Determination

The pH range of the all-gel formulations, which correlates to the normal pH range of the skin, was between 6.3-6.7.

Homogeneity

There were no aggregates formed and all the formulations were found to be homogenous.

Centrifuge Test

The gels maintained their uniformity and there was no observable sediment.

Temperature Change Test

There was no appearance change observed.

Table 2. Evaluation of Anthocyanin gels

Formulation Code	Parameters (n=3)			
	Viscosity in Cps ± S. D	**Spreadability in gm.cm/sec ± S. D**	**Extrudability in % ± S. D**	**Anthocyanin Content in % ± S. D**
F_1	11889.3 ± 9.01	26 ± 0.05	80.2 % ± 0.10	81.8 % ± 0.0005
F_2	11761.3 ± 9.01	40 ± 0.25	79.2 % ± 0.15	88.1 % ± 0.0005
F_3	11660 ± 10	43 ± 0.05	90.6 % ± 0.05	94.3 % ± 0.0005

The viscosity of F_3 was lower than F_1 and F_2, viscosity is inversely proportional to rate of drug release as viscosity increases drug releases decreases. Considering the results of other evaluation parameter of F_3 which was having lower viscosity, better spreadability, excellent extrudability, good Anthocyanin content, highest drug release, hence F3 was considered for clinical evaluation.

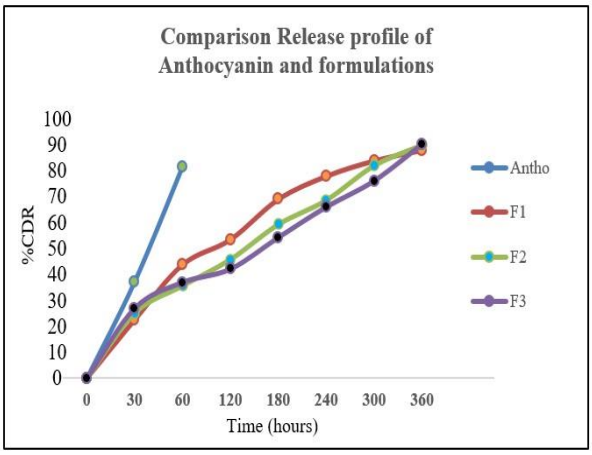

Figure 2. Comparison of cumulative drug release of pure drug and all formulations at the end of 6 hours

Evaluation Studies

Viscosity

All of the gel formulations exhibited good viscosity and the ability to adhere to the application site for an extended period of time. Among these formulations, gels made with sodium carboxy methyl cellulose and chitosan were less viscous than made with carbopol-940.

Spreadability

A small amount of shear was needed to spread the gel. Compared to gels made with carbopol-940 and sodium carboxy methyl cellulose, chitosan-made gels were easier to spread because they had reduced viscosity.

Extrudability

The extrudability values show that the gels have good extrudability. The gel made with chitosan among the formulations had excellent extrudability to the gels made with carbopol-940 and sodium carboxy methyl cellulose.

Anthocyanin Content

All gel formulations exhibited better drug content percentages ranging from 80 to 95 percent. The formulation F3 showed good percentage drug release than other formulations.

In-vitro Drug Release Studies

All gel formulations exhibited good drug release percentages. Gels made with chitosan performed better in terms of release among these formulations than those made with carbopol-940 and sodium carboxy methyl cellulose. The rate of drug release often decreases as viscosity increases.

Clinical Studies

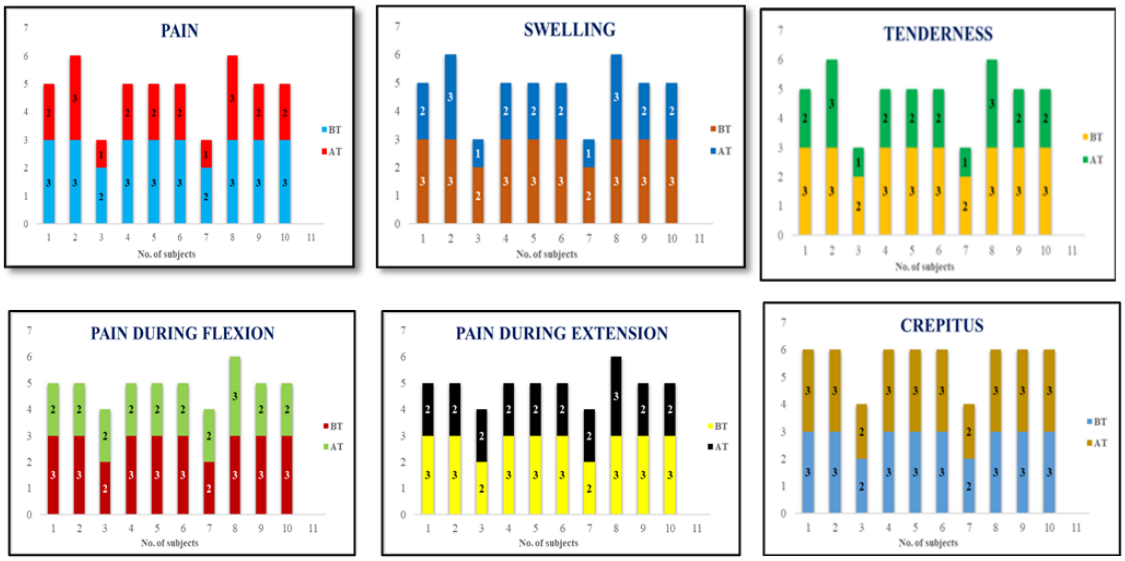

Figure 3. Effect of anthocyanin gel on subjects before treatment (BT) after treatment (AT)

Statistical Analysis: for Paired "t" Test

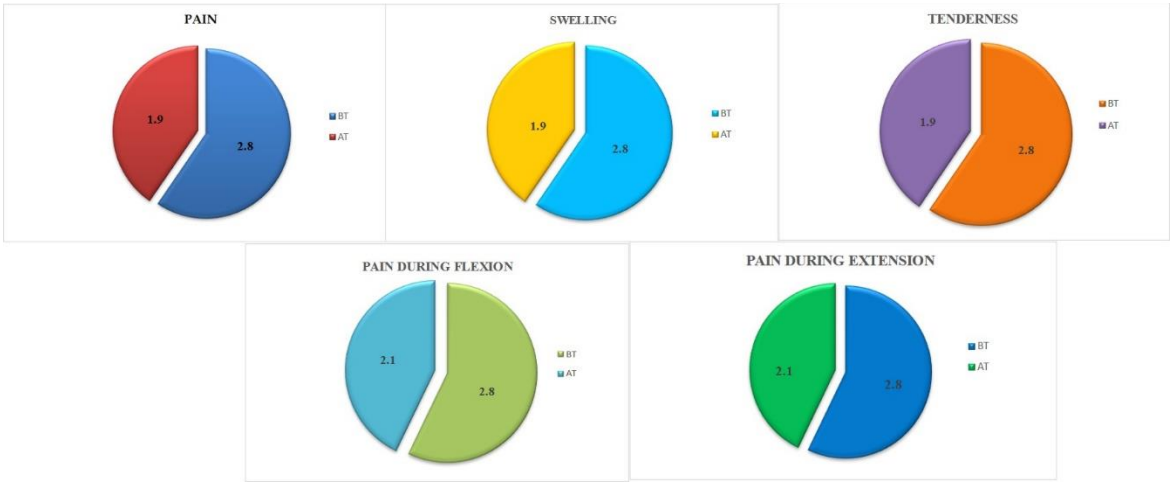

Figure 4. Improvement of parameters within subjects

In the present investigation, extraction of Anthocyanin from *Phaseolus vulgaris* L. for the formulation of topical gel and it's potential for anti-inflammatory activity was evaluated. Anthocyanin-containing topical gel has been shown to have anti-inflammation properties. The formulated gel of 2% concentration using chitosan polymer was applied topically to the subjects in the age-group of 25-70 years for 32 days in BVVS Ayurved Medical College & Hospital Bagalkot.

The subjects diagnosed for Osteoarthritis were included in the study, after obtaining the written consent, the subjects were treated. Subjects were instructed for the procedure of application of gel thrice a day. Further distribution of subjects were made based on the grades of pain, swelling, tenderness, pain during flexion, pain during extension, crepitus for indication of improvement with statistical analysis.

Pain

The effect of Anthocyanin gel on the subjects with pain according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 1.90 after the treatment with percentage relief of 67.85% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Swelling

The effect of Anthocyanin gel on the subjects with swelling according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 1.90 after the treatment with percentage relief of 67.85% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Tenderness

The effect of Anthocyanin gel on the subjects with tenderness according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 1.90 after the treatment with percentage relief of 67.85% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Pain During Flexion

The effect of Anthocyanin gel on the subjects with pain during flexion according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 2.10 after the treatment with percentage relief of 75% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Pain During Extension

The effect of Anthocyanin gel on the subjects with pain during extension according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 2.10 after the treatment with percentage relief of 75% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Crepitus

The effect of Anthocyanin gel on the subjects with crepitus according to the progress of administered formulation as; the mean score which was 2.7 before the treatment remained 2.7 after the treatment which implies same improvement in subjects.

The data for present study were collected from subjects case report and progress chart. All the above results indicated the difference was statistically significant.

ACKNOWLEDGMENTS

I wish to express my deep sincere gratitude to Dr. Mahantesh Salimath, Principal and Dr.Prakash V Naraboli, Professor, Department of Panchakarma of BVVS Ayurved Medical College and Hospital, Bagalkot for their guidance and co-operation to carry out my clinical study.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

Reference no: BVVS/IEC/AMVB-2020-21/767 from Institutional Ethics Committee (IEC for research on human subjects) of BVVS Ayurved Medical College & Hospita, Bagalkot. On August 23rd 2021.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND EVALUATION OF COMPRESSION-COATED TABLETS FOR CHRONOPHARMACEUTICAL DRUG DELIVERY

KRONOFARMASÖTİK İLAÇ TAŞIYICI BASINÇLA KAPLANMIŞ TABLETLERİN HAZIRLANMASI VE DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *This study aims to prepare and evaluate time-controlled drug delivery system of telmisartan. Telmisartan has low aqueous solubility, which is its major drawback. The solubility of the drug enhanced by using solid dispersion method and compression coated chronotherapeutic tablets were formulated.*
Material and Method: *Solid dispersion of telmisartan was prepared by melting method. Direct compression method was used to prepare telmisartan containing core tablets and tablets were coated by compression-coating method. Prepared tablets were characterized in terms of hardness, diameter, and thickness. In order to demonstrate the pulsatile release, the tablets were subjected to USP Apparatus II dissolution test.*
Result and Discussion: *Solid dispersion of telmisartan increased the solubility of telmisartan significantly ($p<0.05$). Compression-coated tablets were obtained with suitable hardness values (149.833±5.862-205.367±3.955 N) and telmisartan was released with lag times of 120-540 min which are suitable for chronopharmaceutic application.*
Keywords: *Chronopharmaceutical, compression-coated, pulsatile release, solid dispersions, telmisartan*

ÖZ

Amaç: *Bu çalışmada telmisartanın çözünürlüğünün artırılması için katı dispersiyonlarının hazırlanması amaçlanmıştır. Telmisartanın en önemli sorunu olan düşük çözünürlüğe sahip olmasıdır. Telmisartanın çözünürlüğü katı dispersiyon yöntemi kullanılarak artırılmış ve basınçla kaplanmış kronoterapötik tabletler formüle edilmiştir.*
Gereç ve Yöntem: *Telmisartanın katı dispersiyonları eritme yöntemi ile hazırlanmıştır. Telmisartan katı dispersiyonlarını içeren çekirdek tabletler doğrudan basım yöntemi ile hazırlanmış ve basınçla*

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kaplama yöntemi ile kaplanmıştır. Hazırlanan tabletler sertlik, çap ve kalınlık açısından karakterize edilmiştir. Tabletlerde pulsatil ilaç salımını göstermek amacıyla USP aparat II çözünme hızı testi yapılmıştır.

Sonuç ve Tartışma: *Telmisartan katı dispersiyonları telmisartanın çözünürlüğünü önemli ölçüde artırmıştır ($p<0,05$). Basınçla kaplanmış tabletler uygun sertlik değerleri (149.833±5.862-205.367±3.955 N) ve elde edilmiştir. Telmisartan, kronofarmasötik uygulamalara uygun şekilde 120-540 dk arasında değişen gecikme sürelerinden sonra hızla salınmıştır.*

Anahtar Kelimeler: *Basınçla kaplama, katı dispersiyon, kronofarmasötik, pulsatil salım, telmisartan*

INTRODUCTION

Circadian rhythm, which is a 24-hour clock related to the sleep-wake cycle, controls the rhythmicity of the biological systems. Biological disorders such as asthma, arthritis, duodenal ulcer, cancer, cardiovascular diseases, diabetes, and hypercholesterolemia can be influenced by circadian rhythm. As a result, they can display daily peaks and troughs [1]. Therefore, certain drug concentrations are required at the site of action at times appropriate to the circadian rhythm [2,3].

Some functions of cardiovascular system like blood pressure, heart rate, stroke volume are associated with circadian rhythm [4]. Capillary resistance and vascular reactivity are higher in the morning and decrease later in the day. In other words, blood pressure is at its lowest during sleep and progressively rises during the early morning hours [2,4,5]. Hence, according to the circadian rhythm of the body, antihypertensive drugs are required in the early hours of the morning [6]. Considering the diseases which have some night or morning symptoms, more effective therapies can be achieved with modified release drug delivery systems [4] rather than conventional systems which release the drug right after the applications [2]. Pulsatile release drug delivery systems are thought to be suitable for chronotherapeutic purposes in order to achieve therapeutic drug concentrations at the time which the symptoms of the diseases arise by bedtime administration of drugs [4]. Pulsatile drug delivery systems, which are synchronized with the circadian rhythm of disease states and body functions, release the drugs immediately like a pulse after a lag time in which no or less than 10% of drug release occurs [2,7]. In this context, by bedtime administration, pulsatile drug delivery systems release the drug after a lag time and show their effects when the symptoms begin, and so keep the patients from unnecessary drug exposure and interruption of night's sleep due to the requirement of night-time dosing [8].

Telmisartan (2-(4-{[4-methyl-6-(1-methyl-1*H*-1,3-benzodiazol-2-yl)-2-proply-1*H*-1,3-benzodiazol-1-yl]methyl}phenyl)benzoic acid) which is a widely used antihypertansive drug belongs to the group of angiotensin II type 1 (AT1) receptor antagonists. However, its low and variable bioavailability after oral administration due to its poor and pH dependent solubility is the major obstacle about telmisartan [9].

Among various other methods including micronization/nanonization, complexation or co-crystals, solid dispersion is one of the most efficient and successful solubility enhancement methods [10]. Solid dispersions are one-phase solid systems that comprising polymeric carrier in which the drug is incorporated [11,12]. In the solid dispersion method, the solubility of the drug is enhanced by altering the crystalline structure to an amorphous state with higher energy [13]. Solid dispersions can be constructed as binary or ternary systems. Binary solid dispersions compose of carrier and drug while ternary systems contain some excipients like surfactants, super disintegrants and, pH modifiers to enhance the solubility and stability of drug [14,15]. pH modifiers are vital components because of that solubility of two-thirds of water soluble active ingredients depends on pH [16]. It is suggested that pH modification of dosage forms can be a good strategy to enhance the solubility of drugs whose solubility are dependent on pH [17]. The solubility of telmisartan is significantly dependent on pH; it is insoluble in acidic mediums but spontaneously soluble in alkaline mediums [18,19], due to its free acid form [17,20]. Therefore, incorporating an alkalizer into telmisartan solid dispersions for changing microenvironmental pH can enhance the solubility of telmisartan, and subsequently minimize the supersaturation in the microenvironment can inhibit drug recrystallization and precipitation, improve solubility, dissolution rate, as well as oral bioavailability of telmisartan [20,21].

The aims of this study were i) to enhance the solubility of telmisartan through the formation of solid dispersion systems, ii) for administration at bedtime, to design pulsatile telmisartan tablets using compression coating method for delayed drug delivery that starts in the morning hours, when symptoms appeared.

MATERIAL AND METHOD

Materials

Telmisartan was obtained from Auctus Pharma Limited, Madhapur-Hyderabad. Poloxamer 188, sodium alginate (SA), hyroxypropyl methylcellulose (HPMC) 100 mPa.s were received from Sigma Aldrich, USA. HPMC 4000 mPa.s was supplied from Fluka, USA and cellulose acetate propionate (CAP) from Aldrich Chemistry, UK. Sodium carbonate was obtained from Riedel-de Haen AG, Seelze-Hannover. Avicel pH 102 and talc were provided from Aklar Kimya, Ankara-Turkey. Crosscarmellose sodium was supplied from JRS Pharma, Rosenberg, Germany and magnesium stearat was from Molychem, Mumbai-India.

Preparation of Solid Dispersions

The solid dispersions were prepared by a modified melting method (Table 1). Poloxamer 188 was melted at 60ºC, above the polymer's melting point and sodium carbonate was added to the molten polymer. After the molten polymer and alkali source were mixed homogeneously telmisartan was added and mixed with magnetic stirrer at 100 rpm for 45 minutes until it became a homogenous mixture. The homogenous mixture was cooled to room temperature to obtain a solid mass and the solidified masses were crushed and passed through 60 mesh sieve/ 250 µm microplate sieve.

Table 1. Composition of solid dispersions

Solid Dispersions	Ratios (telmisartan : poloxamer 188 : sodium carbonate)	Telmisartan (mg)	Poloxamer 188 (mg)	Sodium Carbonate (mg)
SD1	1:9:0	200	1800	-
SD2	1:9:1.5	200	1800	300
SD3	1:9:3	200	1800	600
SD4	1:6:1.5	200	1200	300
SD5	1:6:3	200	1200	600

Determination of Solubility of Pure Telmisartan and Solid Dispersions

The solubility examinations were performed according to the previously described shake flask method [22]. An excess amount of telmisartan, physical mixture, or solid dispersions was added to volumetric flasks containing 10 ml of phosphate buffer pH 7.5 and incubated in an orbital shaker at 100 rpm at 37ºC for 48 hours. Then the content was filtered through 0.45µm filter paper and analyzed at 295 nm with a UV-visible spectrophotometer after appropriate dilutions with phosphate buffer. All measurements were carried out in triplicate. The calculation of drug was performed using the calibration curve which was constructed by plotting the absorbance versus 2.5 to 20 ppm concentrations of telmisartan.

Preparation of Core Tablets

The core tablets were prepared by the direct compression method (Table 2). The ingredients except for talc and magnesium stearate were weighed and mixed for 5 minutes. Talc and magnesium stearate were added to the mixture and mixed for additional 5 minutes. The acquired mixture was compressed using a hydraulic press and 7 mm flat punches under 50 bar pressure for 20 sec.

Table 2. Core tablet formulations

Formulation Code	Telmisartan (mg)	Telmisartan Physical Mixture (mg)	Telmisartan Solid Dispersion (mg)	Avicel pH 102 (mg)	Cross-carmellose Sodium (mg)	Magnesium Stearate (mg)	Talc (mg)	Total (mg)
C1	10	-	-	131	6	1.5	1.5	150
C2	-	100*	-	41	6	1.5	1.5	150
C3	-	-	100*	41	6	1.5	1.5	150

* Equals to 10 mg telmisartan.

Preparation of Compression-coated Tablets

The prepared core tablets were subjected to compression coating using various compositions given in Table 3. The compression-coated tablets were prepared using a hydraulic press and 10 mm flat punches. 140 mg, half of the coating powder (CAP or blends of CAP and HPMC or SA) was filled to the die cavity to make a powder bed and the core tablet was manually placed in the center of the coating powder bed. The remaining half of the coating powder was then poured into the die and compressed at 50 bar for 20 sec.

Table 3. Coating layer compositions

Coating Layer	Formulation Code									
	CCT I	**CCT II**	**CCT III**	**CCT IV**	**CCT V**	**CCT VI**	**CCT VII**	**CCT VIII**	**CCT IX**	**CCT X**
CAP (mg)	280	252	210	140	252	210	140	252	210	140
SA (mg)	-	28	70	140	-	-	-	-	-	-
HPMC (100) (mg)	-	-	-	-	28	70	140	-	-	-
HPMC (4000) (mg)	-	-	-	-	-	-	-	28	70	140

Determination of the Physical Characteristics of the Tablets

Prepared tablets were characterized for hardness, thickness and diameter. The hardness of the core and coated tablets was measured by using Pharma Test hardness tester (model PTB 311, Key, Englishtown, NJ).

In vitro Drug Release Studies for Core and Compression-coated Tablets

In vitro drug release studies of the core and compression-coated tablets were carried out in 900 ml of pH 7.5 phosphate buffer at 37±0.5ºC at 75 rpm using USP dissolution apparatus II as described in USP 36. At specific time points, 5 ml of dissolution medium was withdrawn and replaced with a fresh dissolution medium. Withdrawn aliquots were filtered through 0.45 µm filter paper and analyzed at 295 nm with a UV-visible spectrophotometer. All measurements were carried out in triplicate.

Differential Scanning Calorimetry Studies

Thermal characteristics of telmisartan, poloxamer 188, sodium carbonate, their binary and ternary physical mixtures, as well as binary and ternary solid dispersions, were investigated by differential scanning calorimeter (DSC 60 with software of TA/60 WS, Shimadzu, Japan). Samples of about 2 mg were placed in sealed aluminum pans and heated under a nitrogen flow up to 300ºC, at the heating rate of 10ºC/min.

RESULT AND DISCUSSION

The focus of this study was to develop compression-coated tablets to obtain an immediate

telmisartan release when the highest blood pressure was seen in the early mornings, imitating the circadian cycle of the body after a lag time. For this reason, solid dispersion of telmisartan, a water-insoluble weak acidic drug, was prepared in order to increase the solubility. Then the core tablets containing telmisartan solid dispersion were compression-coated with CAP as the outer layer of coating agent and HPMC or SA as pore-forming agents in order to achieve pulsatile drug release for chronotherapy in hypertension.

Determination of Solubility of Free Telmisartan and Solid Dispersions

In the current study, solid dispersions which include different amounts of poloxamer 188 as carrier with or without the different amounts of sodium carbonate as alkalizer were prepared by melting method, as can be seen in Table 1.

Solubility profiles of the pure telmisartan and drug from SDs are presented in Figure 1. It is seen that the solubility of telmisartan is increased by approximately between 5.7 and 451.2 times in SD formulations with poloxamers 188 and sodium carbonate in comparison with pure drug. The SD containing poloxamers 188 and sodium carbonate in ratio 1:9:3 (SD3 coded solid dispersion) had the highest, while only Poloxamers 188 (SD1 coded formulation) had the lowest solubility of drug, respectively.

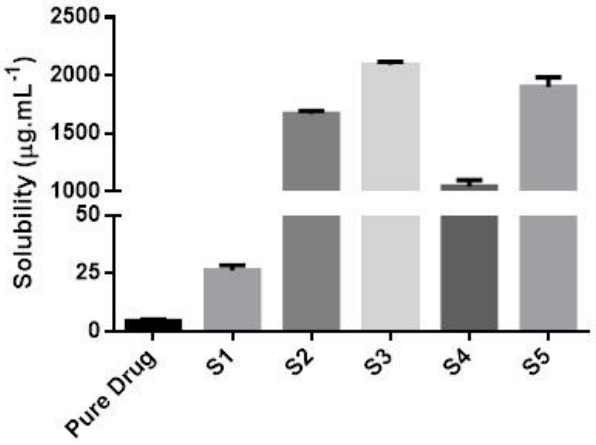

Figure 1. Solubility of pure telmisartan and telmisartan solid dispersions

These considerable differences between binary and ternary solid dispersions have shown that sodium carbonate played a crucial role in the solubilization of telmisartan and increasing amount of sodium carbonate for the same amount of poloxamer has enhanced solubility of telmisartan by changing the pH of the solid dispersion to alkali values. But surprisingly for the same amount of sodium carbonate, increased amount of poloxamer 188 has not significantly improved the solubility of telmisartan. According to these results SD5 which contains telmisartan: poloxamer 188: sodium carbonate 1:6:3 has showed the most promising solubility and so it has been selected for further experiments.

Determination of the Physical Characteristics of the Tablets

The hardness of tablets is the result of bonds between excipients and drug, and this network is formed by compression force during tableting. Moreover, lubricants reduce the friction between the dye and punches to allow smooth tableting with a reduced mechanical strength. Therefore, the hardness of core tablets which include magnesium stearate and talc as lubricant were lower than compression-coated tablets. On the other hand, the thickness and diameter values of all tablets meet the requirements (Table 4).

Table 4. Characterization of core tablets and compression-coated tablets

Formulation	Hardness (N)	Thickness (cm)	Diameter (cm)
C I	158.767±8.467	0.288±0.003	0.706±0.001
C II	56.700±3.517	0.327±0.003	0.704±0.001
C III	62.950±2.475	0.293±0.003	0.703±0.003
CCT I	195.433±2.281	0.475±0.005	1.004±0.001
CCT II	205.367±3.955	0.465±0.005	1.005±0.001
CCT III	179.467±7.132	0.455±0.005	1.005±0.001
CCT IV	149.833±5.862	0.443±0.003	1.010±0.002
CCT V	180.211±4.171	0.410±0.004	1.003±0.002
CCT VI	176.492±6.358	0.408±0.005	1.005±0.001
CCT VII	174.981±4.302	0.407±0.005	1.005±0.001
CCT VIII	178.973±5.180	0.425±0.004	1.002±0.001
CCT IX	179.209±3.129	0.424±0.005	1.005±0.002
CCT X	174.087±6.071	0.421±0.003	1.004±0.001

In vitro Drug Release Studies for Core and Compression-coated Tablets

Core Tablets

Core tablets were prepared with pure telmisartan, physical mixture and solid dispersion of telmisartan, and their dissolution profiles are given in Figure 2.

Telmisartan release from C1- and C2-coded formulations was found to be only 22.747% ± 1.292 and 56.323% ± 0.688, respectively. On the other hand, C3-coded formulation which contains solid dispersion of telmisartan demonstrated almost 100% drug release within the first 45 minutes, due to the enhanced aqueous solubility of telmisartan as a result of ternary solid dispersion. Hence, C3 formulation was selected as core tablet for further experiments.

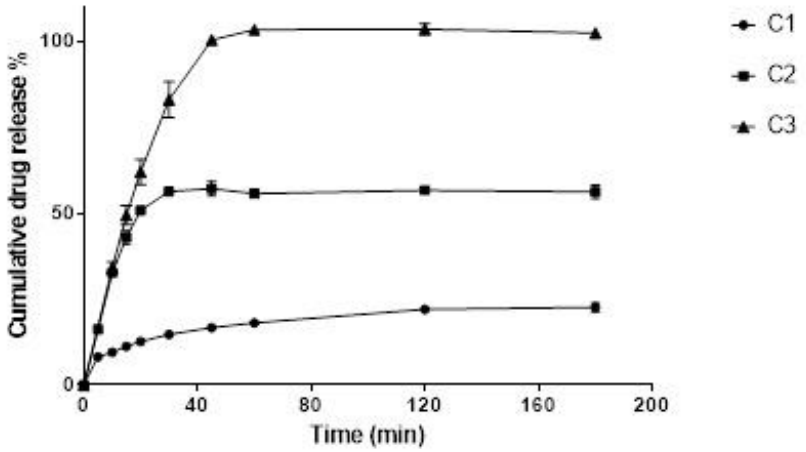

Figure 2. *In vitro* dissolution profiles of core tablets

Compression-coated Tablets

Pulsatile drug delivery systems can achieve chronotherapeutic purposes by mimicking the timing of the body. Being one of the pulsatile drug delivery strategies, compression-coated tablets consist of a core tablet which is made up of active ingredient and excipients and outer layer which determinate the

lag time and release profile according to its content that can be rupturable, swellable or erodible polymers [23,24].

The biggest advantage of these oral time-controlled drug delivery systems is providing a lag time after medication so that patient can benefit from drugs when the symptoms starts to arise special times in circadian cycle, especially diseases like cardiovascular diseases or asthma [25].

A variety of coating material can be used for compression-coating and according to these materials, drug release mechanisms change: rapture coating, swelling/eroding coating and permeation/diffusive coating [1,7,8,26]. Erodible coatings consist of hydrophilic polymers, for example HPMC, hydroxyethylcellulose and hydroxypropylcellulose, which go through swelling, dissolution and/or erosion when exposed to aqueous media and provide modified release of drug from the core. In rupture coating, delayed release of active ingredient is provided by disruption of the coating consisted of mixture of water insoluble polymeric material and water soluble materials as pore-forming agents. Disruption of the coating can be achieved by an adequate increase in core volume or hydration of the swellable polymers in the mixture of coating material together with insoluble polymer [8,26].

In this work, for compression-coating, CAP has been used as the main rupturable, eroding polymer and different grades of HPMC and SA have been used as pore-forming, swellable polymers and the effect of formulation of outer shell consisted of these hydrophobic and more hydrophilic polymers on the lag time and following drug release were investigated.

Cellulose esters are significant parts of the development of new drug delivery technologies due to their low toxicity, endogenous and/or dietary decomposition products, stability, film strength, compatibility with many of drugs, and ability to form micro- and nanoparticles [27].

CAP, a biodegradable and water-insoluble polymer [28] is used as an enteric coating material in capsules and tablet formulations. CAP can be used for coating with organic or aqueous solvent systems or for direct compression [29].

HPMC is a cellulose derivative approved by FDA and has been commonly used as a pharmaceutical additive for various purposes, especially in oral controlled drug delivery systems. HPMC is also a suitable compression-coating material for timed-release systems because of that the swelling upon contact with water and forming a hydrogel behavior of HPMC can control drug diffusion tightly [30]. SA is a hydrophilic polymer which has erodible properties. The erosion property of SA in coating layer leads to breakdown of coating [31]. Swellable polymers, like HPMC, have a crucial role in pulsatile drug delivery systems being either an erodible coating barrier or swelling force to break up rupturable coating layer [26].

The compression-coated tablets showed different release profiles with clear lag times followed by different release phases depending on coating layer compositions. Incorporation of pore-forming agents into coating layer resulted in release profiles with different lag times and release phases. Therefore, incorporation of HPMC or SA in CAP coating layer modulated the lag time and drug release profiles.

It can be a reason for extended lag time that HPMC and SA may form a viscous gel around CAP [32] and drug release from compression-coated tablets started when the outer shell took off by dissolution or erosion of the hydrophilic gel layer formed by HPMC or SA on the surfaces of core tablets [33].

The incorporation of the hydrophilic polymers (both SA and HPMC) into the coating layer contributed to the release of telmisartan. CCT I-coded tablet formulation which contained only CAP as coating agent did not show any telmisartan release while all other formulations which contained different amounts of SA or HPMC released telmisartan after variable lag times without premature release.

CCT II-IV, tablet formulations containing SA, demonstrated pulsatile release of telmisartan after different lag times depending on SA ratio in the coating layer. As can be seen in Figure 3 increased ratio of SA in the coating layer decreased the lag time due to hydration of the coating layer by the hydrophilic characteristic of SA [34]. CCT II-coded tablet with the lowest SA content showed the highest lag time prior to sustained telmisartan release whereas CCT III and IV showed faster drug release within 2 hours.

The mean lag time of the compression-coated tablets containing both HPMC-100 and HPMC-4000 decreased with increase in HPMC concentration from 10% to 25% but increased for 50% level (Figure 4 and 5). This initial decrease in mean lag times up to 25% concentration may be attributed to the dominating pore-forming properties, while further increase to 50% w/w was the consequence of

increased gelling properties of HPMC. During dissolution process, the coating layers which consist of 50% HPMC became elastic as a result of dominated gelling properties over pore-forming properties [35].

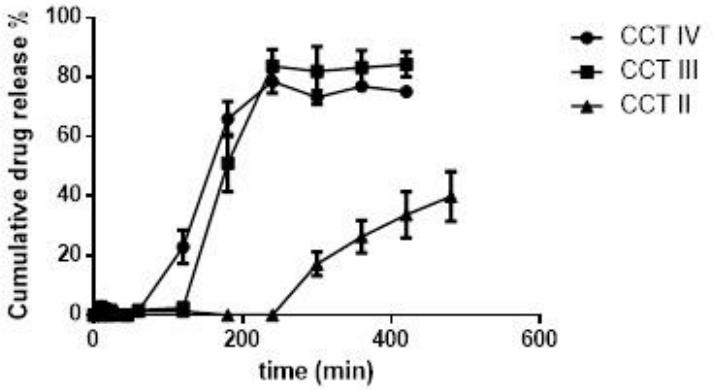

Figure 3. *In vitro* dissolution profiles of SA containing compression-coated tablets

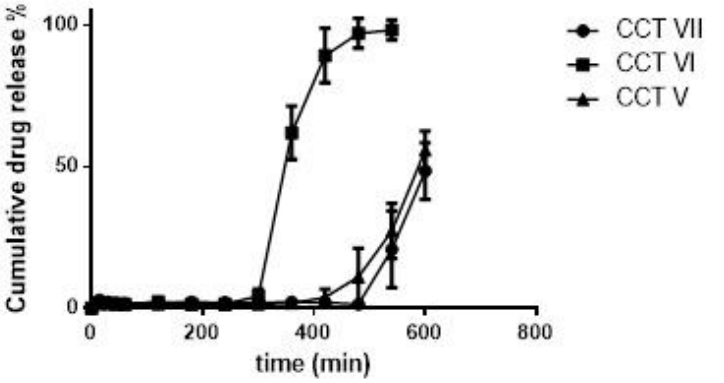

Figure 4. *In vitro* dissolution profiles of HPMC-100 containing compression-coated tablets

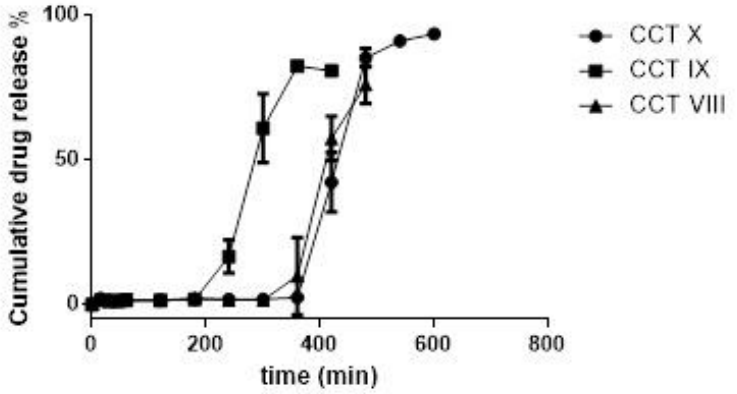

Figure 5. *In vitro* dissolution profiles of HPMC-4000 containing compression-coated tablets

Generally, it is expected that the retardation effect of polymers increases as the viscosity of the polymer increases due to the formed stronger gel with more viscous polymer on the core tablet [36,37]. But interestingly in our work, the lag times obtained from HPMC with higher viscosity grade were shorter than HPMC with lower viscosity grade for all ratios in the coating layer. In fact that swelling property of the polymer increases with increasing viscosity of the polymer [38]. This means HPMC with 4000 mPa.s viscosity swells more than HPMC with 100 mPa.s. The more swelling HPMC (4000 mPa.s) caused to remove the insoluble polymer, CAP coating on the core tablets earlier and release of telmisartan. After the lag time, the outer shell of the CAP coating on the core tablet broke into two halves to result in rapid drug release. The rupturing of the coating layer of the CCT X coded compression coated tablet which is the optimum compression-coated tablet formulation with approximately 6 hours of lag time and following rapid release was visualized by using a colored core tablet, and can be seen in Figure 6.

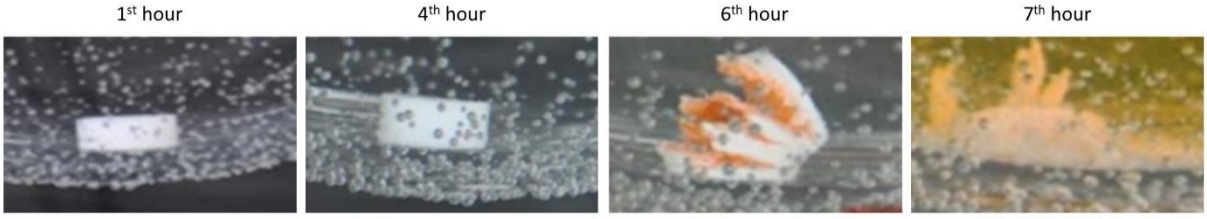

Figure 6. The images of CCT X-coded compression-coated tablets during *in vitro* dissolution tests

Differential Scanning Calorimetry Studies

DSC was used to determine the state of telmisartan in solid dispersion and physical mixtures and to identify possible drug–polymer or drug-alkali interactions. DSC is a thermal analysis which measures change of physical properties like phase transitions of a sample along with temperature against time. While temperature is increasing or decreasing, heat quantity absorbed or radiated by sample as temperature differences between sample and reference are measured [39].

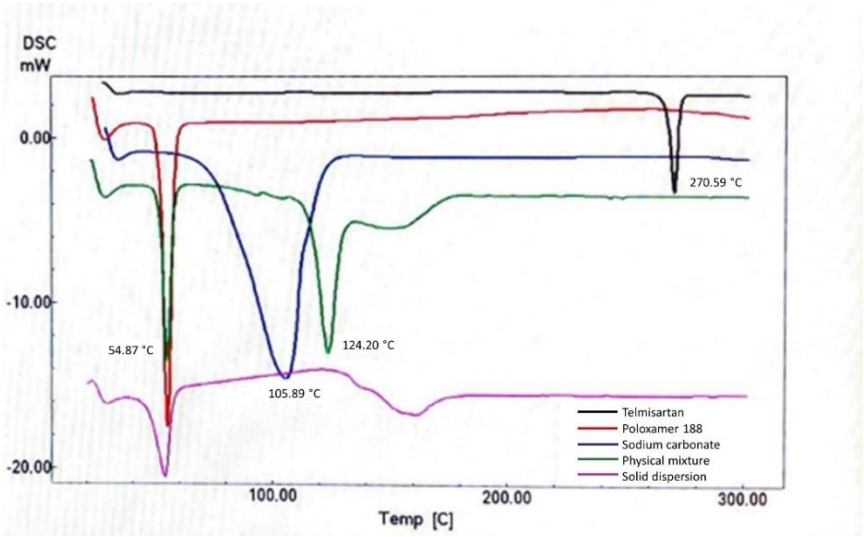

Figure 7. DSC thermograms of the pure telmisartan, poloxamer 188, sodium carbonate, their physical mixture and solid dispersion (SD5)

DSC thermograms of telmisartan, poloxamer 188 as well as their solid dispersions and physical mixture are shown in Figure 7. As can be seen in Figure 7, pure telmisartan, poloxamer 188, and sodium

carbonate showed endothermic peaks at 270.59°C, 54.86°C, and 105.89°C, respectively, corresponding to their melting point and indicating their crystalline structure. However, the sharp endothermic peak of telmisartan has disappeared in the DSC thermograms of solid dispersions and physical mixture. The absence of telmisartan endotherms can be attributed to either drug solubilization in molten polymer before melting point of telmisartan or change into an amorphous state during preparation process [40,41,42]. On the other hand, in the DSC thermogram of the physical mixture the shift of the sodium carbonate peak from 105.89 to 124.20°C may also be due to the relationship between telmisartan and sodium carbonate. These differences have explicated that there was a strong interaction between weak acidic telmisartan and alkali sodium carbonate [17].

This study showed that the solubility of telmisartan can be enhanced by the solid dispersion technique and telmisartan/sodium bicarbonate/poloxamer 188 ternary system can be considered suitable for pulsatile release tablet formulations. Formulations of pulsative compression-coated tablets containing solid dispersion of telmisartan are successfully developed. The tablets provided a desirable lag time followed by rapid and complete drug release to meet the challenges of chronopharmaceuticals. The *in vitro* drug release studies showed that the lag time of the tablet formulation could be modified by several factors such as core composition, the type and ratio of coating materials.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF POTENTIAL DRUG-DRUG INTERACTIONS IN GENERAL PEDIATRIC WARD PATIENTS: A CROSS-SECTIONAL STUDY

GENEL PEDİYATRI SERVİSİ HASTALARINDA POTANSİYEL İLAÇ-İLAÇ ETKİLEŞİMLERİNİN SAPTANMASI: BİR KESİTSEL ÇALIŞMA

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ABSTRACT

Objective: *This study seeks to identify potential drug-drug interactions (pDDIs) in hospitalized patients and compare two commercial drug interaction databases.*
Material and Method: *This prospective cross-sectional study was conducted between February and May 2022 in a tertiary care hospital's general pediatric ward. UpToDate® and Micromedex® Drug Interaction databases were used to determine pDDIs.*
Result and Discussion: *In total, 267 pDDIs were found in 51 pediatric patients' medication lists (181 via UpToDate® and 86 via Micromedex®). The use of at least five different systemic drugs concurrently was statistically significant between groups of patients who experienced at least one pDDI and those who did not. The binary logistic regression analysis showed that a one-drug increase in the total number of drugs a patient received during hospitalization increased the probability of pDDIs by 2.12-fold (CI: 1.321-3.417, p=0.002). The concordance rate between UpToDate® and Micromedex® databases for pDDI determination was 84.31% (kappa coefficient=0.676, standard error=0.102, (p ≤ 0.001)). When the UpToDate® database was assumed as a reference database, the Micromedex® database's sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in determining pDDIs were 79.41%, 94.12%, 96.43%, 69.56%, and 84.5%. To avoid missing pDDIs, utilizing multiple drug interaction databases may be of benefit.*
Keywords: *Children, clinical pharmacist, drug interactions, pediatric patients*

ÖZ

Amaç: *Bu çalışma, hastanede yatan hastalarda potansiyel ilaç-ilaç etkileşimlerini (pDDI'ler) belirlemeyi ve iki ticari ilaç etkileşimi veri tabanını karşılaştırmayı amaçlamaktadır.*

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Gereç ve Yöntem: *Bu prospektif kesitsel çalışma, Şubat ve Mayıs 2022 tarihleri arasında üçüncü basamak bir hastanenin genel pediyatri servisinde yürütülmüştür. pDDI'leri belirlemek için UpToDate® ve Micromedex® İlaç Etkileşimi veritabanları kullanılmıştır.*

Sonuç ve Tartışma: *Elli bir pediatrik hastanın ilaç listesinde toplam 267 pDDI bulunmuştur (UpToDate® aracılığıyla 181 ve Micromedex® aracılığıyla 86). En az beş farklı sistemik ilacın aynı anda kullanımı, en az bir pDDI saptanmış ve saptanmamış hasta grupları arasında istatistiksel olarak anlamlıydı. İkili lojistik regresyon analizi, bir hastanın hastanede yatışı sırasında aldığı toplam ilaç sayısındaki bir ilaç artışının, pDDI olasılığını 2.12 kat artırdığını göstermiştir (GA: 1.321-3.417, p=0.002). pDDI saptnmasında UpToDate® ve Micromedex® veritabanları arasındaki uyum oranı %84.31 olarak bulunmuştur (kappa katsayısı=0.676, standart hata=0.102, ($p \leq 0.001$)). UpToDate® veri tabanı referans veri tabanı olarak kabul edildiğinde, Micromedex® veri tabanının pDDI'leri belirlemedeki hassasiyeti, özgüllüğü, pozitif prediktif değeri, negatif prediktif değeri ve doğruluğu %79.41, %94.12, %96.43, %69.56 ve %84.5 idi. Bir pDDI'yı atlamaktan kaçınmak için çoklu ilaç etkileşimi veritabanlarının kullanılması faydalı olabilir.*

Anahtar Kelimeler: *Çocuklar, ilaç etkileşimleri, klinik eczacı, pediyatrik hastalar*

INTRODUCTION

In pediatric patients, treatment regimens with more than one drug may be required to treat diseases and this may lead to potential drug-drug interactions (pDDIs). One of the most significant drug-related problems that make therapy more challenging among pediatric patients is pDDIs. Due to the fact that the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs vary in children, it is much more important to monitor treatment for drug interactions [1]. Even in children, the cytochrome p450 enzyme system, which is responsible for a significant proportion of drug interactions, varies with age [2].

Drug interactions are frequently encountered, especially in patients with polypharmacy and long hospital stay [3]. In a retrospective cohort study using the American Pediatric Health Information System database, it was reported that 75% of the pediatric patients in intensive care unit were exposed to at least one pDDI, and 51.1% of these interactions were major interactions [1]. In another study involving 42 Children's Hospitals, at least one pDDI was found in approximately half of the 498,956 hospitalizations [4].

In pediatric patients, it is vital not only to identify drug interactions but also to manage them. Drug-drug interaction databases, having become widespread in recent years for managing drug interactions, are time-saving facilitator applications for healthcare professionals. However, these databases differ in sensitivity, specificity and accuracy in detecting drug-drug interactions [5,6]. Besides, the results obtained from these databases and clinicians' evaluations show large discrepancies in clinical importance of interactions [7]. Considering both the difference in databases and the pharmacokinetic difference in pediatric patients, drug interaction management in pediatric patients becomes an issue that needs attention.

This study aims to identify pDDIs in patients hospitalized in a general pediatric ward of a tertiary care hospital and to compare the performance of two different drug interaction databases to identify pDDIs.

MATERIAL AND METHOD

This cross-sectional study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the ethics committee of Inonu University (25th January 2022 - No: 2022/3055). The study conducted between February 2022 and May 2022 in the general pediatric ward of a tertiary care hospital in eastern Türkiye, which has a capacity of 14 beds. Patients who were admitted to the general pediatric ward and whose parental consent was obtained were included in the study. All drug-drug interactions of the patients were determined by two clinical pharmacists in the ward. Information about the sex, age and daily medication lists of the patients were obtained from the electronic database of the hospital. Drug-drug interactions were determined using the UpToDate® Drug Interaction and IBM Micromedex® Drug Interaction databases. In the evaluation of the data, descriptive and advanced statistical analyses were performed using SPSS V25.0.

Statistical Analysis

While continuous variables were indicated by median and interquartile range (IQR), categorical variables were presented by number (n) and percentage (%). The results of statistical tests were deemed statistically significant unless the *p* value of the test was greater than 0.05. The Kolmogorov-Smirnov test was used to determine whether the quantitative data is normally distributed or not. Chi-squared and Mann-Whitney U tests were used to compare categorical and continuous data, respectively. The correlation between the two data was determined through Spearman's rho test. If the correlation coefficient falls between 0.01 and 0.29, 0.30 and 0.70, or 0.71 and 0.99, the correlation is judged to be poor, fair, or strong, accordingly. Risk factors having potential to affect the occurrence of pDDIs were determined by binary logistic regression analysis. Cohen's kappa value was used to determine the concordance rate between the UpToDate® and Micromedex® databases in terms of the databases' ability to identify pDDIs, the severity of identified pDDIs, and the documentation rates of identified pDDIs. If Cohen's kappa value was less than zero, it was concluded that the databases did not agree. If Cohen's kappa value was between 0-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80 or 0.81-1.0; then it was accepted that there was slight agreement, fair agreement, moderate agreement, substantial agreement or almost perfect agreement between the databases, respectively. The UpToDate database categorizes pDDIs into five risk rating categories. These classifications, namely A, B, C, D, and X, indicate: no known interaction, no action needed, monitor therapy, consider therapy modification, and avoid combination, respectively. However, the Micromedex database has no risk rating category classification system. To determine the degree of concordance between the two databases regarding the severity of identified pDDIs, the risk rating categories B, C, D, and X from the UpToDate® database were paired with the minor, moderate, major, and contraindicated severity categories from the Micromedex® database.

Performance of pDDI screening programs was assessed through calculating sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) [8] and their definitions were given in Table 1.

Table 1. Definition of sensitivity, specificity, negative and predictive value in the setting of pDDIs.

Parameter	Definition	Calculation
Sensitivity	The ability to detect clinically important drug-drug interactions.	number of true-positives / (number of true-positives + number of false-negatives)
Specificity	The ability to ignore clinically unimportant drug-drug interactions.	number of true-negatives / (number of true-negatives + number of false-positives)
Positive predictive value (PPV)	When a drug-drug interaction is found, the probability that the drug-drug interaction is clinically important.	number of true-positives / (number of true-positives + number of false-positives)
Negative predictive value (NPV)	When a drug-drug interaction is ignored, the probability that the drug-drug interaction is clinically unimportant.	number of true-negatives / (number of true-negatives + number of false-negatives)

True-positives: at least 1 pDDI was determined by both of the databases
True-negatives: no pDDI was determined by both of the databases
False-positives: at least 1 pDDI was determined by the Micromedex® database while no pDDI was determined by the UpToDate® database
False-negatives: no pDDI was determined by the Micromedex® database while at least 1 pDDI was determined by the UpToDate® database

RESULT AND DISCUSSION

The study comprised a total of 51 pediatric patients, 49% of whom were male. The median (IQR) value of the patients' age was 18 (8-96) months. The median (IQR) length of hospitalization for patients was 7 (5-11) days. The median (IQR) number of different systemic drugs utilized per hospitalized patient

was 6 (3-7). Table 2 lists the admission diagnoses of patients classified by the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10).

Table 2. The diagnoses made at the time of the patients' admission

ICD-10 Code	Number (%)
J00-J99 Diseases of the respiratory system	30 (58.82)
A00-B99 Certain infectious and parasitic diseases	8 (15.69)
R00-R99 Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified	5 (9.80)
Q00-Q99 Congenital malformations, deformations and chromosomal abnormalities	3 (5.88)
U00-U85 Codes for special purposes	1 (1.96)
L00-L99 Diseases of the skin and subcutaneous tissue	1 (1.96)
K00-K93 Diseases of the digestive system	1 (1.96)
H00-H59 Diseases of the eye and adnexa	1 (1.96)
D50-D89 Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism	1 (1.96)

According to the UpToDate® and Micromedex databases, the number of patients having at least one pDDI identified was 34 (66.7%) and 28 (54.9%), respectively. The number of patients with at least one pDDI determined using any of the two databases was 35 (68.6%).

In total, 267 pDDIs were detected on 473 daily medication lists. 181 (67.79%) of the pDDIs were identified using the UpToDate® database, whereas 86 (32.21%) were identified using the Micromedex database. These 267 pDDIs were identified in 129 drug pairings, 50 of which (38.76%) were detected using the Micromedex® database and 79 (61.24%) using the UpToDate® database. Once the overlapping drug pairings between the databases were excluded, 88 different drug pairings remained. Again, once the overlapping pDDIs between the databases were excluded, 195 different pDDIs remained. According to the data obtained from the UpToDate® and Micromedex® databases, the number of pDDIs per daily medication list was found to be 0.38 and 0.18, respectively. According to the UpToDate® database, the following pDDI mechanism-specific distribution rates were determined: 35.91% were pharmacokinetic, 51.93% were pharmacodynamic, and 12.15% were unknown. In addition, the Micromedex® database revealed the following distribution rates for pDDIs in terms of their mechanisms: 63.95 percent of them were pharmacokinetic, 19.77 percent were pharmacodynamic, and 16.28 percent of them were unknown.

The distribution of the pDDIs as regards their severity and documentation rates were given in Table 3.

The UpToDate® database indicated that 64 (35.36%) of the pDDIs did not necessitate any action, 79 (43.64%) required therapy monitoring, 35 (19.34%) required therapy modification, and 3 (1.66%) required avoidance of combination.

According to the UpToDate® database, the top three drug pairs that probably caused at least one pDDI (n, %) were budesonide-clarithromycin (21, 11.6), albuterol-budesonide (20, 11.05), and albuterol-clarithromycin (19, 10.50). Moreover, according to the Micromedex® database, the first three drug pairs that most commonly probably caused at least one pDDI (n, percent) were budesonide-clarithromycin (21, 24.42), clarithromycin-methylprednisolone (10, 11.7), and epinephrine-linezolid (3, 3.49). When the two databases were analyzed together, the first three drug pairs that probably caused at

least one pDDI the most frequently (n, percent) were budesonide-clarithromycin (42, 15.73), clarithromycin-methylprednisolone (21, 7.83), and albuterol-clarithromycin (20, 7.49).

Table 3. The distribution of pDDIs according to severity and documentation rates of two databases

Factor	Rate	UpToDate® n (%)	Micromedex® n (%)
Severity	Minor	35 (19.34)	3 (3.49)
	Moderate	134 (74.03)	53 (61.63)
	Major	12 (6.63)	26 (30.23)
	Contraindicated	NA	4 (4.65)
Documentation	Fair	99 (54.70)	55 (63.95)
	Good	58 (32.04)	28 (32.56)
	Excellent	24 (13.26)	3 (3.49)

NA: not applicable

Table 4 shows the ten most frequently observed pDDIs. The classification of the drug groups associated with pDDIs frequently is given in Table 5. The Anatomical-Chemical Classification System (ATC) was used to classify the drugs.

It was determined that there was a positive-oriented fair association between concomitant usage of at least 5 different systemic drugs and number of pDDIs (correlation coefficient=0.644, $p<0.001$ for the UpToDate® database; correlation coefficient=0.572, $p<0.001$ for the Micromedex® database).

It was observed that there was a positive-oriented strong association between the total number of different systemic drugs administered during hospitalization and number of pDDIs (correlation coefficient=0.738, $p<0.001$ for the UpToDate® database; correlation coefficient=0.710, $p<0.001$ for the Micromedex® database).

Statistically, the number of pDDIs categorized according to the risk categories of the UpToDate® database is not affected by sex ($p>0.05$). The effect of concomitant usage of at least five different systemic drugs on the number of pDDIs is statistically significant for risk rating categories B, C, and D ($p \leq 0.001$), but not for group X ($p>0.05$).

It was found that sex has no statistically significant effect on the number of pDDIs detected regarding their degree of importance in the UpToDate® database ($p>0.05$). The concomitant usage of at least 5 different drugs was however found to has statistically significant effect on the distribution of pDDIs according to risk rating categories of UpToDate® database except for X category (p values for A, B, C, D, and X categories is NA, <0.001, <0.001, =0.001, and >0.05, respectively).

Sex of the patient has no statistically significant effect on severity of pDDIs according to both UpToDate® and Micromedex® databases ($p>0.05$). The concomitant usage of at least 5 different systemic drugs has statistically significant effect over the number of pDDIs according to severity categories of the UpToDate® which consists of minor, moderate, and major (p values for minor, moderate, and major severity were <0.001, <0.001, and =0.039, respectively). The effect of concomitant usage of at least 5 different systemic drugs was statistically significant only for the number of pDDIs whose severities were moderate according to the Micromedex® database ($p \leq 0.001$).

A binary logistic regression analysis was performed to determine the factors that could potentially affect the occurrence risk of pDDIs. In the analysis, the effects of the length of hospitalization and the total number of drugs to which the patient was exposed were examined. Increasing the total number of drugs a patient received during hospitalization by one could increase the odds of identifying the risk of pDDIs by 2.12-fold (95% confidence interval [CI]: 1.321-3.417, p=0.002).

Table 4. The first 10 of the most commonly encountered pDDIs determined through the UpToDate® and the Micromedex® databases

UpToDate®				Micromedex®			
Drug pair	**Risk Category/ Severity**	**Docume-ntation Rate**	**Comment**	**Drug Pair**	**Severity**	**Docume-ntation Rate**	**Comment**
Budesonide (inh)-clarithromycin (iv)	D/Moderate	Good	Clarithromycin can increase the serum level of budesonide.	Budesonide (inh) -clarithromycin (iv)	Moderate	Fair	Clarithromycin can increase the serum level of budesonide.
Albuterol (inh)-budesonide (inh)	B/Moderate	Fair	Budesonide can increase the hypokalemic effect of albuterol.	Clarithromycin (iv)-methylprednisolone (iv)	Moderate	Good	Clarithromycin can increase the side effects of methylprednisolone.
Albuterol (inh)-clarithromycin (iv)	B/Minor	Fair	The QT prolongation risk can increase with concomitant usage	Epinephrine (nasal)-linezolid (iv)	Contrain dicated	Fair	Increased hypertensive effect can be seen with concomitant usage.
Albuterol (inh)-epinephrine (nasal)	C/Moderate	Fair	Sympathomimetics can increase adverse/toxic effects of the other sympathomimetics.	Clarithromycin (iv)-valproate (iv)	Moderate	Fair	Increased levels of valproate can be seen with concomitant usage.
Albuterol (inh)-methylprednisolone (iv)	B/Moderate	Fair	Methylprednisolone can increase the hypokalemic effect of albuterol.	Amikacin (iv)-ibuprofen (po)	Moderate	Good	Increase in exposure of amikacin can be seen with concomitant usage.
Clarithromycin (iv)-methylprednisolone (iv)	C/Moderate	Excellent	Clarithromycin can increase the serum level of methylprednisolo-ne.	Budesonide (inh)-ibuprofen (po)	Major	Fair	Increase in the risk of gastrointestinal system bleeding and ulcer can be seen.
Amikacin (iv)-ceftriaxone (iv)	C/Moderate	Excellent	Cephalosporins can decrease the serum levels of aminoglycosides. Cephalosporins can increase nephrotoxic effects of aminoglycosides.	Clarithromycin (iv)-clonazepam (po)	Major	Fair	Clarithromycin increases the toxicity of clonazepam.
Albuterol (inh)-linezolid (iv)	D/Major	Fair	Linezolid can increase hypertensive effect of albuterol.	Albuterol (inh)-digoxin (po)	Moderate	Good	Concomitantly use of the two decreases serum digoxin levels.
Epinephrine (nasal) -linezolid (iv)	X/Major	Fair	Linezolid can increase hypertensive effect of epinephrine.	Albuterol (inh)-furosemide (iv)	Moderate	Fair	Concomitantly use of the two can result in hypokalemia and ECG changes.
Albuterol (inh)-azithromycin (iv)	B/Minor	Fair	The QT prolongation risk can increase with concomitant usage	Amikacin (iv)-piperacillin/tazobactam (iv)	Minor	Good	Decrease in the efficacy of amikacin can be seen with concomitant usage.

inh: per inhalation, iv: intravenous, po: per oral

Table 5. The ATC groups of the drugs the most commonly caused pDDIs

UpToDate®		Micromedex®	
Drug Group (ATC)	**n (%)**	**Drug Group (ATC)**	**n (%)**
Drugs for Obstructive Airway Diseases	112 (30.94)	Antibacterials for systemic use	68 (39.53)
Antibacterials for Systemic Use	101 (27.90)	Antiepileptics	25 (14.53)
Antiepileptics	49 (13.54)	Drugs for obstructive airway diseases	25 (14.53)
Corticosteroids	25 (6.91)	Corticosteroids	13 (7.56)
Nasal Preparations	17 (4.70)	Cardiac glycosides	6 (3.49)
Psycholeptics	10 (2.76)	Anti-inflammatory and antirheumatic products	5 (2.91)
Analgesics	9 (2.49)	Nasal preparations	4 (2.33)
Drugs for Functional Gastrointestinal Disorders	7 (1.93)	Psycholeptics	4 (2.33)

Table 6 lists factors that may be related with the presence of pDDIs.

Table 6. The relationship between various factors and the determination of pDDIs

Factors	**The roup that at least one pDDI was encountered (n=35)**	**The group that no pDDI was encountered (n=16)**	***p* value**
Age (months) [median (min-max)]	18.00 (8.00 – 43.50)	72.00 (9.50 – 99.00)	0.324[a]
Sex n (%)			0.485[b]
Male	16 (45.71)	9 (56.25)	
Female	19 (54.29)	7 (43.75)	
The duration of hospitalization (days) [median (min-max)]	7.00 (5.50 – 11.50)	6.50 (5.00 – 8.00)	0.194[a]
Concomitant usage of at least 5 different systemic drugs n (%)	28 (80.00)	3 (18.75)	<0.001[b]

[a]Mann-Whitney U test [b]Chi-squared test

The concordance rate between the UpToDate® and Micromedex® databases was 84.31% in terms of their ability to identify pDDIs (kappa coefficient=0.676, standard error=0.102, ($p \leq 0.001$).

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the Micromedex® database in determining pDDIs were calculated to be 79.41%, 94.12%, 96.43%, 69.56%, and 84.31% when the UpToDate® database was assumed as the reference database. The main reason we assume the UpToDate® database as a reference is that it categorizes drug interactions into risk rating categories and gives a higher number of drug interactions compared to the Micromedex® database.

The concordance rates between the drug interactions databases were given in Table 7.

Drug-drug interactions are major issues that require attention, as they result in prolonged hospitalizations, increased health care expenses, severe drug responses, and treatment failure. Inappropriate drug use is an important risk factor for the development of adverse reactions [9]. For this reason, pDDIs are issues that should be considered by healthcare professionals for the well-being of the patient.

In a previously conducted study, Hassanzad et al. [10] found 845 pDDIs via the Lexi-Interact® database among 176 prescriptions obtained from 176 pediatric patients. In our study, the number of pDDIs was 181 out of 473 daily medication lists obtained from 51 pediatric patients via the UpToDate® database, which was quite different from those of Hassanzad et al. The explanation of this could be our relatively small sample size. In addition, while Hassanzad et al. evaluated the prescriptions of the patients only on the second day of hospitalization, we evaluated the daily medication lists of the patients

on each day of their hospitalization. Maybe in our case, interday drug variability was lower than those of Hassanzad et al.

Table 7. The concordance rates between the UpToDate® and the Micromedex® databases in terms of the severities and documentation rates of the determined pDDIs

Parameter	Degree	Concordance Rate (%)	Kappa Coefficient	Standard Error	*p* value
The Severity of the Interaction	Minor	54.90	-0.026	0.072	0.724
	Moderate	84.31	0.689	0.098	< 0.001
	Major	74.51	0.485	0.106	< 0.001
	Contraindicated	96.08	0.646	0.233	< 0.001
Documentation Rate	Fair	88.24	0.765	0.090	< 0.001
	Good	78.43	0.571	0.107	< 0.001
	Excellent	60.78	0.071	0.082	0.355

A study which was conducted among 384 pediatric patients in a university hospital, the frequency rate of pDDIs was found as 45.8% according to the Micromedex® database [11]. In another study that employed the Micromedex® database and included all patients younger than 21 years of age hospitalized between January 2011 and December 2011 in U.S. children's hospitals, the rate of pDDIs was 49%. [4]. In our study, the frequency of pDDIs in our analysis of 51 pediatric patients was 54.9% (n=86) according to the Micromedex® database.

Getachew et al. [11] categorized pDDIs based on their mechanisms and found that the biggest proportion (50%) belonged to the pharmacokinetic group, as did our study (63.95%) according to the Micromedex® database. Tavousi et al. [12] categorised pDDIs based on their mechanisms and found that the biggest proportion belonged to the pharmacodynamic category (56.1%), as did our analysis (51.93%) according to the UpToDate® database.

In a study done by Bebitoglu et al. [12], a total of 634 pDDIs were identified in hospitalized pediatric patients over the course of one year by using Lexi-Interact database. The following were the rates of interactions based on risk rating categories: 42.7% of the interactions were in category A, 44.8% were in category B, 8.4% were in category C, and 4.1% were in category D. We found 181 pDDIs using UpToDate®. None were A, 35.36% B, 43.65% C, 19.34% D, and 1.66% X risk rating category. We observed fewer interactions than Bebitoglu et al. [13] since duration of our study was shorter.

Getachew et al. [11] found that the proportion of pDDIs with minor, moderate, and severe severity was 39%, 51%, and 10%, respectively, out of a total of 393 pDDIs according to the Micromedex® database. Ismail et al. [12] was found the minor, moderate, major and contraindicated severity rates of pDDIs as 35.4%, 41.5%, 21.9%, and 1.2%, respectively according to Micromedex® database among 260 pDDIs in pediatric patients. However, in our study the minor, moderate, major, and contraindicated severity rates were found as 3.49%, 61.63%, 30.23%, and 4.65% respectively in total 86 pDDIs according to the Micromedex® database. We think that this observed difference in the distribution of interaction severity may be due to the difference in the drugs used in the treatment of the patients and the patient profile.

Choi et al. [14] have conducted a study in 115 pediatric patients and obtained 592 pDDIs according to the Micromedex® database in 258 drug pairs. However, in our study, we found 86 pDDIs in 50 different drug pairs in 51 pediatric patients according to the Micromedex® database. The difference in the number of drug pairs can be explained by the inclusion of different drug pairs in our study.

In a study including 88 pediatric patients, the Micromedex® database identified ampicillin-amikacin as the most common drug pair causing pDDIs [15]. In another study consisting of 115 pediatric oncology patients, according to the Lexi-interact database, aminoglycosides and cephalosporines were found to be the most common drug pair causing pDDIs [16]. In our study, the most common drug pair causing pDDIs was determined as budesonide-clarithromycin according to both databases. When the

two studies are compared, it is seen that antibiotics are the drug pairs that cause the most of the interactions.

In a study including 510 pediatric patients, antimicrobials were identified as the class of drugs that caused the most severe pDDIs [13]. In another study including 124 pediatric patients, the drug groups that the most frequently associated with pDDIs have been found as nervous system drugs and antiinfectives for systemic use [3]. In our study, however, antibacterials for systemic use, antiepileptics, and drugs for obstructive airway diseases were most frequently associated with pDDIs.

The number of pDDIs increases with age and the number of prescribed drugs, especially as the number of antiepileptic and immunosuppressant drugs increases [17]. As being parallel with our study, Getachew et al. [11] found that there was a positive association between total number of drugs and the likelihood of pDDIs [11]. Although the study suggested that age had a substantial effect on the incidence of pDDIs, particularly in the 2–6 year age group [11], this was not the case in our research. Maybe this is because we have not categorized the age further. In another study, the association between the number of drugs and the number of pDDIs was analyzed, and it was shown that an increase in the total daily number of drugs was associated with an increase in the number of pDDIs in both adult and pediatric populations [17]. Also in the same study, the frequency of pDDIs which belongs to risk rating category C and D was highest among prescriptions containing 3-4 drugs [13]. In a prior study done in a pediatric intensive care unit, compared to those with <5 distinct drugs daily, those with 5–9 distinct drugs daily had 5 times higher likelihood of any pDDIs exposure, and those with ≥10 distinct drugs daily had 37 times higher likelihood of any pDDIs exposure [1]. In our study, concomitantly usage of at least 5 different systemic drugs was associated with higher occurrence of pDDIs.

Ismail et al. [12] performed logistic regression analysis to define various factors associated with the occurrence of pDDIs; the relationship between the occurrence of pDDIs and hospitalization lasting longer than five days, female sex, and use of at least five drugs were found to be statistically significant. In the present study, we observed that a one-drug increase in the overall number of drugs a patient received during hospitalization increased the likelihood of identifying pDDIs by 2.12-fold.

While the concomitant usage of at least 5 different systemic drugs was significantly affected the number of pDDIs according to the risk rating category of B, C and D of UpToDate® database; it was observed that concomitant usage of at least 5 different systemic drugs was not significantly associated with risk rating category X. This maybe a consequence of our relatively small sample size.

Tecen-Yucel et al. [18] evaluated Lexicomp, Micromedex® and Medscape databases in adult renal transplant recipients and compared the 3 databases in terms of compatibility of the severity of the interactions detected and found that minor and moderate interactions showed poor agreement, while major interactions showed mild and severe interactions showed moderate agreement. In our study, it was found that the interactions of minor severity showed poor agreement, the interactions of moderate severity showed substantial agreement, the interactions of major severity showed moderate and contraindicated interactions showed substantial agreement between Micromedex® and Lexicomp databases.

Reis et al. [6] have analyzed the accuracy of the Micromedex® and Lexi-Interact (Lexicomp) databases by assuming Stockley's Drug Interactions 8th edition as the standard for identifying drug interactions. They found sensitivity, specificity, positive predictive value and negative predictive value for Drug-reax system as 88%, 91%, 88%, 91% and for Lexi-Interact as 87%, 88%, 88%, 87%; respectively. We used the UpToDate® (Lexicomp) database as the reference standard in our study, and we discovered that the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the Micromedex® database in terms of determining pDDIs were 79.41%, 94.12%, 96.43 %, 69.56%, and 84.51%, respectively.

Our research has some limitations. Because the study is only interested in pDDIs, more research is needed to illuminate these interactions from a clinical aspect. The study was limited to one center and a small patient population; a larger sample size is required to draw more generalizable conclusions about pDDIs.

There are numerous studies in the literature regarding assessment of pediatric pDDIs. Although patient population remains the same across these literatures, the treatment protocols may be different from each other. Hence, the pDDIs encountered are differed as well. As a result, the ability of drug

interaction databases to detect pDDIs may vary. Here in this study, we aimed to put an emphasis on this diversity seen among various drug interaction databases through a university experience. We expect that when more studies similar to the current study is published, awareness of pDDIs in vulnerable patient populations such as pediatrics will grow.

In conclusion, the active participation of clinical pharmacists in the healthcare team could aid in the determination of pDDIs. In addition, the utilization of multiple drug interaction databases may be effective in preventing the omission of a pDDI.

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AUTHOR CONTRIBUTIONS

Concept: Z.Ü.G.; Design: N.Ö.; Control: N.Ö., Z.Ü.G.; Sources: - ; Materials: - ; Data Collection and/or Processing: H.M., A.Ç.; Analysis and/or Interpretation: H.M., A.Ç.; Literature Review: H.M., A.Ç.; Manuscript Writing: H.M., A.Ç., N.Ö.; Critical Review: N.Ö., Z.Ü.G.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

This cross-sectional study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the ethics committee of Inonu University (25th January 2022 - No: 2022/3055).

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

2,5(6)-DİSÜBSTİTÜEBENZİMİDAZOL TÜREVİ LİGANDLARI TAŞIYAN PLATİN KOMPLEKSLERİNİN ANTİKANSER AKTİVİTELERİNİN ARAŞTIRILMASI

INVESTIGATION OF THE ANTICANCER ACTIVITIES OF PLATIN COMPLEXES BEARING 2,5(6)-DISUBSTITUTEDBENZIMIDAZOLE DERIVATIVE LIGANDS

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ÖZ

Amaç: *Sisplatin, klinikte kanser hastalarının tedavisinde başarılı bir şekilde kullanılmasına rağmen hastalarda rezistans gelişimi ve ciddi toksik yan etkiler görülmektedir. Bu dezavantajların önüne geçmek için bu çalışmada 2,5(6)-disübstitüebenzimidazol türevi taşıyıcı ligandlar kullanarak platin(II) komplekslerinin sentezlenmesi ve sitotoksik etkilerinin test edilmesi amaçlanmıştır.*
Gereç ve Yöntem: ***L1-L6*** *taşıyıcı ligandları Philips yöntemine göre elde edilmiştir.* ***K1-K4*** *kompleksleri, K_2PtCl_4 ve uygun ligandın ısıtılıp karıştırılması ile sentezlenmiştir. Sentez edilen* ***L1-L6*** *ve* ***K1-K4****'ün kimyasal yapıları elementel analiz, İnfrared ve ^{1}H Nükleer Manyetik Rezonans ile aydınlatılmıştır.* ***L1-L6*** *taşıyıcı ligandları ve* ***K1-K4*** *komplesklerinin, MTT yöntemi ile MCF-7 ve DU-145 hücre hatlarına karşı in vitro sitotoksik etkileri test edilmiştir.*
Sonuç ve Tartışma: *IC_{50} değerlerine göre 2 numaralı konumunda –CH_3 sübstitüenti taşıyan ligandlar, nonsübstitüe ligandlara göre test edilen hücre hatlarında daha etkili bulunmuştur. Ayrıca 5(6) numaralı konumunda flor sübstitüe* ***L5*** *ve* ***L6*** *en etkili benzimidazol türevi olarak test edilmiştir. Test edilen kompleksler içerisinde* ***K3****, MCF-7 hücre hattına karşı 32.75 µM IC_{50} değeri ile DU-145 hücre hattında ise 18.842 µM IC_{50} değeri ile en etkili kompleks olarak bulunmuştur.*
Anahtar Kelimeler: *Benzimidazol, platin(II)kompleksleri, sisplatin, sitotoksik aktivite*

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ABSTRACT

Objective: *Although cisplatin is used successfully in the treatment of cancer patients in the clinic, resistance development and serious toxic side effects are seen in patients. In order to avoid these disadvantages, in this study, it was aimed to synthesize platinum(II) complexes using 2,5(6)-disubstitutedbenzimidazole derivative carrier ligands and to test their cytotoxic effects.*
Material and Method: ***L1-L6*** *carrier ligands were synthesized according to the Philips method.* ***K1-K4*** *complexes were synthesized by heating and mixing K_2PtCl_4 and the appropriate ligand. The chemical structures of synthesized* ***L1-L6*** *and* ***K1-K4*** *were elucidated by elemental analysis, Infrared and ^1H Nuclear Magnetic Resonance. In vitro cytotoxic effects of* ***L1-L6*** *carrier ligands and* ***K1-K4*** *complexes against MCF-7 and DU-145 cell lines were tested by MTT method.*
Result and Discussion: *Ligands with $–CH_3$ substituent in position 2 according to IC_{50} values were found to be more effective in tested cell lines than non-substituted ligands. Also, fluorine substituted* ***L5*** *and* ***L6*** *at position 5(6) were tested as the most effective benzimidazole derivatives. Among the tested complexes, it was found to be the most effective complex with an IC_{50} value of 32.75 μM against the* ***K3*** *MCF-7 cell line and with an IC_{50} value of 18.842 μM in the DU-145 cell line.*

Keywords: *Benzimidazole, cisplatin, cytotoxic activity, platinum(II)complexes*

GİRİŞ

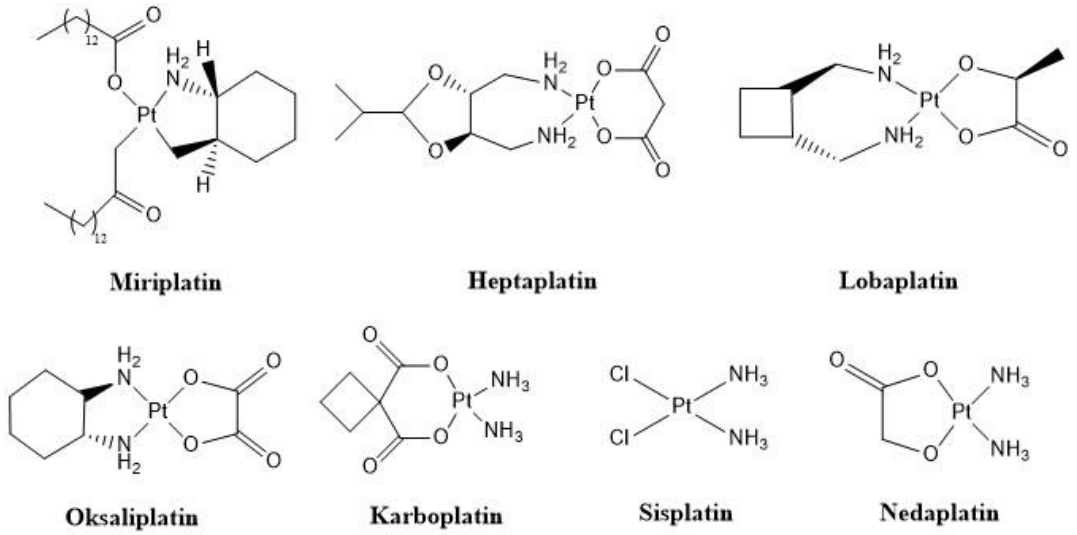
Kanser dünya çapında kardiyovasküler hastalıklardan sonra en önde gelen mortalite sebeplerinden bir tanesidir [1]. Türkiye'de Sağlık Bakanlığının raporuna göre 2030 yılında 22 milyon yeni kanser vakasının ortaya çıkması ön görülmektedir [2]. Önemli bir halk sağlığı problem olan kanser, ülkelerin sosyoekonomik koşulları ile hasta ve yakınlarının psikososyal durumlarını olumsuz olarak etkilemektedir. Bu nedenle erken teşhis ve etkili tedavi yöntemleri ile mortalite ve morbidite oranının düşmesi kanseri tedavi edilebilir hastalıklar arasına koymaktadır. Bu amaçla bilim insanları, tedavisi güç veya direnç gelişmiş kanser türlerini tedavi edebilen kemoterapötik ilaç geliştirme çalışmalarına hız kesmeden devam etmektedirler [3,4].

Kanser tedavisinde kullanılan ve en etkili kemoterapötik gruplardan birisi platin kompleksleridir. Bu gruptan ilk kullanılan ilaç olan sisplatin (*cis*-diammindikloroplatin(II)) 1844 yılında Michael Peyrone tarafından sentezlenmiştir [5]. Rosenberg tarafından yapılan bir çalışmada antikanser etkisi tesadüfen bulunmuş ve 1978 yılında sisplatin kanser tedavisinde kullanılmaya başlanmıştır [6,7]. Sisplatin, testis, ovaryum, akciğer, baş-boyun, mesane ve küçük hücreli olmayan akciğer tümörleri gibi çeşitli tümör türlerine karşı klinikte etkin olarak kullanılmaktadır [8,9].

Sisplatin günümüzde klinik kemoterapi ilaçlarının reçetelerinde diğer platin ilaçları ile birlikte hastaların tedavisinde kullanılan ilaçların %50'lik bir bölümünü oluşturmaktadır [10,11]. Sisplatin, karboplatin ve oksaliplatin olmak üzere üç platin(II) kompleksi tüm dünyada, bölgesel olarak ise Japonya'da nedaplatin ve miriplatin, Kore'de heptaplatin ve Çin'de lobaplatin kompleksleri klinik kullanım için onaylanmıştır (Şekil 1) [12].

Klinikte başarılı bir şekilde kullanılan sisplatinin nefrotoksisite, ototoksisite, hepatotoksisite, bulantı ve kusma gibi yan etkiler kullanımını kısıtlamaktadır [13,14]. Ayrıca tedavi sırasında görülen rezistans gelişiminin de önüne geçebilmek için sisplatin yapısında bulunan ayrılan ve/veya taşıyıcı ligandlarının değiştirilmesi ile yeni platin kompleksleri araştırılmaya devam edilmektedir [15]. Sisplatin yapısındaki taşıyıcı amonyak ligandının farklı heterosiklik amin grupları ile yer değiştirilmesi sonucunda rezinstans gelişen kanser türlerine karşı etkili olan veya hiç etki görülmeyen kanser türlerine karşı terapötik etkinliği artmış kompleks geliştirme çalışmaları hız kesmeden devam etmektedir [15-19]. Bu amaçla organizmanın tanıdığı çeşitli aminoasitler, glukozaminler ve peptidleri taşıyan veya endojen bileşiklerin biyoizosterleri taşıyıcı ligand olarak kullanılmaktadır [20-22].

Heteroaromatik bisiklik halka sistemine sahip olan benzimidazol, yapısında bulunan asidik ve bazik özelliğe sahip azot atomlarından dolayı amfoterik karakter göstermektedir. DNA yapı taşını oluşturan pürin bazlarına, birçok kofaktör, aminoasitler ve vitamin B12 gibi biyoaktif moleküllerinin yapısında bulunmasından dolayı organizmanın tanıdığı aromatik bir halka sistemidir [22-25]. Benzimidazol halka sistemine sahip ilaçların antikanser, antihelmintik, antifungal, antiviral, antienflamatuvar, antihistaminik, antipsikotik gibi terapötik etkinlikleri bulunmaktadır [25-30].



Şekil 1. Klinikte kullanılan platin(II) kompleksleri

Literatür taramalarında benzimidazol halkasının 1, 2 ve 5 numaralı konumları sübstitüe edilerek farklı hastalıkların tedavisi için molekül tasarlama çalışmalarının yapıldığı görülmektedir [23-33]. Bu çalışmada da benzimidazol halkasının 5 numaralı konumunda F/Cl/CH_3 ve 2 numaralı konumunda da H/CH_3 sübstitüenti bulunan altı adet 5(6)-floro/kloro/metil-2-hidrojen/metilbenzimidazol türevleri Philips yöntemi kullanılarak sentezlenmiştir [34]. Sentez edilen **L1-L6**'nın kimyasal yapıları aydınlatıldıktan sonra potasyumtetrakloroplatinat ile dört adet literatüre kayıtlı olan $[Pt(L1)_2Cl_2].H_2O$ (**K1**), $[Pt(L2)_2Cl_2].1.5\ H_2O$ (**K2**), $[Pt(L3)_2Cl_2]$ (**K3**) ve $[Pt(L4)_2Cl_2].H_2O$ (**K4**) sentezlenerek MCF-7 (meme kanseri) ve DU-145 (prostat kanseri)'e karşı 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolium bromür (MTT) yöntemi ile *in vitro* sitotoksik etkileri test edilmiştir.

GEREÇ VE YÖNTEM

Kimyasal Çalışmalar

Sentez çalışmalarında kullanılan 4-floro/kloro/4-metil-1,2-fenilendiamin, potasyum tetrakloroplatinat, sodyumbikarbonat ve solvanlar analitik niteliktedir (Merck veya Aldrich). İnce tabaka kromatografisi (İTK) çalışmalarında 254 nm UV ışığı altında görüntülenecek aluminyum plaklar kullanıldı (Merck). **L1-L6** ve **K1-K4**'ün FTIR-ATR spektrumlarının alınmasında Perkin Elmer Spectrum FT-IR Spektrometresi (4000-600 cm^{-1} aralığı) kullanıldı. Bileşiklerin NMR spektrumları ise dötero dimetilsülfoksit (DMSO-d_6) içerisinde Bruker 400 MHz NMR Spektrometresinde alındı. Kimyasal kayma değerleri δ skalasında değerlendirildi. Sentezlenen **K1-K4** komplekslerinin elementel analizleri (C, H, N), Leco-932 Cihazı kullanılarak yapıldı.

2,5(6)-Disübstitüebenzimidazol Türevi Bileşiklerin Genel Sentez Yöntemi (Phillips Yöntemi) (L1-L6) [34]

0.01 mol sentezlenmek istenen 2,5(6)-disübstitüebenzimidazole göre 4-floro/kloro/metil-o-fenilendiamin türevi ile 0.02 mol formik asit veya asetik asit balon içerisine alındıktan sonra üzerine 100 ml 5 N HCl ilave edildi. Reaksiyon karışımı 8-24 saat arasında değişen sürelerde ısıtılmasıyla sentezlendi. Reaksiyonun tamamlanması İTK ile izlendi. Reaksiyonun tamamlandığı anlaşıldıktan sonra balon içeriği buz banyosu içerisinde nötralize edildi. Çökelek süzüldükten sonra soğuk su ile yıkandı. Kristalizasyon solvanı olarak su veya su-etanol kullanıldı. Oluşan kristal halindeki bileşik süzülerek kurutuldu.

Taşıyıcı Ligandların Karakterizasyonu

*5(6)-Klorobenzimidazol (**L1**)* Erime noktası: 118-120°C (125-126°C [36, 37], 124-126°C [38],

122-123°C [39]); FTIR-ATR: 3080-2564 (-N-H ve =C-H, gerilimleri), 1623-1283 (C=N ve C=C gerilim, N-H ve C-H eğilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 8.34 (s, 1H), 7.70 (d, J= 2 Hz, 1H), 7.66 (d, J=8.4 Hz, 1H), 7.29 (dd, J=8.4 Hz, J= 2 Hz, 1H) ppm.

*5(6)-Kloro-2-metilbenzimidazol (**L2**)* Erime noktası: 201-202°C (197-199°C [38], 199-200°C [39]); FTIR-ATR: 3100-2533 (-N-H, =C-H ve –C-H gerilimleri), 1618-1278 (C=N ve C=C gerilim, N-H ve C-H eğilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 7.51 (d, J= 2 Hz, 1H), 7.47 (d, J= 8.8 Hz, 1H), 7.14 (dd, J=8.8, J= 2 Hz, 1H), 2.49 (s, 3H, CH_3) ppm.

*5(6)-Metilbenzimidazol (**L3**)* Erime noktası: 112-113°C (115-116°C [37]); FTIR-ATR: 3230-2557 (-N-H, =C-H, –C-H gerilimleri), 1618-1245 (C=N ve C=C gerilim, N-H ve C-H eğilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): 9.53 (s, 1H), 7.75 (d, J= 8.8 Hz, 1H), 7.65 (s, 1H), 7.41 (d, J=8.4 Hz, 1H), 2.49 (s, 3H, CH_3) ppm.

*5(6)-Metil-2-metilbenzimidazol (**L4**)* Erime noktası: 198-200°C (201-202°C [40], 203-204°C [41]); FTIR-ATR: 3146-2563 (-N-H, =C-H, –C-H gerilimleri), 1630-1281 (C=N ve C=C gerilim, N-H ve C-H eğilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): 7.33 (d, J= 8 Hz, 1H), 7.23 (s, 1H), 6.90 (d, J= 8 Hz, 1H), 2.43 (s, 3H, CH_3), 2.37 (s, 3H, CH_3) ppm.

*5(6)-Fluorobenzimidazol (**L5**)* Erime noktası: 132-133°C (128-132°C) [42], 130-132°C [43]); FTIR-ATR: 3085-2573 (-N-H, =C-H, –C-H gerilimleri), 1631-1294 (C=N ve C=C gerilim, N-H ve C-H eğilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): 8.52 (s, 1H), 7.66 (dd, J= 8.8 Hz, J=4.8 Hz, 1H), 7.46 (dd, J=9.2Hz, J=2.4 Hz, 1H), 7.14 (td, J=9.8 Hz, J=2,4 Hz, 1H) ppm.

*5(6)-Fluoro-2-metilbenzimidazol (**L6**)* Erime noktası: 177°C (177-179°C [41], 176-178°C [44]); FTIR-ATR: 3115-2587 (-N-H, =C-H, –C-H gerilimleri), 1632-1248 (C=N ve C=C gerilim, N-H ve C-H eğilim), 770 (sübstitüebenzen =C-H plan dışı eğilim); ^{1}H-NMR (DMSO-d_6): 12.20 (s, 1H), 7.40 (dd, J= 8.8, J=4.8 Hz, 1H), 7.21 (dd, J=9.6, 2.4 Hz, 1H), 6.91 (td, J=9.2, J=2.8 Hz, 1H), 2.44 (s, 3H, CH_3) ppm.

K1-K4 Platin(II) Komplekslerinin Genel Sentez Yöntemi [35]

K_2PtCl_4'ün (0.6 mmol) 5 ml distile su içerisindeki çözeltisine sentez edilmek istenilen komplekse göre seçilen **L1**-**L4** türevi taşıyıcı-ligand (1.10 mmol) etanol-distile su karışımında (7:3 ml) çözülerek ilave edildi. Reaksiyon karışımı 40-60°C'ye kadar karanlıkta karıştırıldı. Reaksiyon ortamının pH'sı 0.1 M sodyum bikarbonat çözeltisi ile yaklaşık pH 7'ye ayarlandı. Reaksiyonun tamamlandığı İTK ile anlaşıldıktan sonra oluşan çökelek vakumda süzüldü. Ardından oluşan çökelek sırayla su, etanol ve eter ile yıkanarak temizlendi.

*[dikloro-di(5(6)-klorobenzimidazol)platin(II).H_2O (**K1**) [Pt(**L1**)$_2$$Cl_2$].$H_2O$.* Verim: %16.37; Erime Noktası: > 400; FTIR-ATR: 3210-2980 (-N-H, =C-H, O-H gerilimleri), 3183 (N-H gerilim), 1622-1296 (C=N ve C=C gerilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 13.64 (s, 2H, 2x N-H, D_2O değişimi yapılmıştır), 8.91 (d, J=1.6 Hz, 1H), 8.85 (s, 1H), 7.89 (dd, J= 1.6 Hz, J= 2 Hz, 1H), 7.83-7.77 (m, 1H), 7.61 (dd, J=1.6 Hz, J= 1.6 Hz, 1H), 7.57-7.53 (m, 1H), 7.32-7.25 (m, 2H) ppm; Elementel analiz: $C_{14}H_{10}Cl_4N_4Pt.H_2O$ Hesaplanan C, 28.54; H, 2.05; N, 9.51. Bulunan: C, 28.79; H, 2.00; N, 9.79.

*[dikloro-di(5(6)-kloro-2-metilbenzimidazol)platin(II).1.5 H_2O (**K2**) [Pt(**L2**)$_2$$Cl_2$].1.5 H_2O.* Verim: % 18.3; Erime Noktası: > 400; FTIR-ATR: 3184-2986 (-N-H, =C-H, –C-H ve O-H gerilimleri), 1623-1221 (C=N ve C=C gerilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): 13.44 (bs, 2H, 2x N-*H*, D_2O değişimi yapılmıştır), 8.27-7.96 (m, 2H), 7.62- 7.23 (m, 4H), 3.04-2.45 (m, 6H, 2x CH_3) ppm; Elementel analiz: $C_{16}H_{14}Cl_4N_4Pt.1.5\ H_2O$ Hesaplanan C, 30.69; H, 2.73; N, 8.94; Bulunan C, 30.12; H, 2.41; N, 8.63.

*[dikloro-di(5(6)-metilbenzimidazol)platin(II) (**K3**) [Pt(**L3**)$_2$$Cl_2$].* Verim: % 28.28; Erime Noktası: > 400; Elementel analiz: $C_{16}H_{16}\,Cl_2N_4Pt$; FTIR-ATR: 3277-2981 (-N-H, =C-H ve –C-H gerilimleri), 1597-1248 (C=N ve C=C gerilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 13.26 (s, 2H, 2x N-H, D_2O değişimi yapılmıştır), 8.76-8.71 (m, 2H), 7.72-7.57 (m, 2H), 7.36-7.25 (m, 2H), 7.06-6.98 (m, 2H), 2.48-2.30 (m, 6H, 2x CH_3) ppm; Elementel analiz: $C_{16}H_{16}\,Cl_2N_4Pt$ Hesaplanan C, 36.24; H, 3.04; N, 10.56; Bulunan C, 35.98; H, 3.03; N, 10.54.

*[dikloro-di(5(6)-metil-2-metilbenzimidazol)platin(II).H_2O (**K4**) [Pt(**L4**)$_2$$Cl_2$].$H_2O$.* Verim: % 46.26; Erime Noktası: > 400; FTIR-ATR: 3166-2981 (-N-H, =C-H, –C-H gerilimleri), 1602-1226 (C=N ve C=C gerilim) cm^{-1}; ^{1}H-NMR (DMSO-d_6): 13.04 (bs, 2H, 2x N-H, D_2O değişimi yapılmıştır), 7.97-

7.85 (m, 2H), 7.28- 7.00 (m, 4H), 2.81-2.74 (m, 6H, 2x C*H*$_3$), 2.48-2.43 (m, 6H, 2x C*H*$_3$) ppm; Elementel analiz: $C_{18}H_{20}Cl_2N_4Pt.H_2O$ Hesaplanan C, 37.51; H, 3.85; N, 9.72; Bulunan C, 37.65; H, 3.47; N, 9.86.

Biyolojik Çalışmalar

Çalışmada ticari olarak satın alınmış MCF-7 meme kanseri hücre hattı (T.C. Tarım ve Orman Bakanlığı Şap Enstitüsü, Hücre Kayıt No: 00092502) ve DU-145 prostat kanseri hücre hattı (ATCC, HTB-81) kullanıldı. Test edilen sentez maddeleri ve sisplatin, hücrelere uygulanacak besi yerindeki konsantrasyonu 1/1000 olacak şekilde DMSO ile çözüldü. Testlerde kimyasal madde ve malzeme olarak RPMI 1640 (Sartorius, 01-106-1A) sığır fetus serumu (FBS, Gibco, 10082147), L-glutamin (Thermo, 25030081), amfoterisin (Thermo, 15290018), penisilin+streptomisin (Thermo, 15140130), tripsin-EDTA (17-161E, Lonza), DMSO (AI0231, Amresco), DPBS-10X (14080-055, Thermo) kullanıldı. MCF-7 ve DU-145 hücreleri 37°C'deki su banyosunda çözüldükten sonra hücreler 15 ml'lik falkon tüpe konulup üzerine katkılı medyum eklendi ve süspansiyon santrifüj edildikten sonra pellet üzerine katkılı medyum eklenerek homojen hücre süspansiyonu T25 flasklara ekildi. Hücreler 37°C sıcaklık ve %5 CO_2 ortamına sahip inkübatörde 3 günde bir medyumu değiştirilerek çoğaltıldıktan sonra MCF-7 ve DU-145 hücreleri %70-80 konfluent olunca pasajlandı ve fosfat tampon solüsyonu (PBS) ile yıkandı. Test için yeterli miktarda hücre elde edildikten sonra MTT deney protokülü uygulandı.

MTT Deney Protokolü

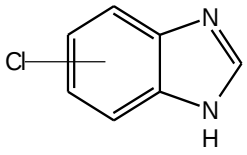
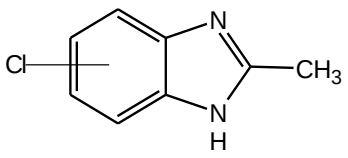
Sentezlenen maddelerin sitotoksik etkileri MTT analizi ile değerlendirildi [45,46]. MTT analizinde **L1-L6** ve **K1-K4**'ün 2.5, 5, 10, 20, 40, 80 ve 160 µM ve sisplatinin 1.56, 3.125, 6.25, 12.5, 25, 50 ve 100 µM konsantrasyonları analiz edildi. MTT analizi için 96 kuyucuklu plakalara her bir konsantrasyon için n=3 olacak şekilde kuyucuk başına $8.7x10^3$ hücre ekim yapıldı. Ekimden 24 saat sonra hücrelerin üzerindeki medium çekildi ve belirlenen konsantrasyonlarda **L1-L6**, **K1-K4** ve sisplatin içeren medium eklendi. 72 saat sonra hücrelerin üzerindeki medyum uzaklaştırıldı ve her bir kuyucuğa 200 µl medyum ve 50 µl 5 mg/ml MTT içeren medyum eklendi. Hücreler 37°C sıcaklıkta 4 saat inkübe edildi. Daha sonra hücrelerin üzerindeki medyum çekildi ve her her bir kuyucuğa 200 µl DMSO ve pH değeri 10.5 olan glisin tamponundan 50 µl eklendi. Plakalar bekletilmeden ELISA cihazında 570 nanometre (nm) dalga boyunda ölçüldü.

IC_{50} değerinin hesaplanmasında doz-absorbans eğrisi Microsoft excel programı kullanılarak elde edildi.

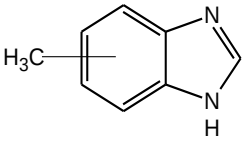
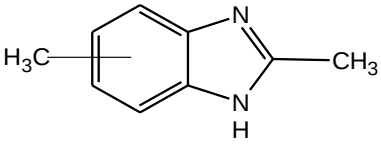
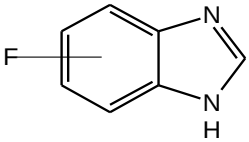
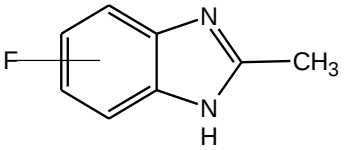
Sitotoksik Aktivite Test Sonuçları

Bu çalışmada sentez edilen **L1-L6** ve **K1-K4**'ün sitotoksik aktiviteleri MTT yöntemi ile MCF-7 ve DU-145 hücre hatlarına karşı test edildi [45,46]. **L1-L6** ve **K1-K4**'ün 2.5, 5, 10, 20, 40, 80 ve 160 µM; sisplatin 1.56, 3.125, 6.25, 12.5, 25, 50, 100 µM konsantrasyonlardaki IC_{50} değerleri Tablo 1'de, MCF-7 ve DU-145 hücre hattına karşı canlılık değerleri Tablo 2'de verilmektedir.

Tablo 1. L1-L6, K1-K4 ve sisplatin MCF-7 ve DU-145 hücre hattına karşı IC_{50} değeri (µM)

Ligand veya Kompleks No	Formül	MCF-7	DU-145
L1		239.73	513.22
L2		187.85	228.38

Tablo 1 (*devamı*). L1-L6, K1-K4 ve sisplatin MCF-7 ve DU-145 hücre hattına karşı IC_{50} değeri (µM)

L3		268.93	383.79
L4		161.35	207.57
L5		68.29	81.27
L6		59.45	61.12
K1	[Pt(**L1**)$_2$Cl$_2$]	67.28	34.92
K2	[Pt(**L2**)$_2$Cl$_2$]	75.62	89.07
K3	[Pt(**L3**)$_2$Cl$_2$]	32.75	18.84
K4	[Pt(**L4**)$_2$Cl$_2$]	66.72	62.82
Sisplatin		62.43	49.96

Tablo 2. L1-L6, **K1-K4** ve sisplatin MCF-7 ve DU-145 hücre hattına karşı % canlılık değerleri

% Canlılık (µM ±SS)						
C(µM)	**L1**		**L2**		**L3**	
	MCF-7	**DU-145**	**MCF-7**	**DU-145**	**MCF-7**	**DU-145**
160	61.25 ± 1.46	80.42 ± 1.22	54.76 ± 1.63	65.22 ± 4.71	59.70 ± 2.38	74.69 ± 10.96
80	85.63 ±3.20	83.56 ±1.20	67.93 ± 6.19	69.07 ± 5.01	64.91 ± 2.54	81.62 ± 1.67
40	89.60 ± 2.84	87.61 ± 1.77	73.49 ± 5.63	83.95 ± 0.68	65.57 ± 1.38	83.31 ± 0.92
20	92.73 ± 1.41	89.26 ± 1.47	74.14 ± 3.12	87.47 ± 3.51	68.02 ± 3.61	86.16 ± 2.54
10	89.06 ± 0.81	91.06 ± 2.23	76.91 ± 1.98	88.65 ± 0.28	70.71 ± 0.59	87.48 ± 2.78
5	90.73 ± 0.99	92.24 ± 0.94	83.15 ± 5.52	90.56 ± 1.99	73.12 ± 3.33	90.84 ± 2.37
2.5	93.53 ± 0.51	94.74 ± 1.88	81.12 ± 1.68	90.72 ± 0.73	73.47 ± 0.78	95.12 ± 3.80
	L4		**L5**		**L6**	
160	52.27 ± 2.73	59.69 ± 5.86	14.02 ± 1.15	3.38 ± 1.15	18.46 ± 1.01	18.29 ± 0.72
80	63.40 ± 7.42	72.74 ± 5.54	38.92 ± 5.06	48.27 ± 5.06	38.74 ± 0.95	42.73 ± 3.52
40	71.26 ± 2.03	87.46 ± 4.83	63.77 ± 0.68	83.28 ± 0.68	58.36 ±1.99	55.63 ±0.63
20	80.14 ± 0.39	87.90 ± 3.75	68.74 ± 1.82	86.53 ± 1.82	64.77 ± 1.70	61.58 ± 0.48
10	82.32 ± 4.78	89.36 ± 0.96	69.65 ± 1.70	89.90 ± 1.70	66.32 ± 2.20	64.02 ± 2.53
5	83.13 ± 1.30	89.49 ± 5.23	78.94 ± 0.19	91.13 ± 0.19	67.11 ± 3.00	68.43 ± 1.57
2.5	81.65 ± 1.68	89.00 ± 4.03	84.16 ± 2.51	93.16 ± 2.51	68.88 ± 1.14	76.05 ± 1.75

Tablo 2 (*devamı*). L1-L6, **K1-K4** ve sisplatin MCF-7 ve DU-145 hücre hattına karşı % canlılık değerleri

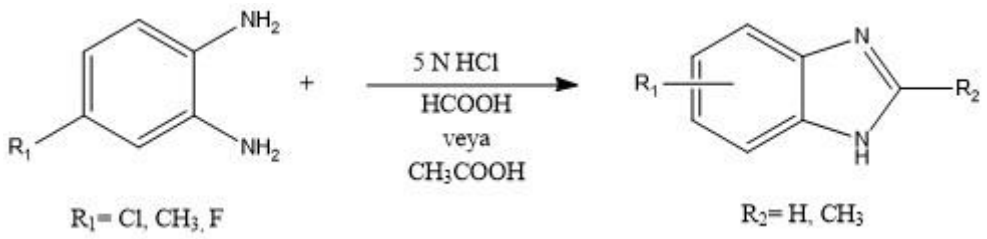
	K1		**K2**		**K3**	
160	10.15 ± 0.70	3.96 ± 0.01	12.27 ± 1.45	4.44 ± 0.11	08.75 ± 0.02	3.39 ± 0.04
80	40.37 ± 1.29	8.50 ± 0.19	45.97 ± 1.75	53.36 ± 2.74	10.89 ± 0.34	3.90 ± 0.55
40	55.70 ± 2.73	25.95 ± 1.36	72.52 ± 4.64	92.58 ± 1.25	28.85 ± 0.69	5.82 ± 0.19
20	73.11 ± 1.23	63.77 ± 1.89	75.82 ± 6.77	97.17 ± 0.66	65.10 ± 3.97	33.94 ± 1.19
10	80.00 ± 1.85	80.39 ± 1.56	74.41 ± 1.43	98.12 ± 0.58	68.06 ± 2.65	46.06 ± 4.54
5	81.74 ± 4.74	86.68 ± 0.91	80.24 ± 6.38	94.17 ± 3.90	68.57 ± 2.12	79.34± 4.67
2.5	80.12 ± 1.58	93.31 ± 1.65	82.34 ± 3.28	96.05 ± 0.13	82.16 ± 4.27	86.37 ± 2.49
	K4		**C(µM)**		Sisplatin	
160	07.23 ± 0.36	03.00 ± 0.22	100		34.76 ± 1.82	15.09± 1.10
80	18.76 ± 0.44	14.83 ± 3.04	50		50.60± 0.51	38.50± 2.20
40	63.72 ± 2.28	54.85 ± 3.01	25		59.70± 2.49	54.90± 3.00
20	71.93 ± 5.70	74.89 ± 2.25	12.5		76.91± 1.21	83.77± 4.00
10	87.06 ± 6.34	88.06 ± 0.80	6.25		83.53± 1.85	93.72± 0.47
5	94.00 ± 6.66	96.40 ± 1.50	3.125		86.53± 3.49	98.46± 0.12
2.5	95.68 ± 2.85	94.85 ± 1.57	1.56		91.02± 2.34	99.23± 0.47

SONUÇ VE TARTIŞMA

Azot taşıyan heterosiklik bileşikler organizmada ve birçok ilaç etken maddesinin yapısında bulunduğu için medisinal kimya alanında bu bileşikler üzerine yapılan çalışmalar giderek artmaktadır. 1 ve 3 numaralı konumlarında iki adet azot atomu taşıyan benzimidazol ve türevleri antihipertansif, antikanser, antiviral, antidiyabetik, antimikrobiyal, antiinflamatuar, antikonvülsanlar, antiülser vb. gibi birçok farmakolojik aktiviteye sahiptir [47,48].

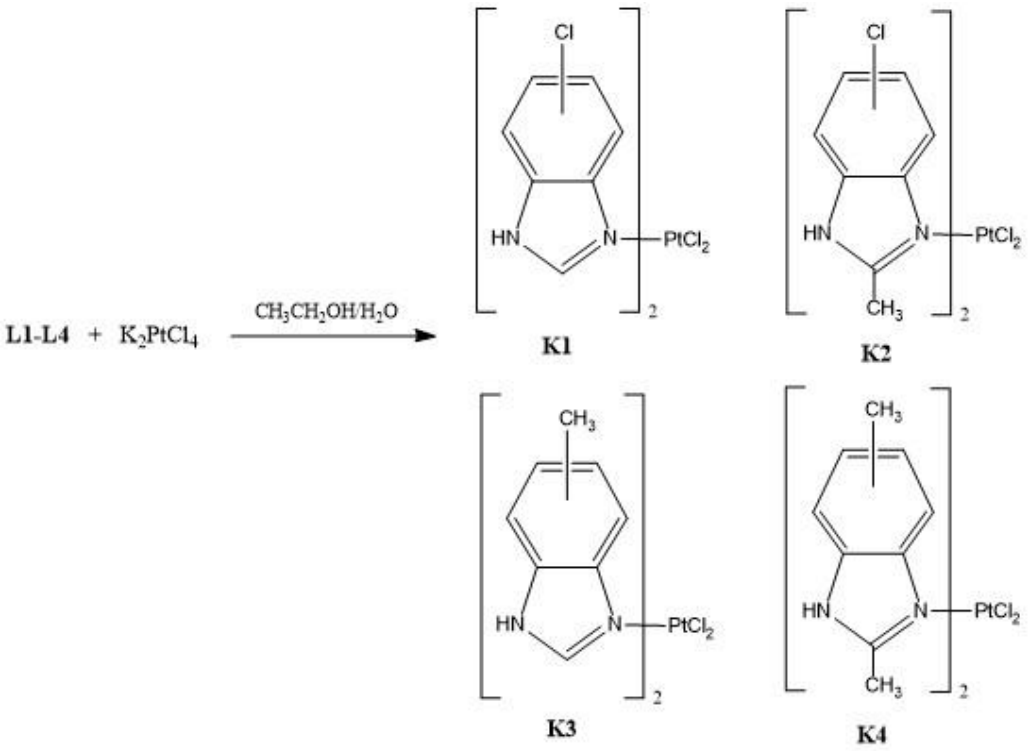
Klinikte kullanılan ilk platin(II) kompleksi bileşik olan sisplatin antikanser etkisinin tesadüfi keşfinden günümüze kadar klinikte en çok reçetelenen ilaç olma özelliğini korumaktadır. Klinik kullanım dezavantajlarını azaltmak için sisplatinin taşıyıcı ve ayrılan ligandının farklı gruplar ile yerdeğiştirilmesi sonucu binlerce platin kompleksi sentezlenmiş ve sitotoksik etkileri test edilmiştir. [49,50].

Bu çalışmada birçok ilaç molekülünün farmakofor kısmını oluşturan 6 adet benzimidazol türevi bileşik Phillips yöntemi ile sentezlenmiştir [34]. Flor atomu, medisinal kimyada ilaç molekülü geliştirmede en önemli biyoizosterlerden birisi olma özelliğini taşımaktadır. Florun, H atomuna benzemesine rağmen karbon atomu ile oluşturduğu C-F bağı, C-H bağına göre daha kuvvetli ve lipofilisitesi daha yüksektir. Ayrıca iyonize olabilen proton taşıyan fonksiyonel gruba sahip bileşiklerin pKa'sını düşürmektedir. Birçok enzim veya reseptörün aktif bölgesine bağlanabilen, H atomuna sahip bileşik ile karşılaştırıldığında, benzer veya daha yüksek farmakolojik etki gösteren florsübstitüe bileşikler tasarlanmaktadır [51]. Yaptığımız literatür taramaları sonucunda antikanser aktivitesi olabilecek benzimidazol halkasının 5 numaralı konumuna F, Cl veya CH_3 ve 2 numaralı konumuna da H/CH_3 olan 6 adet 5(6)-floro/kloro/metil-2-hidrojen/metilbenzimidazol taşıyıcı ligandların sentez şeması Şekil 2'de verilmektedir. **L1-L6**'nın erime noktaları, FTIR ve ^{1}H-NMR spektrumları literatürde kayıtlı olan veriler ile karşılaştırılmıştır. Elde edilen veriler literatür ile uyumludur [36-44].



Şekil 2. Ligand L1-L6'nın sentez şeması

Sentez edilen taşıyıcı ligandlardan 5(6)-flurobenzimidazol **(L5)** ve 5(6)-fluro-2-metilbenzimidazol **(L6)**'ın etanol/su, 0.5 N HCl veya DMF çözücülerinde K_2PtCl_4 ile kompleksleşme reaksiyonları yapılmıştır. Değişen süre ve sıcaklıklarda reaksiyon şartları denenmiş ancak ^{1}H-NMR sonuçları göre saf olarak ürün izole edilemediği anlaşılmıştır. Bu komplekslerin sentezi daha sonraki çalışmalarımızda yapılmak üzere bu çalışmaya dahil edilmemiştir. Diğer taşıyıcı ligandlar **L1-L4** ve K_2PtCl_4'den hareketle kapalı formülü $[Pt(\mathbf{L1})_2Cl_2].H_2O$, $[Pt(\mathbf{L2})_2Cl_2].1.5H_2O$, $[Pt(\mathbf{L3})_2Cl_2]$ ve $[Pt(\mathbf{L4})_2Cl_2].H_2O$ olan Nzeyimana ve arkadaşları tarafından daha önceden sentezlenen 4 adet **K1-K4** platin (II) komplekleri, literatüre kayıtlı yöntem kullanılarak bu çalışma yeniden sentezlenmiştir (Şekil 3) [35].



Şekil 3. K1-K4'ün sentez şeması

K1-K4'ün kimyasal yapılarının belirlenmesi için **L1-L4**'ün aynı şartlarda FTIR ve ^{1}H-NMR spektrumları alınmıştır. Ek olarak sentez edilen **K1-K4**'ün elementel analiz verileri kullanılmıştır. Komplekslerin kimyasal yapısı, kompleksleşme ile **L1-L4** kimyasal yapısında bulunan belirleyici gruplara ait piklerdeki değişiklikler ve kayma ile belirlenmiştir. Elementel analiz verileri göre **L1-L4** tek dişli ligand olarak potasyum tetrakloroplatinat ile 1:2 Platin: **L1-L4** oranında platin(II) kompleksi oluşturmak üzere reaksiyona girdiği belirlenmiştir.

K1-K4'ün kimyasal yapılarının aydınlatılmasında 3 numaralı konumdan platine bağlandığının kanıtlanması için IR ve ^{1}H-NMR spektrumlarının incelenmesi sırasında N-H grubuna ait sinyallerin varlığı ve yerleri üzerinde durulmuştur. Elde edilen IR ve ^{1}H-NMR verileri kompleksleşmenin, sübstitüebenzimidazol halkasının 3 numaralı piridin azotu üzerinden olduğunu desteklenmiştir.

L1-L6'ya ait spektrumda 3100-2530 cm^{-1} bölgesinde kuvvetli, yayvan multiplet absorbsiyon bandları görülmektedir. **K1-K4**'e ait IR spektrumlarında **L1-L4**'ün spektrumunda görülen bu yayvan pikin yerine daha keskin pikler görülmektedir. Bu bulgu, **L1-L4**'ün yapısında bulunan "imino hidrojeni" nin serbest olarak bulunduğunu kanıtlamaktadır. Bu bulgu, bazı azol türevi taşıyıcı ligandların çeşitli metaller ile yapmış oldukları kompleksler için verilmiş literatür verileri ile uygunluk göstermektedir

[53-55]. N-H gerilim bandlarındaki bu keskinleşme, kompleks oluşumu ile pirol azotuna ait protonun tautomerisinin engellenmiş olmasından kaynaklanmaktadır [56].

K1-K4'ün yapısının aydnlatılması için ^{1}H-NMR spektrumu kompleksler organik çözücülerde çözünmediği için DMSO-d_6 içinde çözülerek alınmıştır. **L1-L4** ve **K1-K4**'ün sahip olduğu protanlara ait piklerdeki kaymalar ve yarılmalar beknenen alanda gözlenmiştir [35]. 2,5(6)-sübstitüebenzimidazol halkasının pirol azotuna ait proton, N^1-C-N^3 boyunca eşit olarak delokalize olduğundan ^{1}H-NMR spektrumunda bu sinyal bazen görülmemektedir [57]. Sentez edilen ligandlardan sadece **L6** ligandına ait N-H protonu 12.20 ppm singlet olarak gözlenmiştir. **K1-K4**'e ait N-H protonları sırasıyla δ 13.64, 13.44, 13.26 ve 13.04 ppm de görülmektedir.

Bu çalışmada sentez edilen ligand **L1-L6** ve kapalı formülü **$[Pt(L1)_2Cl_2].H_2O$, $[Pt(L2)_2Cl_2]$ .1.5 H_2O, $[Pt(L3)_2Cl_2]$** ve **$[Pt(L4)_2Cl_2].H_2O$** olan **K1-K4**'ün MCF-7 (meme kanseri) ve DU-145 (prostat kanseri) hücre hatlarına karşı MTT yöntemi ile sitotoksik etkileri test edilmiştir. **K1-K4**'ün 160-2.5 µM ve sisplatinin 100-3.125 µM arasındaki konsantrasyonlarının test edilen hücre hatlarına karşı IC_{50} değerleri Tablo 1 ve % canlılık değerleri Tablo 2'de verilmektedir.

IC_{50} değerlerine göre 2 numaralı konumda metil taşıyan ligandlar, nonsübstitüe ligandlara göre daha etkili bulunmuştur. 5(6) numaralı konumda flor sübstitüsyonuna sahip **L5** ve **L6** test edilen MCF-7 hücre hattında 68.29 ve 59.45 µM ve DU-145 hücre hatlarına karşı ve 81.27 ve 61.12 µM aktiviteye sahiptir. Diğer ligandlar **L1-L4** MCF-7 hücre hattına karşı sırasıyla 239.73, 187,85, 268.93 ve 161.35 µM IC_{50} değerlerine sahipken DU-145 hücre hatlarına karşı sırasıyla 513.22, 228.38, 383.79 ve 207.57 µM IC_{50} değerine sahip olduğu test edilmiştir. 5(6)-Floro sübstitüe benzimidazol ligandları **L5** ve **L6** test edilen MCF-7 ve DU-145 hücre hatlarına karşı **L1-L4** taşıyıcı ligandlarından daha düşük IC_{50} değerlerine sahip olduğu bulunmuştur.

L3 taşıyıcı ligandına sahip **K3** kompleksi MCF-7 hücre hattına karşı 32.75 µM IC_{50} değeri ile DU-145 hücre hattında ise 18.84 µM IC_{50} değeri ile en etkili kompleks olarak test edilmiştir. MCF-7 hücre hattına karşı diğer en etkili kompleksler sırasıyla 66.72, 67.28 ve 75.62 µM IC_{50} değerlerine sahip **K4, K1** ve **K2** kompleksleri olarak bulunmuştur. Test edilen DU-145 hücre hattında ise 34.92, 62.82 ve 89.07 µM IC_{50} değerlerine sahip **K1**, **K4** ve **K2** kompleksleri bulunmuştur. Taşıyıcı ligandların tam tersi olarak 2 numaralı konumda metil taşımayan kompleksler **(K1** ve **K3),** metil taşıyan komplekslerden **(K2** ve **K4)** daha etkili bulunmuşlardır.

MTT test sonuçlarına DU-145 ve MCF-7 hücre hatlarına karşı **K3** ve DU-145 hücre hattına karşı **K1** test edilen kompleksler içerisinde siplatinden daha etkili olarak bulunmuştur. Diğer sentez edilen kompleksler **K2** ve **K4** test edilen her iki hücre hattına karşı sisplatin ile karşılaştırılabilir etkiye sahip olduğu görülmektedir.

TEŞEKKÜR

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YAZAR KATKILARI

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

OKSİRESVERATROLÜN L929 FİBROBLAST HÜCRELERİNDEKİ OKSİDATİF STRES HASARI ÜZERİNE OLUMLU ETKİLERİ

POSITIVE EFFECTS OF OXYRESVERATROL ON OXIDATIVE STRESS DAMAGE IN L929 FIBROBLAST CELLS

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ÖZ

Amaç: *L929 fibroblast hücrelerinde H_2O_2 etkisiyle oluşturulan hücre hasarının bertaraf edilmesinde oksiresveratrolün rejeneratif etkisinin değerlendirilmesi amaçlanmıştır.*
Gereç ve Yöntem: *Oksiresveratrolün total antioksidan seviyesi (TAS), total oksidan seviyesi (TOS), oksidatif stres indeksi (OSİ), toplam fenolik madde seviyeleri farklı derişimlerde ölçülmüştür. Oksiresveratrol fibroblast hücrelerinde IC_{50} değeri MTT yöntemiyle belirlenmesinin ardından 12.5 - 400 µM konsantrasyonlarında H_2O_2 ile meydana getirilen oksidatif stres hasarındaki rejeneratif etkisi in-vitro ortamdaki hücre proliferasyonunun ölçümü xCELLigence cihazıyla gerçekleştirilmiştir. Ayrıca hücre hasarındaki yara iyileşme düzeylerini belirlemek amacıyla 3.125 - 25 µM derişimlerinde scratch analizi yapılmıştır.*
Sonuç ve Tartışma: *Oksiresveratrolün 0.5 mg/ml'de TAS değeri 3 ± 0.3 ve TOS değeri 0.77 ± 0.52; (OSİ) değeri 0.02 ± 0.09 olarak bulunmuştur. Oksiresveratrolün 0.625 mg/ml'den 10 mg/ml'ye farklı dozlarındaki total fenolik madde (TFM) derişimleri düşük konsantrasyondan yüksek konsantrasyona daha yüksek TFM'ye sahip olduğu gözlemlenmiştir. Oksiresveratrolün L929 fibroblast hücreleri IC_{50} değeri 214.2 µM; H_2O_2'e maruz bırakılan L929 fibroblast hücrelerindeki hücre hattında oksiresveratrol IC_{50} değeri 109.7 µM olduğu belirlenmiştir. Oksiresveratrolün 12.5 µM konsantrasyonunun xCELLigence cihazıyla ölçülmesinde en fazla hücre proliferasyonu gözlenmiştir. Scratch analizinde H_2O_2 ile hasarlanmış hücrelerdeki 12.5 µM oksiresveratrol konsantrasyonunda 24. Saat için %62, 48. Saat için %88'lik bir hücre proliferasyonuyla diğer dozlardan daha fazla iyileşme gözlenmiştir. 12.5 µM konsantrasyonu hem proliferasyon, hem de scratch analizinde en etkili konsantrasyon olduğu belirlenmiştir. Oksiresveratrol; düşük konsantrasyonlarda gösterdiği antioksidan kapasiteyle H_2O_2 aracılığıyla meydana getirilen*

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hücresel hasarda, L929 fibroblast hücrelerini oksidatif stresten korur, fibroblast canlılığına ve migrasyona bir güç çarpanı etkisi oluşturur. Bu sonuçlar oksiresveratrolün; serbest oksijen radikallerinin oluşturduğu akut ya da kronik hastalıkların engellenmesinde hücre düzeyinde etkili olabileceğini göstermiştir. Hücre düzeyinde gerçekleştirilecek daha kapsamlı çalışmalar, in-vivo çalışmalarla oksidatif stres kaynaklı hastalıkların tedavisinde oksiresveratrolün potansiyel bir molekül olabileceği düşünülebilir.
Anahtar Kelimeler: *Oksiresveratrol, OSİ, TAS, TFM, TOS*

ABSTRACT

Objective: *It was aimed to determine the regenerative effect of oxyresveratrol in eliminating the cell damage caused by the effect of H_2O_2 in L929 fibroblast cells.*
Material and Method: *Total antioxidant capacity (TAC), total oxidant capacity (TOC), oxidative stress index (OSI), total phenolic substance levels of oxyresveratrol were measured at different concentrations. After the IC_{50} value of oxyresveratrol fibroblast cells was determined by the MTT method, the regenerative effect on oxidative stress damage induced by H_2O_2 at 12.5 - 400 μM concentrations was performed with the xCELLigence device to measure cell proliferation in-vitro. In addition, scratch analysis was performed at concentrations of 3.125 - 25 μM in order to determine the wound healing levels in cell damage.*
Result and Discussion: *The TAC value of oxyresveratrol at 0.5 mg/ml was 3 ± 0.3 and the TOC value was 0.77 ± 0.52; (OSI) value was found to be 0.02 ± 0.09. It has been observed that the total phenolic substance (TPS) concentrations of oxiresveratrol at different doses from 0.625 mg/ml to 10 mg/ml have higher TPS from low concentration to high concentration. Oxyresveratrol L929 fibroblast cells IC_{50} value 214.2 μM; The IC_{50} value of oxyresveratrol was determined to be 109.7 μM in the cell line of L929 fibroblast cells exposed to H_2O_2. The highest cell proliferation was observed when measuring the 12.5 μM concentration of oxiresveratrol with the xCELLigence device. Scratch analysis showed a greater improvement than other doses, with a cell proliferation of 62% at the 24th hour and 88% at the 48th hour at a concentration of 12.5 μM oxyresveratrol in H_2O_2-damaged cells. The 12.5 μM concentration was determined to be the most effective concentration in both proliferation and scratch analysis. Oxyresveratrol; with its antioxidant capacity at low concentrations, L929 protects fibroblast cells from oxidative stress in cellular damage caused by H_2O_2, and creates a power multiplier effect on fibroblast viability and migration. These results indicate that oxyresveratrol; showed that it can be effective at the cellular level preventing of acute or chronic diseases caused by free oxygen radicals. It can be thought that oxyresveratrol may be a potential molecule in the treatment of oxidative stress-induced diseases with more comprehensive studies to be carried out at the cellular level and in-vivo studies.*
Keywords: *OSI, oxyresveratrol, TAC, TPS, TOC*

GİRİŞ

Canlılardaki homeostazinin değişmesiyle serbest radikaller oksidatif stres meydana getirmektedir. Reaktif oksijen türleri (ROT), yüksek aktiviteye sahip moleküllerdir. ROT, öncelikle mitokondride ve diğer hücre elemanlarındaki normal metabolik faaliyetler neticesi ya da iskemi, reperfüzyon, yaşlılık, radyoaktivite, oksijen basıncı, inflamasyon, kimyasal etkenlere maruziyet gibi sebeplerle oluşmaktadır [1-3]. Oksidatif stres, kanser, diyabet, kardiyovasküler ve nörolojik hastalıklar, ateroskleroz ve inflamatuvar bozukluklar vb. birçok hastalık oluşumuna sebep olmaktadır [4-7]. Oksidatif stresin azaltılmasında antioksidanların önemli bir fonksiyonu vardır. Antioksidanlar; kansere, diyabete, kalp-damar hastalıklarına neden olan ve aşırı düzeyde reaktif olan serbest radikalleri etkisizleştirebilen kimyasallardır. Organizmanın ihtiyaç duyduğu antioksidanlar endojen ya da eksojen yollardan karşılanmaktadır. Eksojen yolla alınan fenolik bileşikler doğal antioksidanlardır ve fenolik asit, flavonoid ve fenolik polimer bu grupta yer almaktadır [8]. Kronik dejeneratif hastalıklar, özellikle de kalp-damar hastalıklarına karşı polifenollerin koruyucu etkisini kanıtlar nitelikteki çalışmalar artmaktadır [9,10]. Oksidatif stresin azaltılmasında flavonoidler de kullanılmaktadır [11]. Flavonoidlerden biri olan oksiresveratrol, gıdalarda bulunan bir dış antioksidan fenolik bileşiktir [12].

Oksiresveratrol, *Artocarpus lakoocha*'nın [13] öz odununda, bundan üretilen konvansiyonel ilaç 'Puag-Haad'da [14] ve beyaz dutta [15] var olan mulberroside A'nın aglikonunda vardır [16].

Oksiresveratrol, kuvvetli tirozinaz inhibisyonu yapmakta ve melanom inhibisyonuna neden olmaktadır [17]. Oksiresveratrolün; fotoprotektif aktivitesi, [18] prooksidan aktivitesi, [19] antiviral aktivitesi [20,21], antiherpetik etkisi [22] *in-vitro* olarak gösterilmiştir. Aynı zamanda kuvvetli antioksidan, antiinflamatuar, nöroprotektif ve nörodejeneratif bozukluklar, kanserle mücadele tedavisinde aday ilaç olarak kullanımı tavsiye edilmektedir [23-26]. Çalışmamızda, oksiresveratrolün antioksidan ve oksidan seviyesinin ve toplam fenolik içeriğinin tespitinin ardından hidrojen peroksitle oluşturulmuş L929 fibroblast hücrelerinde oksiresveratrolün MTT analizi ve xCELLigence cihazı aracılığıyla hücre proliferasyonuna etkisi sitotoksisitesi; scratch analiziyle ise yara iyileşmesindeki rolü tespit edilmek istenmiştir.

GEREÇ VE YÖNTEM

Araştırma Kimyasalları

Dimetil Sülfoksit (DMSO) [Sigma-Aldrich]; Trypsin/EDTA(1X) [Gibco]; L929 Mediumu [ATCC]; Dulbecco's Modified Eagles Medium (DMEM) [Lonza]; H_2O_2 [Sigma-Aldrich]; 2,5-Diphenyltetrazolium Bromide (MTT) [Sigma-Aldrich]; Tripan Mavisi(%0.5) [Sigma-Aldrich]; Oksiresveratrol [Sigma-Aldrich]; L-Glutamin [Gibco]; Penisilin-Streptomisin-Amfoterisin(PSA) [Lonza]; Fetal Bovin Serum (FBS)(%10) [Gibco]; TAS Assay Kit [RAD]; TOS Assay Kit [RAD]; Folin-Ciocalteu reaktif [Sigma-Aldrich] kimyasalları köşeli parantez içerisinde belirtilen ticari firmalardan temin edilmiştir.

Hücre Kültürü Hazırlığı

37°C su banyosunda hücre hattı çözüldüğünde flakon su banyosundan çıkarılarak %70 etanolle asepsi işlemine tabi tutulmuştur. Flakon içeriği 1000 rpm, 5 dk. santrifüj edilmiş ve hücre hattı 15 dakika inkübe edilerek pH değeri 7.0 -7.6 aralığında olması sağlanmıştır.

Kültür ortamı %0.25 Tripsin-0.53 mM EDTA çözeltisiyle yıkanmıştır. Flakona Tripsin-EDTA çözeltisi eklenmiş ve hücre tabakasının dağılmasında sonlandırılmıştır. Yeni kültür kapları hücre süspansiyonuna uygun biçimde eklenmiştir. Hücrelerin çoğalmasında flasklar %5 CO_2, %90 nem, 37°C'de inkübasyona tabi tutulmuştur. Her hafta 2-3 defa yenilenerek hücre yoğunlukları %85 üstüne çıkıncaya dek sürdürülmüştür. %89 DMEM; %10 FBS; %1 PSA besiyerleri kullanılmıştır.

16'lı plate kuyucukları en az mililitredeki hücre sayıları $1x10^5$ olacak şekilde ekilmiştir. xCELLigence cihazındaki e-plate kuyucukları toplam hacimi 100 µl besiyerine denk gelecek şekilde ekim gerçekleştirilmiştir. Herbir kuyucukta 1960 µl (hücre ve besiyeri) + 40 µl oksiresveratrol bulunacak şekilde hesaplanmıştır. Herbir kuyucuk %5CO_2, %90 nem, 37°C koşullarında inkübe edilmiştir. 24 saat inkübasyondan sonra oksiresveratrol uygulanmıştır.

TAS ve TOS Tayini

TAS solüsyonları ve örnekler eklenerek 10 dakika sonra spektrofotometrik ölçüm yapılmıştır. Yöntemin işleyişi; numunedeki antioksidanlar, solüsyonlardaki koyu yeşil renge sahip 2,2'-azinobis-3-etilbenzotiazolin-6-sülfonikasit (ABTS•+) katyonu indirgenip ABTS molekülüne dönüşümüyle tayin edilmektedir. Yöntemdeki kalibrasyon E vitaminine benzeyen ve "Trolox Equivalent" olarak isimlendirilen stabil antioksidan standardıyla gerçekleştirilmiştir. Ölçümü gerçekleştirilen TAS mmol Trolox Eq/L şeklinde verilmiştir [27].

TOS solüsyonları ve örnekler eklenerek 10 dakika sonra spektrofotometrik ölçüm yapılmıştır. Yöntemin işleyişi; numunedeki oksidanların analizdeki solüsyonları Fe^{+2} iyon komplekslerini okside edip Fe^{+3} şekle dönüşümüyle ilerlemektedir. Yöntemin kalibrasyonu hidrojen peroksitle yapılıp, sonuçlar µmol H_2O_2 Eq/L ile ifade edilmiştir [28].

OSİ Tayini

Oksidatif yük belirteci OSİ; TOS sonuçlarının TAS sonuçlarına bölümüyle bulunur. OSİ değerleri aşağıda belirtilen formülle hesaplanmıştır [29].

$$\text{Oksidatif Stres İndeksi} = \frac{\text{Total Antioksidan Seviyesi}}{\text{Total Oksidan Seviyesi X 10}}$$

TFM Tayini

Folin-Ciocalteu (FC) yöntemi; total fenolik madde miktar tayininde çoğunlukla kullanılmaktadır [30]. TFM ölçülmesinde, Gallik Asit (GA) standardı kullanılmış; 0.625 - 10 mg/ml oksiresveratrol konsantrasyonlarına denk gelen TFM bulunmuştur [31]. GA, %70'lik metanolde 1 mg/ml çözelti hazırlanıp; 8 mg oksiresveratrol 10 ml saf su içinde seyreltilmiştir. 1:10 suyla seyreltilip FC reaktifi hazırlanmıştır. 7.5 g Na_2CO_3, 90 ml su içinde çözülerek suyla 100 ml'ye ulaştırılmıştır. Oksiresveratrol konsantrasyonları tekrarlı şekilde eppendorf tüplerine eklenip üzerlerine, 125 µl FC reaktifi ilave edilmiştir. 5 dakika 30ºC'de inkübasyon sonrasında karışım plate yüklenmiştir. Kuyucuklara 10 µl Na_2CO_3 eklenip, 90 dakika 30ºC'de inkübe edilip ve 765 nm'de ölçülmüştür. TFM değerleri; oksiresveratrol örneklerinin miligramı başına miligram Gallik Asit Eşdeğeri (GAE) şeklinde verilmiştir.

MTT ile IC_{50} Tayini

Çalışmamızda 0.4 mM H_2O_2 ile hücresel hasara maruz kalan L929 hücre hatları üzerinde oksiresveratrolün 2.5-1000 µM konsantrasyonları zamana bağlı IC_{50} değerlendirilmesinde yararlanılmıştır. IC_{50}; H_2O_2 maruziyeti olan ve olmayan numunelerde ve farklı saatlerde belirlenmiştir. Hücre hatlarına oksiresveratrol eklenmesinin ardından, H_2O_2'e 3 saat maruz kalan hücreler; plateleri %5 CO_2'li, neme sahip atmosferde 37°C'de 24 saat, 48 saat ve 72 saat inkübasyon sonrasında çıkarılmıştır. Plateler; 24., 48. ve 72. saatlerde kuyucukların üstündeki bölgeler uzaklaştırılmıştır. Sonrasındaki işlemler karanlıkta yapılmıştır. Kuyucuklara 100 µl MTT çözeltisi ilave edilmiştir. Hücreler 37°C ve %5 CO_2 koşullarında 3 saat inkübe edilmiştir. İnkübatörden çıkan platelerin üzerindeki süpernatant atılmıştır. Hücrelere 200 µl izopropilalkol eklenmiştir. Spektrofotometrede ölçümler 570 nm'de alınmıştır. IC_{50} değerleri GraphPad Prism7.0 programından faydalanılarak bulunmuştur.

xCELLigence Cihazı ile Hücre Proliferasyon Tayini

xCELLigence Real Time Cell Analysis (RTCA) ile ölçümde %5 CO_2, %90 nem, 37ºC şartlarına sahip inkübasyon seçilmiştir. Önce kuyucuklara 50 µl besiyeri eklenmiştir. Ardından kuyucuklarda $5x10^3$ hücre bulunacak biçimde 50 µl (hücre+besiyeri) çözeltisi eklenmiştir. Stok oksiresveratroller DMEM (%10 FBS-%1 PSA) karışımıyla dilüe edilerek 12.5-400 µM konsantrasyonlarına seyreltilmiştir. Konsantrasyonlar IC_{50} değerleriyle belirlenmiştir. Hücrelerin ekilmesinden 24 saat sonra, plate tabanlarına tutunmuş hücre üstündeki süpernatant atılmış ve oksiresveratrol çözeltileri kuyucuklarda 100'er µl olacak şekilde eklenmiştir. Kontrol grubu içinde üç kuyucuk üzerine yalnızca 100 µl DMEM ilave edilmiştir. Oksiresveratrol uygulandıktan 1 saat sonra H_2O_2 (0.4 mM) herbir kuyucuğa uygulanarak hücre hasarı indüklenmiştir. Oksiresveratrol uygulanmadan önceki 24 saat ve oksiresveratrol uygulandıktan sonraki 72 saatte kuyucukların hücre canlılığı ve proliferasyonu xCELLigence RTCA sistem programıyla 15 dakika arayla gerçek zamanlı empedans ölçümlerinin alınabileceği biçimde ayarlanmış, 96 saatlik veriler kaydedilmiş ve hücre-zaman proliferasyonunu gösteren Şekil 5'deki grafiklere ulaşılmıştır.

Scratch Tayini

L929 hücreleri, mikroplakalara her bir kuyucukta 1 ml besiyerinde $2x10^4$ hücre olacak şekilde ekilmiştir. Hücreler iki gün 37°C'de, %90 nemlilikte ve %5 CO_2'de inkübe edilmiştir. Olympus CKX53 inverted mikroskopta incelererek hücre yoğunluğu %70-80'e ulaştığında kuyular 200 µl steril pipet ucu vasıtasıyla kuyu tabanlarına birbirlerine paralel olacak şekilde aynı yönde boşluk (yara) açılmıştır. Kuyucuklardaki besiyerleri uzaklaştırılmış ve PBS ile kuyucuklar 2 defa yıkanmıştır. 25-3.125 µM oksiresveratrol, %10 FBS L929'da hazırlanmış ve yara açılan kuyulara 1 ml'ye tamamlanmıştır. Kontrol için bir kuyuya oksiresveratrol bulunmayan besiyerinden 1 ml ilave edilmiştir. Sonrasında kuyucukların

görüntüleri kaydedilmiş ve 0. saat, 24. saat, 48. saatteki mesafe görüntüleri kaydedilmiştir. Yara kapanma yüzdeleri aşağıdaki formül aracılığıyla hesaplanmıştır [32].

$$\%\text{Proliferasyon} = \frac{\text{Başlangıçtaki hasarın mesafesi-t anındaki ölçülmüş hasarın mesafesi}}{\text{Başlangıçtaki hasarın mesafesi}} \text{ X } 100$$

İstatistiki Analiz

Çalışmamız sonucunda elde edilen verilerin istatistiki analizleri SPSS yazılımı (SPSS25.0 for Windows, IBM Co. II, USA) aracılığıyla yapılmıştır. ANOVA normal dağılımlı serilerinde uygulanmıştır. Post-Hoc ve Duncan çoklu karşılaştırma testlerinde uygulanmıştır. Normal dağılımlı veri analizlerinde eşleştirilmiş örneklem t-Testi uygulanmış ve veriler ortalama ± standart sapma biçiminde sunulmuştur. İstatistiki anlamlılık düzeyi $p<0.05$ değeri baz alınmıştır.

SONUÇ VE TARTIŞMA

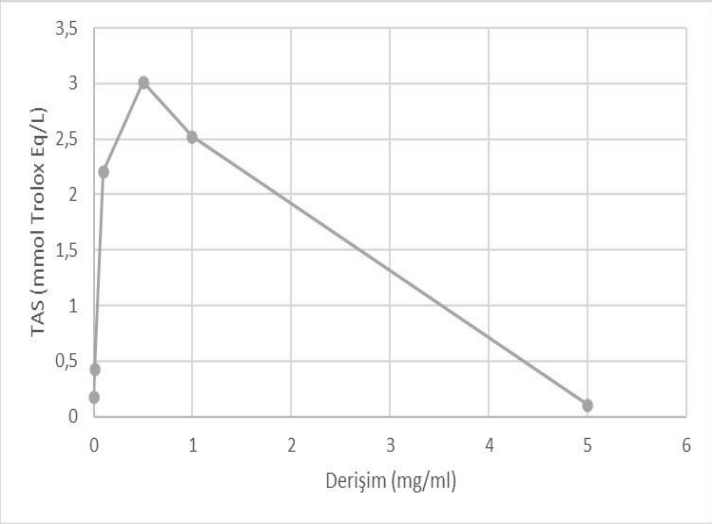
TAS, TOS ve OSİ Analiz Verileri

TAS, TOS ve OSİ verileri Tablo 1' de ve Şekil 1, Şekil 2, Şekil 3'de belirtilmiştir.

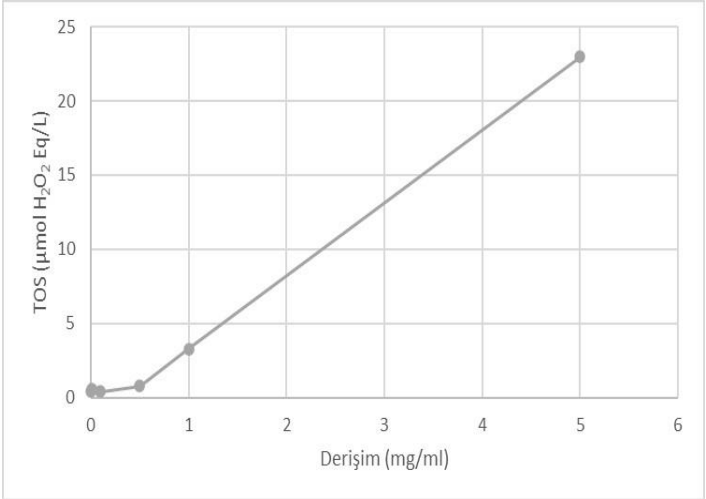
Tablo 1. Oksiresveratrole ait TAS, TOS ve OSİ verileri

Konsantrasyon (mg/ml)	TAS (mmol/l)	TOS (µmol/l)	OSİ
0.0001	0.17 ± 0.03	0.39 ± 0.03	0.22 ± 0.03
0.01	0.42 ± 0.08	0.59 ± 0.08	0.14 ± 0.03
0.1	2.20 ± 0.13	0.40 ± 0.07	0.01 ± 0.00
0.5	3.00 ± 0.30	0.77 ± 0.52	0.02 ± 0.09
1.0	2.52 ± 0.18	3.26 ± 0.19	0.12 ± 0.07
5	0.10 ± 0.09	22.96 ± 2.57	37.92 ± 1.7

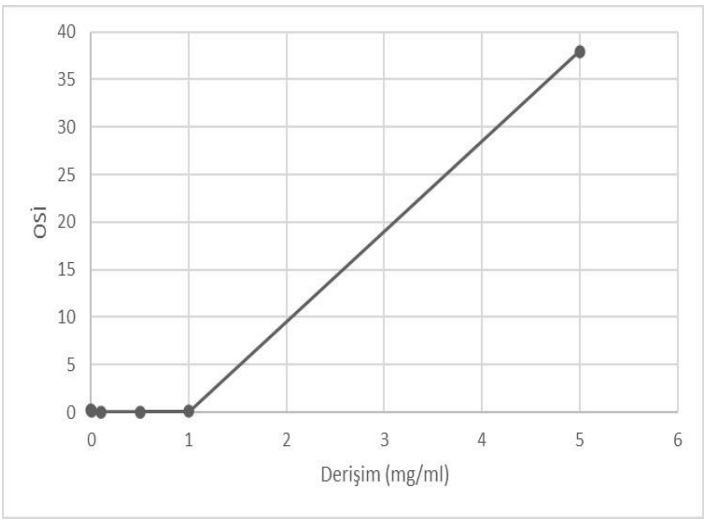
Veriler Ortalama ± SD olarak belirtilmiş ve analiz maddeleri 3 tekrarlı çalışılmıştır ($p<0.001$).



Şekil 1. Oksiresveratrolün TAS değerlerinin konsantrasyonlara bağlı değişimleri



Şekil 2. Oksiresveratrolün TOS değerlerinin konsantrasyonlara bağlı değişimleri



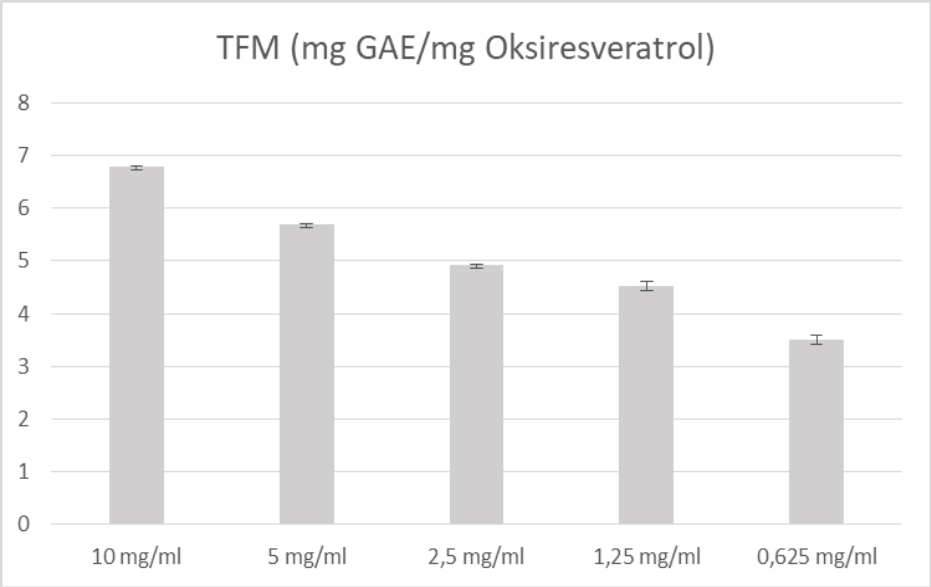
Şekil 3. Oksiresveratrolün OSİ değerlerinin konsantrasyonlara bağlı değişimleri

TFM Tayini Verileri

Oksiresveratrolün seyreltilerek 10-0.625 mg/ml'ye değişen konsantrasyonlardaki TFM verileri Tablo 2'de ve Şekil 4'de ise Gallik Asit Eşdeğeri (GAE) cinsinden değerleri görülmektedir. Tablo 2'de ve Şekil 4'de de gözlendiği gibi, oksiresveratrolün düşük konsantrasyondan yüksek konsantrasyona doğru gidildikçe daha yüksek TFM'ye sahip olduğu gözlemlenmiştir ve bu istatistiksel olarak anlamlıdır ($p<0.001$).

Tablo 2. Oksiresveratrol TFM içeriği

Oksiresveratrol Derişimleri (mg/ml)	**Oksiresveratrol mg GAE/mg**
0.625	3.50 ± 0.08^{d}
1.25	4.52 ± 0.09^{c}
2.5	4.90 ± 0.04^{c}
5	5.68 ± 0.03^{b}
10	6.77 ± 0.05^{a}



Şekil 4. Oksiresveratrolün TFM içeriği ($p<0.001$).

MTT Tayini IC_{50} Verileri

L929 hücrelerinin 96 kuyulu mikroplakalara hücre ekiminin ardından 2,5 µM ile 1000 µM arası 20 farklı oksiresveratrol derişimi ve kontrol olarak ise oksiresveratrol içermeyen besiyeri kullanılmıştır. 24., 48. ve 72. saatteki deney grupları oluşturulmuştur. Konsantrasyonlar üçer defa tekrarlanarak MTT canlılık tayini 570 nm'de yapılmıştır. Hücre canlılık yüzdesi aşağıdaki formülle hesaplanmıştır:

$$\text{Hücre canlılığı yüzdesi}(\%) = \frac{\text{Örneğin Absorbansı}}{\text{Kontrolün Absorbansı}} \times 100$$

L929 fibroblast hücreleri MTT tayiniile ulaşılan verilerle oksiresveratrolün; hem H_2O_2'e maruz bırakılan hem de H_2O_2'maruz bırakılmayan L929 hücrelerindeki IC_{50} değerleri bulunmuş ve Tablo 3'de belirtilmiştir.

Tablo 3. IC_{50} değerleri

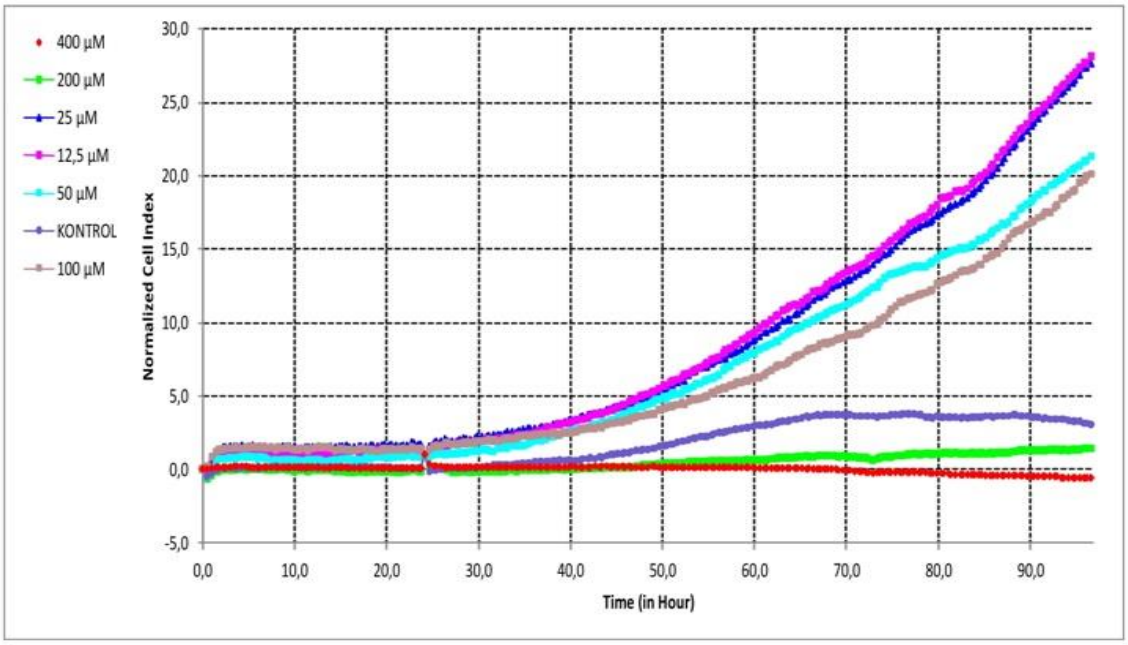
Zaman	Oksiresveratrol(µM)	Oksiresveratrol+H_2O_2(µM)
24. saat	151.2	101.7
48. saat	192.8	90.1
72. saat	214.2	109.7

IC_{50} verileri; maddelerin maksimum biyolojik cevabının yarısının inhibe olmasını sağlayan konsantrasyonu ifade etmektedir. Araştırmamızda MTT tayinindeki hücre canlılığının %50 oranındaki düşüşü tespit edilmiştir. Tablo 3'de hücre hatlarına yalnızca oksiresveratrol ya da oksiresveratrol+H_2O_2 uygulandığında elde edilen IC_{50} verileri gözlenmektedir.

xCELLigence ile Proliferasyon Verileri

L929 hücre hatlarının 72 saatte tüm birimlerdeki hücre prolifererasyonu, hücre empedans verileri Şekil 5'de gösterilmiştir. Hücrelerin kuyucuklara ekiminden 24 saat sonra oksiresveratrol uygulanmıştır. 1 saatin ardından H_2O_2'e maruz bırakılarak hücrelerde hasar oluşturulmuştur. Daha sonra 72 saat hücrelerin proliferayonu takip edilmiştir. İlk 24 saatte, her ne kadar 400 µM, 200 µM oksiresveratrolün

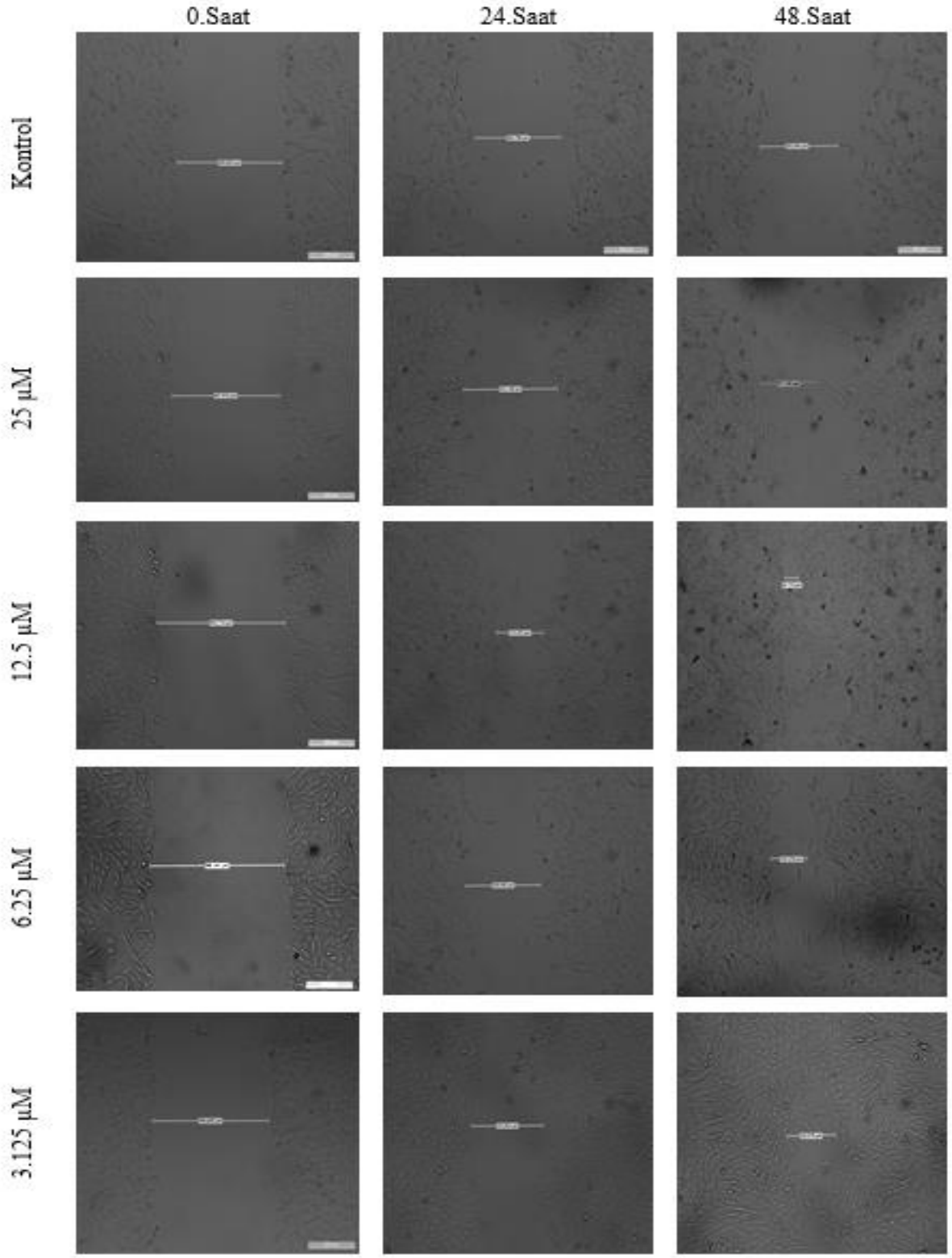
uygulamasının kontrol grubuna göre proliferasyonu düşürdüğü; 100 µM, 50 µM, 25 µM, 12.5 µM konsantrasyonlarında proliferasyonu yükselttiği görülse de bütün konsantrasyonlarda çok fazla bir değişiklik gözlenmemiş ve bu istatistiksel olarak anlamlıdır ($p<0.001$). İkinci 24 saatte konsantrasyonlardaki değişiklikler görülmeye başlanmış; 400 µM, 200 µM oksiresveratrol konsantrasyonlarında kontrol grubuna göre proliferasyon düşmüş; 100 µM, 50 µM, 25 µM, 12.5 µM konsantrasyonlarında proliferasyon yükselmiştir. Üçüncü 24 saatte konsantrasyonlardaki değişiklikler daha da görülür hale gelmiş; 400 µM, 200 µM oksiresveratrol konsantrasyonlarında kontrol grubuna göre proliferasyondaki düşüş çok fazla değişmemiş; 100 µM, 50 µM, 25 µM, 12.5 µM konsantrasyonlarındaki proliferasyonlardaki yükselişler öncekilere nazaran daha fazla olduğu gözlemlenmiştir. Araştırmamızda kullanılan en küçük konsantrasyona tekabül eden 12.5 µM, L929 hücrelerdeki proliferasyonda en üst düzeye ulaştırmış ve yükselen konsantrasyona nazaran fibroblast hücre proliferasyonlarını ilerleyen zamana oranla düşürmüştür.



Şekil 5. xCELLigence ölçüm grafikleri ($p<0.001$).

Scratch Tayini Proliferasyon Verileri

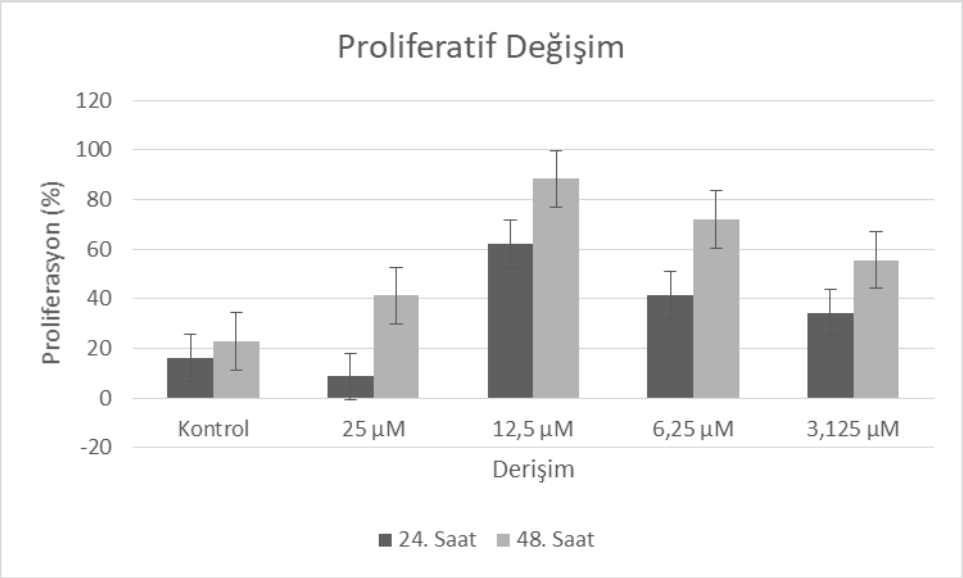
L929 hücreleri mikroplakalara ekilmiş ve 24 saat sonra mikroplak kuyucuklarına paralel şekilde çizgiler yapılmıştır. Dört ayrı oksiresveratrol konsantrasyonu kullanılmış ve hücrelerin değişik zamanlardaki migrasyonlarının mikroskobik görüntüleri Şekil 6'da sunulmuştur. Hasarlı hücrelerde değişik oksiresveratrol konsantrasyonu için proliferatif davranışları sunulmuştur (Şekil 7, Tablo 4). 12.5 µM oksiresveratrol konsantrasyonu için 24. saatteki %62, 48. satteki %88'lik hücre proliferasyonuyla öteki numunelerden daha yüksek iyileşme görülmüştür. Oksiresveratrolün 25 µM konsantrasyonu ise 24. saatteki %8, 48. satteki %41'lik hücre proliferasyonuyla görece daha düşük düzeyde bir proliferasyon düzeyine ulaşılmıştır. Araştırmamızdaki tayin işlemleri sırasında istatistiki olarak eşleştirilmiş örneklem t-Testi'nden faydalanılarak istatistiki anlamlılık tespit edilmiştir (Şekil 7, $p<0.01$). 50 µM, 100 µM, 200 µM, 400 µM oksiresveratrol konsantrasyonlarının yükselişiyle beraber hücrelerdeki proliferasyon inhibisyona dönüşmüştür.



Şekil 6. Scratch tayinindeki mikroskop görünümleri

Tablo 4. Hasarlı hücrelerdeki proliferatif değişme

Derişim (µM)	0. Saatteki Hasarın Mesafesi (µm)	24. Saatteki Hasarın Mesafesi (µm)	48. Saatteki Hasarın Mesafesi (µm)	24. Saatteki Proliferasyonu (%)	48. Saatteki Proliferasyonu (%)
Kontrol	456.60	382.75	352.64	16.17	22.76
3.125	496.88	325.92	220.57	34.40	55.60
6.25	580.44	339.04	162.22	41.58	72.05
12.5	553.71	210.32	64.11	62.01	88.42
25	461.91	422.10	271.10	8.61	41.30



Şekil 7. Oksiresveratrolün proliferatif değişimi

Araştırmamızda farklı konsantrasyonlardaki oksiresveratrolün, H_2O_2'e maruz kalmasıyla hücrelerde hasar oluşturulmuş; L929 hücre hattındaki proliferatif, sitotoksik etkilerin kıyaslanması hedeflenmiştir. Yapılan araştırmanın birisinde; viral enfeksiyonda, yara iyileşmesinde oluşan inflamatuar yanıt oksidatif stres düzeyinin indirilmesinin ilk emarelerinin antioksidan seviyesindeki artış olduğu gözlenmiştir [33]. Çalışmamızda da, yukarıdaki çalışmaya benzer bir yara iyileşme durumu tıpkı enfeksiyon iyileşmesindekine benzer oksiresveratrolün antioksidan kabiliyetinden yararlanılarak oksidatif stresin azaltılabileceği düşünülmektedir.

Bir başka araştırmada; TFM ile TAS değerleri arasında doğru orantı bulunduğu ifade edilirken; [34,35] bir başka araştırmacı grubu ise TFM ile TAS arasındaki bağda doğru orantının bulunmayacağını belirterek öngörülenden daha karmaşık bir sitemi olduğunu belirtmişlerdir [36,37]. Araştırmamızda oksiresveratrolün 10 mg/ml'den 0.625 mg/ml'ye kadar farklılaşan konsantrasyonlardaki TFM içeriği düşük konsantrasyonlardan yüksek konsantrasyonlara doğru daha yüksek TFM'si gözlemlenmiş, bu istatistiksel olarak ($p<0.001$) ve ulaşılan veriler TFM miktarı yüksek numunelerin yüksek antioksidan kapasitesi olacağı görüşüne katkı sağlamaktadır.

Oksiresveratrolün literatürde; TOS, TAS, OSİ değerleri çalışmamız öncesinde araştırılmamıştır. Farklı bitkilerdeki araştırmalarda TAS verileri; *Silybum marianum, Allium calocephalum, Rhus coriaria var. zebaria, Mentha longifolia, Rosa canina, Salvia multicaulis ve T. spicata* sırasıyla 5.767; 5.853; 7.342; 3.628; 4.602; 6.434 ve 8.39 mmol/l şeklinde gösterilmiştir. TOS verileri sırayla 12.144; 16.288; 5.170; 4.046; 6.294; 22.441 ve 6.53 µmol/l şeklinde gösterilmiştir. OSİ verileri sırayla 0.211; 0.278; 0.071; 0.112; 0.138; 0.349 ve 0.07 şeklinde gösterilmiştir [38-43]. Araştırmamızdaki OSİ verileri yukarıdaki araştırmalara nazaran daha düşük seviyede olduğu gözlemlekmektedir. Bu kapsamda, oksiresveratrol yukarıdaki bitkilere göre endojen antioksidan moleküllere karşı direncinin fazla

olduğunu düşündürmektedir. Oksiresveratrolün; güçlü antioksidan olması düşük OSİ düzeylerine neden olduğunu düşündürmektedir. Yukarıdaki araştırmalarla kıyaslandığında oksiresveratrol için TAS, TOS değeri; *S. marianum, A. calocephalum, R. coriaria, M. longifolia, R. canina, S. multicaulis, T. spicata, B. rapa.* vb. bitkilerden düşüktür. Araştırmamızda oksiresveratrolün 0.5 mg/ml derişiminde yüksek TAS değerine ulaşırken; daha yüksek deirşimlerde ise belkide yapısında meta-hidroksi fonksiyonel grubu barındırmasından dolayı TAS değerinde düşüş gözlenmiştir. Oksiresveratrol konsantrasyon değişimiyle, antioksidan nitelikten oksidan niteliğe geçişi görülmüştür. Oksiresveratrolün özelliğindeki bu değişim molekül yapısında orto-hidroksi fonksiyonel gruba sahip olmayan oksiresveratrolün; ABTS radikalini indirgeme yeteneği yukarıdaki bitkilerden daha düşük olabileceğinin bir göstergesi olduğunu düşündürmektedir. Araştırmamızdaki TOS ve OSİ verileri ise yukarıdaki araştırmalarla paralellik göstermektedir.

Yapılan araştırmaların bazılarında, resveratrolün IC_{50} değerinin 10 - 100 nmol/l olduğu ve bu değerin kanserler arasında farklı olabileceğini göstermiştir [44]. Diğer bir araştırmada; resveratrolün IC_{50} değeri, 24 saat inkübasyonda 87.5 µM, 48 saat inkübasyonda 85.5 µM konsantrasyonlarda olduğunu göstermiştir [45]. Araştırmamızda hücre hattına sadece oksiresveratrol uygulandığında; IC_{50} değeri sırayla 24 saatte 151.2 µM, 48 saatte 192.8 µM ve 72 saatte 214.2 µM bulunmuş; H_2O_2'e maruz kalan hücre hatlarında oksiresveratrol uygulandığında, IC_{50} değeri sırayla 24 saatte 101.7 µM, 48 saatte 90.1 µM ve 72 saatte 109.7 µM bulunmuştur. Elde edilen veriler ışığında resveratrolle benzer özellikler içermesi nedeniyle oksiresveratrolün de antikanserojen etkili olabileceğini düşündürmektedir.

xCELLigence cihazıyla proliferasyon ölçülmünde; hücre ekiminin ardından 24 saat sonra H_2O_2'e üç saat maruz bırakılarak hücreler hasarlanmış ve sonrasında 400 µM, 200 µM,100 µM, 50 µM, 25 µM, 12.5 µM oksiresveratrol konsantrasyonları L929 hücre hatlarına uygulanarak 48 saat proliferasyon takip edilmiştir. 400 µM ve 200 µM oksiresveratrol uygulanması kontrol grubunda 48 saat proliferasyonu düşürdüğü gözlemlenmiştir. 100 µM, 50 µM, 25 µM, 12.5 µM konsantrasyonları kıyasla diğer yüksek konsantrasyonlara nazaran oksiresveratrol uygulandığında ise kontrol grubunda 48 saat proliferasyonu yükselttiği gözlemlenmiştir. Araştırmamızdaki 12.5 µM konsantrasyonu, L929 proliferasyonunda en yüksek düzeye ulaştırmıştır. 12.5 µM'lık konsantrasyon; xCELLigence cihazındaki ölçümde ve scratch analizinde en yüksek proliferasyonu oluşturmuştur.

Yapılan bir araştırmada; antioksidan özellikteki *A. Speciosa* uygulanan L929 fibroblast hücreleri 3.125-1.56 µg/ml derişimlerinde proliferasyonun araştırmamızdaki gibi 72. saatte de devam ettiği bulunmuştur [46]. Araştıramızdaki 12.5 µM oksiresveratrol konsantrasyon yüksek proliferasyonu işaret ederek L929 hücre hatlarında da uyumlu neticelere ulaşılmıştır. Bundan dolayı da oksiresveratrolün de resveratrol benzeri antikanserojen olabileceği yeni araştırmalarla desteklenebilecektir.

Duman'ın yaptığı araştırmada, yara iyileşmesinde HEC1A hücrelerinde D vitamininin migrasyona etkisi alfakalsidolün, kalsitriolün yüksek konsantrasyonları için yara mesafesini ilk 24 saate düşürmüştür [47]. Pereira ve arkdaşlarının araştırmasında*; L. Pacari* yaprağının HELp 0.1-0.03 mg/ml konsantrasyonlarında L929 hücre hatlarındaki migrasyonu hızlı hale getirerek proliferasyonu arttırmıştır [48]. Araştırmamızda da; 12.5 µM oksiresveratrol konsantrasyonunda 24. saatteki %62, 48. satteki %88'lik proliferasyonla öteki numunelerden daha çok iyileşmeye sebep olmuştur. 25 µM oksiresveratrol konsantrasyonunda ise 24. saatteki %8, 48. satteki %41'lik proliferasyonla diğer konsantrasyonlara göre daha düşük proliferasyon düzeyine ulaşılmıştır.

TFM ile TAS arasındaki bağ TFM içeriği yüksek numunelerin yüksek TAS değeri olacağını desteklediği belirlenmiştir. Oksiresveratrolün sitotoksitite analizleriyle etkili konsantrasyonları, IC_{50} değeri bulunmuş ve elde edilen veriler oksiresveratrole antioksidan özellik yüklediği belirlenmiştir.

Araştırmamızdaki 12.5 µM oksiresveratrol konsantrasyonu; hem xCELLigence cihazı ölçümünde hem de scratch analizinde en yüksek proliferasyona ulaştırmıştır. MTT hücre canlılık analizlerinde ise 640 µM oksiresveratrol konsantrasyonu proliferasyonda daha yüksek bir seviyeye ulaştırmıştır. Proliferasyonların belirlenmesinde; anlık ölçüm alınabilmesi, kontaminasyon riskinin düşük seviyede oluşu ve sürecin kontrolünün mümkün oluşu sebebiyle xCELLigence cihazıyla tayinlerin ileride MTT metoduna nazaran daha çok kullanılabileceğini düşündürmektedir. Bundan dolayı da oksiresveratrolün hücre proliferasyonunda kullanımında gerekli konsantrasyonları daha ideal değerlerde belirlenebilecektir.

Oksiresveratrolün, L929 hücre hatlarında ROT oluşturabilecek maddelerin toksisitesini düşürmede yararlanılabileceği düşünülmektedir. Ancak bunun oluşturulabilmesinde sitotoksisitenin meydana geliş süreçlerininde incelendiği daha kapsamlı araştırmalara ihtiyaç duyulabilecektir. Aynı zamanda oksiresveratrolün antioksidan, oksidan özellikleri *in-vivo* araştırmalarla desteklenmeli ve etki mekanizmaları da aydınlatılmalıdır. Oksiresveratrolün bu özellikleri; antioksidan yokluğunda meydana gelebilen rahatsızlıklar için geliştirilecek kişiye özgü tedavilerde, bu tedavilere katkı sağlayan tamamlayıcı terapötik bir made olarak yaralanılabileceği düşünülmektedir.

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Makale yazarları için gerçek, potansiyel ya da algılanan çıkar çatışması bulunmadığını beyan ederler.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

INTERVENTIONS IN INTERNAL MEDICINE WARDS WITH SCOPE OF CLINICAL PHARMACY RESIDENCY PROGRAM: A RETROSPECTIVE STUDY

KLİNİK ECZACILIK UZMANLIK PROGRAMI KAPSAMINDA İÇ HASTALIKLARI SERVİSLERİNDEKİ MÜDAHALELER: RETROSPEKTİF BİR ÇALIŞMA

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ABSTRACT

Objective: *Drug-related problems are a common health problem in hospitalized patients, affecting optimal patient outcomes. The aim of the study is to classify the drug-related problems detected by clinical pharmacy resident in hospitalized patients during their rotations and evaluate their interventions to the healthcare team.*

Material and Method: *This is a retrospective study in which the drug-related problems of the patients hospitalized in the internal medicine wards between April and November 2018 were examined during the rotations of the clinical pharmacy resident. Patients' demographics, prescribed medicine, laboratory findings were recorded. Drug-related problems are classified with the Pharmaceutical Care Network Europe Version 9.1 system.*

Result and Discussion: *Ninety-two patients were included in the study. The median age of patients was 60.5 years and 59.8% of them were female. Most of the patients (63%) had three or more comorbidities. The most frequent comorbidities were hypertension (21.70%) and diabetes mellitus (10.64%). One hundred forty-seven drug-related problems were detected in 57 patients (62%). Potential drug-drug interactions (55.78%), errors in dosing timing instructions (9.52%), and inappropriate drug use according to guidelines (8.16%) were the most common causes of drug-related problems. The acceptance rate of interventions for resolving drug-related problems was 65%. The most common drug-related problems in this study were due to drug selection. The acceptance rate of recommendations for drug-related problems was lower than in the literature. However, this initial acceptance rate can be considered successful in a center where clinical pharmacy services have not been established.*

Keywords: *Clinical pharmacy, drug-related, internal medicine*

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ÖZ

Amaç: *İlaçla ilgili sorunlar, hastanede yatan hastalarda optimal hasta sonuçlarını etkileyen yaygın bir sağlık sorunudur. Çalışmanın amacı, klinik eczacılık asistanının rotasyonları sırasında hastanede yatan hastalarda tespit ettiği ilaçla ilgili sorunları sınıflandırmak ve sağlık ekibine yapılan girişimleri değerlendirmektir.*

Gereç ve Yöntem: *Bu çalışma, Nisan-Kasım 2018 tarihleri arasında iç hastalıkları servislerinde yatan hastaların klinik eczacılık asistanının rotasyonları sürecinde ilaçla ilgili sorunlarının incelendiği retrospektif bir çalışmadır. Hastaların demografik bilgileri, reçete edilen ilaçlar, laboratuvar bulguları kaydedildi. İlaçla ilgili sorunlar, Avrupa Farmasötik Bakım Ağı Versiyon 9.1 sistemi ile sınıflandırıldı.*

Sonuç ve Tartışma: *Çalışmaya 92 hasta dahil edilmiştir. Hastaların ortanca yaşı 60.5'tir ve %59.8'i kadındı. Hastaların çoğunda (%63) üç veya daha fazla ek eşlik eden hastalık vardır. En sık eşlik eden hastalıklar hipertansiyon (%21.70) ve diabetes mellitus'tur (%10.64). Hastaların 57'sinde (%62) 147 adet ilaçla ilgili sorun saptandı. Potansiyel ilaç-ilaç etkileşimleri (%55.78), doz zamanlama talimatlarındaki hatalar (%9.52) ve kılavuzlara göre uygunsuz ilaç kullanımı (%8.16) ilaçla ilgili sorunların en yaygın nedenleri olarak belirlenmiştir. İlaçla ilgili sorunların çözümüne yönelik önerilerin kabul oranı %65'tir. Bu çalışmada en yaygın karşılaşılan ilaçla ilgili sorunlar ilaç seçiminden kaynaklanmaktadır. İlaçla ilgili sorunlar için önerilerin kabul oranı literatüre kıyasla daha düşüktür. Ancak klinik eczacılık hizmetinin henüz kurulmadığı bir merkezde bu ilk kabul oranları başarılı olarak kabul edilebilir.*

Anahtar Kelimeler: *İç hastalıkları, ilaçla ilgili, klinik eczacılık*

INTRODUCTION

Drug-related problems (DRPs) are a common health issue among inpatients that can negatively impact patient outcomes, increase morbidity and mortality, and increase healthcare costs. To improve patient outcomes, it is important to prevent, detect, and resolve DRPs [1,2]. A review of studies found that when clinical pharmacists were involved in clinical rounds and medication reconciliation, there were reductions in DRPs and shorter hospital stays [3]. The involvement of pharmacy students in healthcare team meetings, pharmacist-patient interactions, medication reconciliation, and post-discharge counseling and follow-up has been shown to improve patient outcomes [4,5].

Many studies on DRPs in inpatients have been conducted, with reported rates varying depending on factors such as location. In studies conducted with pharmacy students in Turkey and Lebanon, at least one drug-related problem (DRP) was found in 80% and 15% of patients, respectively. The main causes of DRPs are drug and dose selection, with common risk factors including renal failure, polypharmacy, and prolonged hospital stays [6-9]. Studies have shown that student interventions to resolve DRPs are well accepte [7-10].

Clinical pharmacy has been present in Turkey since 1991, but clinical pharmacists are not widely employed in hospitals. Most research in Turkey has been conducted as part of undergraduate and graduate programs. A clinical pharmacy residency program was established in 2018 under the Ministry of Health, in which, clinical pharmacy residents (CPRs) rotate through 15 different clinics and make recommendations to the healthcare team to resolve DRPs. These recommendations are generally accepted, but there is currently no system in place to record them.

The aim of this study is to examine the interventions made by a clinical pharmacy resident (CPR) to the healthcare team for identifying, preventing, and resolving DRPs internal medicine wards during the CPR's rotations.

MATERIAL AND METHOD

Study Design and Sample Size

This retrospective study was conducted in the 40 beds internal medicine wards of a training and research hospital in Turkey. Patients hospitalized in endocrinology (2 months), rheumatology (1 month), gastroenterology (1 month), nephrology (1 month) and chest diseases (2 months) services were

examined. In this study, DRPs detected by CPR during rotations within the clinical pharmacy residency program scope were evaluated retrospectively between April and November 2018. The detected DRPs, which include issues related to dosage, indication, treatment duration, drug-drug interactions, therapeutic drug monitoring, dosage form, and administration route, and the recommendations made by the CPR were presented to the healthcare team. The DRPs were classified according to the Pharmaceutical Care Network Europe (PCNE) Version 9.1 DRP classification system.

This study included patients aged 18 years and older who used at least one drug, were hospitalized for more than 24 hours, and were followed by CPR. Eligible patients were included in the study without randomization to eliminate potential selection bias. Patients who could not reach sufficient information and data were excluded from the study by the researchers. One CPR conducted the study. The CPR made interventions for DRPs during patient visits and discussed them with the patient's primary physician. The CPR documented implementation and acceptance of suggestions and interventions are recorded routinely. The CPR participated in weekday patient rounds and made interventions during the day. These interventions covered any stage of the medication treatment process and included recommendations such as discontinuation/addition of the drug, alternative therapy, or dose adjustment.

Data Collection

The study was collected data on patients' demographic information, medication use and medical history, laboratory test results and length of hospital stay. The appropriateness of drug selection and dosage was assessed by comparing to local and international guidelines. Treatment was considered appropriate when drug and dose selection complied with these guidelines. In the study, UpToDate (Wolters Kluwer Health Inc.) and Micromedex® Drug Information (Truven Health Analytics Inc.) were primarily used as references for clinical decision making, drug administration, and drug-drug/nutrition/disease interactions. Lexicomp was used through the UpToDate database to identify drug-drug interactions. Only contraindicated and major drug interactions were classified as DRPs. The Sanford Guide to Antimicrobial Therapy (Antimicrobial Therapy, Inc. Sperryville, USA) was used for information on dosage and indication of antimicrobial drugs. This study used CKD-EPI to calculate the estimated glomerular filtration rate (eGFR). eGFR values below 60 ml/min/1.73 m2 were classified as "decreased GFR" at the hospital where the study was performed. Also, normal values of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) parameters are 10-37 IU/L and 10-40 IU/L, respectively. Patients with AST and ALT values above normal values were classified as having "increased AST-ALT".

Statistical Analysis

The study determined if the distribution of continuous variables were normal by using the Kolmogorov-Smirnov test, and as the data were not normally distributed, the relationship between patient variables and the number and presence of DRPs was analyzed using Pearson's correlation. The statistical analyses were performed using the IBM SPSS Statistics for Windows, Version 25.0. (Armonk, New York: IBM Corp.) software package. All data were considered statistically significant at $p<0.05$ and with a 95% confidence interval.

RESULT AND DISCUSSION

One hundred nineteen patients were evaluated for eligibility and included 92 patients in the study. The median age (interquartile range [IQR]) of the patients was 60.5 (39.25-73.00) years, and 59.8% were female. The median (IQR) values of the number of medications used by the patients at admission and during discharge were 8 (6-10) and 7 (5-9), respectively. Most of the patients (63%) had three or more comorbidities. The most common comorbidities were hypertension (21.70%) and diabetes mellitus (10.64%) (Table 1). Most patients were admitted to the department of chest diseases (39.13%) and gastroenterology (20.65%).

Table 1. Demographic information of the patients

Variable	Numbers
Gender (n, %)	
Female	55 (59.8)
Male	37 (40.2)
Age, Median (IQR)	60.5 years (39.25-73.00)
Admission wards to hospital (n, %)	
Nephrology	9 (9.78)
Rheumatology	11 (11.96)
Endocrinology and Metabolic Diseases	17 (18.48)
Gastroenterology	19 (20.65)
Chest Diseases	36 (39.13)
Comorbidities (n, %)	
Hypertension	51 (21.70)
Diabetes mellitus	25 (10.64)
Chronic kidney disease	12 (5.11)
Heart failure	10 (4.26)
Coronary artery disease	10 (4.26)
Hyperlipidemia	10 (4.26)
Hypothyroidism	7 (2.98)
Atrial fibrillation	7 (2.98)
Other	103 (43.82)
Charlson Comorbidity Index, Median (IQR)	3 (0-5)
Body mass Index, Mean (± Standard Deviation)	27.28 (± 6.37)
Length of Hospitalization Stay, Median (IQR)	13 (7.75-19)
Kidney Function, (n, %)	
Normal	63 (68.5)
Decreased GFR	24 (26.1)
Hemodialysis	4 (4.3)
Peritoneal dialysis	1 (1.1)
Liver Function (n, %)	
Normal	85 (92.4)
Increased AST-ALT	3 (3.3)
Cirrhosis	2 (2.2)
Hepatitis	2 (2.2)
Number of Medications in Hospitalization, Median (IQR)	8 (6-10)
Number of Medications in Discharge, Median (IQR)	7 (5-9)
Discharge Status (n, %)	
Discharge	87 (94.5)
Referral to Another Service	2 (2.2)
Transfer to the intensive care unit	2 (2.2)
Death	1 (1.1)

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GFR: glomerular filtration rate, IQR: Interquartile Range

In this study, 147 DRPs were detected in 57 (62%) patients. The mean (standard deviation) number of DRPs per patient was 1.59 (1.87). The most common causes of DRP were inappropriate drug selection (76.19%) and inappropriate dose selection (14.28%). The most common agents causing DRPs were quetiapine (7.72%), tramadol (6%), calcium carbonate (5.57%), methylprednisolone (4.29%), aspirin (3.86%) and pantoprazole (3.43%). Potential drug-drug interactions (pDDIs) (55.78%), errors in dose timing instructions (9.52%), and inappropriate drug use according to guidelines (8.16%) were the main causes of DRPs (Table 2). The rate of contraindicated pDDIs was 7.4%.

Table 2. Distribution of main causes of DRPs

Causes	n (%)	Medications Causing Drug-Related Problems (n)*
1. Drug selection	**112 (76.19)**	
C1.1 Inappropriate drug according to guidelines/formulary	12 (8.16)	oxybutynin (2), tenofovir (2)
C1.2 No indication for drug	7 (4.76)	pantoprazole (1), rabeprazole (1)
C1.3 Inappropriate combination of drugs. or drugs and herbal medications. or drugs and dietary supplements	82 (55.78)	methylprednisolone-calcium carbonate (6), quetiapine-tramadol (4), fluconazole-quetiapine (3)
C1.4 Inappropriate duplication of therapeutic group or active ingredient	2 (1.36)	Magnesium oxide (1), Vitamin D (1)
C1.5 No or incomplete drug treatment in spite of existing indication	9 (6.12)	Vitamin B12 (2), Folic acid (2)
2. Drug form	**10 (6.80)**	
C2.1 Inappropriate drug form/formulation (for this patient)	10 (6.80)	pantoprazole (5), linezolid (2)
3. Dose selection	**21 (14.28)**	
C3.2 Drug dose of a single active ingredient too high	7 (4.76)	tramadol (2), ceftazidime (1)
C3.5 Dose timing instructions wrong. unclear or missing	14 (9.52)	levothyroxine (3), nifedipine (2)
4. Treatment duration	**1 (0.68)**	
C4.2 Duration of treatment too long	1 (0.68)	flavoxate (1)
9. Other	**3 (2.04)**	
C9.1 No or inappropriate outcome monitoring (incl. TDM)	3 (2.04)	hydroxychloroquine (2)
Total DRP	**147**	**100**

C: Cause, DRP: Drug-related problem
*The active ingredients that most commonly cause drug-related problems are given.

The major and contraindicated pDDIs were mainly methylprednisolone-calcium carbonate (n=6) and atorvastatin-gemfibrozil (n=1), respectively. Details of pDDIs and levels of evidence were shown in Figure 1.

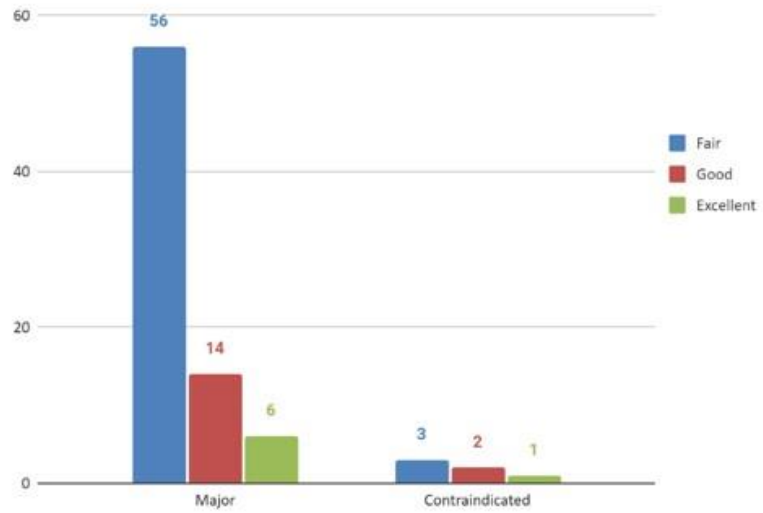

Figure 1. Frequency of potential drug-drug interactions by level of evidence (n=82)

The CPR made 80 recommendations for resolving 147 DRPs. The healthcare team accepted 52 (65%) of the recommendations. Forty-one (51.25%) of all recommendations were accepted and fully implemented. The CPR' recommendations were made only at the level of the patient's primary physician and face-to-face. Interventions were mainly made as stopping the drug (35%) and changing the instructions for the use of the drug (30%). Interventions for stopping the drug (30%) and changing the

instructions for use of the drug (15%) were accepted mainly by the healthcare team (Figure 2).

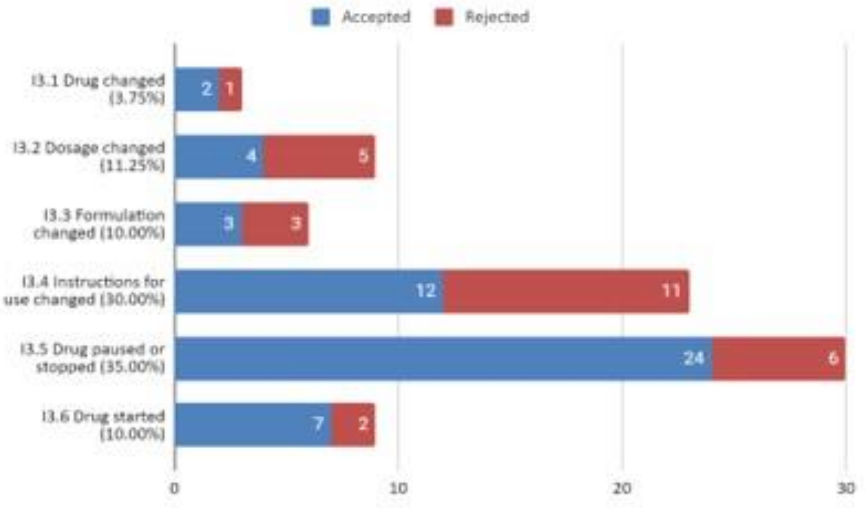

Figure 2. Classification of clinical pharmacist intervention types for DRPs (n=80)

No statistically significant difference was found when DRP numbers were compared in terms of age, gender, length of hospital stay, kidney failure, liver failure. A correlation was found between the number of DRPs and number of medications at hospitalization, number of medications at discharge, length of hospital stay and Charlson comorbidity index (Table 3).

Table 3. Correlation between patient variables and drug-related problem numbers

Variables	**Number of DRP**	
	R	**p**
Number of Medications in Hospitalization	0.417*	< 0.001
Number of Medications in Discharge	0.531*	< 0.001
Total Hospitalization Days	0.280*	0.008
Charlson Comorbidity Index	0.115	0.275

R = correlation coefficient

The clinical pharmacy residency program, which started in Turkey for the first time in 2018, graduated its first specialists in 2021. In this context, universities have designed rotation programs for CPRs in nearly 15 different clinics for two years to complete their clinical training. In this process, CPRs tried to provide both their theoretical knowledge and skills in clinical practice by participating in routine patient rounds with a multidisciplinary team. During their clinical rotations, they participated in their clinics' training, patient rounds and case discussions. While trying to improve their education, they reviewed the treatments of inpatients and presented their recommendations for DRPs to the healthcare team. In this study, interventions for DRPs, determined by the CPR in the rotation of the internal medicine wards during the first seven months of clinical pharmacy residency training in Turkey and presented to the healthcare team, were examined.

Researchers have demonstrated that the recommendations made by pharmacy students during clinical rotations are crucial in enhancing patient care and decreasing costs in various clinics [4,5,11-13]. Studies have highlighted that the suggestions made by student pharmacists in the internal medicine ward aid in solving DRPs, preventing them, reducing costs, and positively impacting patient outcomes [7,8,14-16]. It is acknowledged that healthcare teams increasingly accept students who participate in CPR programs in Turkey, and physicians accept their recommendations [17].

A study conducted by a pharmacy student and a clinical pharmacy specialist in an internal medicine ward in Turkey found that the mean number of DRPs per patient (1.6) was consistent with the average number of DRPs in this study (1.6) [6]. Other studies have reported that between 15.4-80% of patients had at least one DRP [6,7,18]. The DRP rates among inpatients may vary due to factors such as the clinics where the studies were conducted, the level of knowledge of the researchers, the clinical environment, and the different DRP classification systems used.

Studies conducted by internal medicine wards and pharmacy students primarily focused on the proportion of interventions and acceptance rates. The acceptance rate of the recommendations made by the CPR in this study (65%) was lower than reported in the literature (68-96%) [7-10,14-16,19-21]. Studies that report high acceptance rates of clinical pharmacy recommendations were conducted in locations where the practice is well-established. The low acceptance rate in this study could be attributed to the hospital's lack of routine clinical pharmacy services and the uncertainty of the healthcare team's attitude towards clinical pharmacists' interventions. Additionally, postgraduate studies in clinical pharmacy in Turkey are typically conducted in the later stages of students' education [9,20,21]. In contrast, this study examines the acceptance rate during the initial residency training phase. Another potential reason for the low acceptance rate could be the level of knowledge and experience of the CPR during the rotation process.

Studies in internal medicine wards, similar to this study, have found that DRPs primarily result from drug and dose selection [6-8,14,22]. As stated in previous studies, the most common cause of DRPs is from pDDIs with a rate of 29.4-65% [6,7,9,18,23]. As reported by Vinluan et al., the rate of pDDIs was low as the medical treatment records were routinely examined for drug-drug interactions in the electronic environment [8,22]. This study found that high doses (4.76%) and inappropriate drug selection (8.16%) according to guidelines, which frequently cause DRPs, are consistent with other studies. However, DRP was not detected in this study due to the low dose compared to other studies [6–8,24]. Like other studies, interventions made to the healthcare team that were highly accepted include dose adjustment, changing the drug's instructions for use, changing the drug, stopping the drug, and adding new drugs [7,8]. While pDDIs are the most common types of DRPs encountered in studies, most drug interactions are described as "potential" and the outcome of pDDI is followed. Therefore, only a few recommendations have been made for pDDIs. The acceptance of clinical pharmacists' recommendations, particularly for dosing and drug selection, indicates that pharmacists' competencies and services in this area are generally accepted.

In this study, 8.1% of DRPs were caused by inappropriate drug use according to guidelines, which is consistent with other studies that have reported rates of 2-29.5% [6,7,9,25]. Clinical pharmacists play a role in optimizing patients' treatment and providing updated treatments to the healthcare team. Another common cause of DRPs in this study was errors in the dose timing instructions (9.5%). Other studies have reported different rates (1-19.6%) of DRPs caused by errors in dose timing instructions [7,9,25]. Abunahlah et al. did not specify such a DRP [6]. It is important to use medications at the appropriate time for maximum effect. This DRP may have been caused by lack of knowledge and workload. Studies have reported various rates since there is insufficient consistency in the systems used to classify DRPs, which can affect the detection of DRPs [7].

Several factors can influence the number and type of DRPs, such as modifiable factors like length of hospital stay and polypharmacy, and non-modifiable factors such as age, diagnosis, and comorbidities. This study found that patient's age, gender, renal status, and hepatic status did not have an impact on the presence of DRPs. Similar to the study by Blix et al., this study also found that age and gender did not affect the existence of DRPs [22]. However, most studies have shown that polypharmacy is a risk factor that affects DRPs. This study also found a positive correlation between the number of DRPs and length of hospital stay, the number of medications at admission, the number of medications at discharge, and number of multiple comorbidities [6,18,22,26]. Extended hospitalization and various comorbidities, which are known to affect DRPs, are risk factors that lead to polypharmacy, ultimately resulting in an increase in DRPs.

A clinical pharmacist working in hospital wards can provide services such as consulting with physicians, reviewing prescriptions, and preventing DRPs [27,28]. Furthermore, a clinical pharmacist's participation in patient rounds can detect and control most DRPs [28]. Consulting with doctors and

nurses provided by clinical pharmacists can decrease cost, length of hospital stay, and mortality rate [27,29,30].

This study highlights the role of the CPR in detecting and resolving DRPs in internal medicine wards during the ongoing residency rotation in a hospital where clinical pharmacy services were not routinely offered before. The limitations of this study include its short duration and the limited number of patients. Additionally, the absence of a staff clinical pharmacist in the hospital for the CPR to consult during training is a significant limitation. Conducting such studies with a larger number of residents, for a longer period and in the presence of a clinical pharmacy specialist would better reflect the impact of solution proposals and clinical pharmacy activities for DRPs in the rotation process. Since this study was conducted in a single center, the findings cannot be generalized to other internal medicine wards.

This study found that at least one DRP was detected in over half of the patients hospitalized in the internal medicine wards. The most common causes of DRPs were inappropriate drug selection and dose selection. The study also found that DRPs were influenced by the number of medications at admission and discharge, the number of comorbidities, and length of hospital stay. The healthcare team's acceptance rate of these interventions was lower than what has been reported in literature. However, considering that clinical pharmacy services were not routinely provided in this hospital, this acceptance rate can be considered successful as a starting point. Clinical pharmacists and other healthcare professionals should work collaboratively to minimize DRPs while delivering pharmaceutical care. Pharmaceutical care programs integrating CPR into multidisciplinary patient care teams will increase therapeutic success. This research has taken its place among the limited number of studies conducted by CPR in the literature on the identification, resolution, and prevention of DRPs in the internal medicine wards.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

The ethics committee approval has been received from Marmara University non-interventional clinical research ethics committee (No: 09.2022.272, Date: 11.02.2022).

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

LACTARIUS DELICIUS VE *LACTARIUS SALMONICOLOR* MANTARLARININ FENOLİK BİLEŞİKLERİ VE ANTİOKSİDAN ETKİLERİNİN DEĞERLENDİRİLMESİ

EVALUATION OF PHENOLIC COMPOUNDS AND ANTIOXIDANT EFFECTS OF LACTARIUS DELICIUS AND LACTARIUS SALMONICOLOR MUSHROOMS

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ÖZ

Amaç: *Bu çalışmada, L. delicius ve L. salmonicolor mantarlarından elde edilen etanollü özütlerin fenolik içerikleri ve antioksidan enzim aktivitelerine olan etkilerinin incelenmesi amaçlanmıştır.*
Gereç ve Yöntem: *Mantar özütlerinin fenolik bileşiklerinin miktarı Folin-Ciocalteu, Alüminyum klorür kolorimetrik ve Yüksek Performanslı Sıvı Kromatografi (YPSK) yöntemleri ile analiz edilmiştir. Ayrıca, mantar özütlerinin antioksidan aktivite tayini DPPH yöntemi ile gerçekleştirilmiştir. Bununla birlikte mantarların etanollü özütlerinin glutatyon-S-transferaz (GST), glutatyon peroksidaz (GPx) ve katalaz (CAT) enzimleri üzerine olan etkileri araştırılmıştır.*
Sonuç ve Tartışma: *Elde edilen sonuçlar, L. delicius mantarından elde edilen etanollü özütün L. salmonicolor'a göre daha yüksek oranda fenolik bileşik içerdiğini ve antioksidan kapasitesinin de daha yüksek olduğunu göstermiştir.*
Anahtar Kelimeler: *Antioksidan, fenolik bileşikler, Lactarius delicius, Lactarius salmonicolor*

ABSTRACT

Objective: *In this study, it was aimed to investigate the phenolic content and antioxidant activities of ethanolic extracts obtained from L. delicius and L. salmonicolor mushrooms.*
Material and Method: *In this study, the amount of phenolic compounds of mushroom extracts were analyzed by Folin-Ciocalteu, Aluminum chloride colorimetric and High Performance Liquid Chromatography (HPLC) methods. In addition, the antioxidant activity of the mushroom extracts were determined by the DPPH method. The activity of ethanolic extracts of these mushrooms were*

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also investigated on glutathione-S-transferase (GST), glutathione peroxidase (GPx) and catalase (CAT) enzymes.
Result and Discussion: *The results showed that the ethanolic extract obtained from L. delicius mushroom contains higher phenolic compounds and higher antioxidant capacity than L. salmonicolor.*
Keywords: *Antioxidant activity, Lactarius delicius, Lactarius salmonicolor, phenolic compounds*

GİRİŞ

Lactarius, birkaç yenilebilir mantar arasında yer alan ektomikorizal mantarların bir cinsidir. Özellikle süt şapkası olarak bilinen cinsin türleri, kesildiğinde veya hasar gördüğünde salgıladıkları lateks (sütlü sıvı) ile karakterize edilir. Bu cinste yaklaşık 500'den fazla mantar türü teşhis edilmiştir. Bu mantar cinsine ait olan türler çoğunlukla Kuzey yarımkürede dağılım göstermektedir. *Lactarius deliciosus*, Kuzey yarım küredeki en değerli mantarlar arasında yer alırken, *L. indigo* veya *L. deterrimus* gibi diğer türlerin tadı konusunda görüşler farklılık gösterir. Rusya, Tanzanya ve Çin'de çeşitli türlerin yiyecek olarak toplandığı bildirilmiştir. Aynı zamanda, *L. turpis* veya *L. helvus* gibi bazı *Lactarius* türleri toksik mutajenik bir bileşik içerdiği için toksik mantarlar olarak bilinmektedir [1,2].

L. deliciosus Russulaceae familyasında ve *Lactarius* cinsinde yer alan yenilebilir mantar türüdür. Halk arasında safran süt kapağı ve kızıl çam mantarı olarak bilinmektedir. Genellikle Bulgaristan, İspanya, Yunanistan, İtalya, Kıbrıs, Fransa, Akdeniz bölgelerinde dağılım göstermektedir. Türkiye'de ise İzmir'den başlayarak Antalya'ya kadar yayılım göstermektedir. *L. deliciosus* mantarı ayrıca sonbahar döneminde genellikle Kastamonu, Bolu ve Mengen etrafında iğne yapraklılar altında yüksek oranda görünmektedir. *L. deliciosus*, başarıyla yetiştirilen birkaç ektomikorizal mantar türünden biri olarak da tanınmaktadır [3].

Halk arasında Kanlıca mantarı olarak bilinen *L. salmonicolor*, Russulaceae familyası ve *Lactarius* cinsinin başka bir türüdür. Bu mantarın şapka kısmının çapı yaklaşık 5-15 cm civarındadır. Mantarın rengi turuncudur, ama bazen açık sarı rengine değişmektedir. Genellikle, çam ormanı, çayırlıklarda ve yapraklı ağaç ormanlarında görülmektedir. Türkiye'de ise Karadeniz bölgesinde yayılış göstermektedir. Mantarın tadı acımsıdır fakat lezzetlidir [4].

Bu çalışmada, Karadeniz bölgesinde halk pazarlarından temin edilen *L. deliciosus* ve *L. salmonicolor* türlerinden hazırlanan etanollü özütlerin flavonoid ve fenolik madde miktarları, antioksidan özellikleri, ayrıca antioksidan savunma sisteminde görev alan glutatyon-S-transferaz (GST), glutatyon peroksidaz (GPx) ve katalaz (KAT) enzimleri üzerindekietki potansiyellerinin araştırılması hedeflenmiştir.

GEREÇ VE YÖNTEM

Mantarların Özütlemesi

Çalışma kapsamında sunulan *L. deliciosus* ve *L. salmonicolor* mantarları Ekim ve Kasım aylarında Kastamonu halk pazarından satın alındı. Toplanan örneklerin tür teşhisleri Prof. Dr. Ilgaz AKATA tarafından yapıldı. Özütleme işlemi için %99'luk etanol kullanıldı. Bu uygulama için, her ekstraksiyon aşamasında, her iki mantar türünden ayrı ayrı 10'ar gr tartılarak, sıvı azot yardımıyla havanda öğütüldü. Öğütülen mantar örneklerinin üzerine ağırlığının 10 katı kadar (100 ml) %99'luk etanol ilave edildi. Karışım yaklaşık 4 saat manyetik balıkla karıştırıldı. Örnekler karıştırıldıktan sonra yaklaşık 24 saat +4°C'de inkübe edildi. İnkübasyon sonrası, karışım Watmann No.1 kağıdından süzüldü. Ardından elde edilen karışımın etanolü 40°C'de uygun basınçta (337 mbar) rotary evaporatör yardımıyla uçuruldu. Evaporasyon sonrasında özütler liyofilizatör yardımıyla kurutuldu ve toz haline getirildi. Hazırlanan örnekler deneylerde kullanılana kadar -20°C'de saklandı [5].

Mantar Özütlerinin Fenolik Madde Miktarı Tayini

Mantarlardan elde edilen etanollü özütlerin fenolik bileşik tayini için spektrofotometrik metot kullanıldı. Bu yöntemde Folin-Ciocalteu solüsyonuna ilaveten %2'lik sodyum karbonat solüsyonu ve fenolik madde standardı olarak gallik asit (GA) kullanıldı. Reaksiyon sonunda oluşan renk değişimi 750

nm'de spektrofotometre de kolorimetrik olarak ölçüldü [6].

Mantar Özütlerinin Flavonoit Miktar Tayini

Mantar örneklerinden elde edilen etanollü özütlerin toplam flavanoit miktarı alüminyum klorür kolorimetrik metodu ile analiz edildi. Bu metotta %95'lik etil alkol, %10'luk alüminyum klorür ve 1 M sodyum asetat solüsyonları kullanıldı. Çalışmada, Woisky ve Salatino tarafından 1998 yılında modifiye edilmiş olan alüminyum klorür kolorimetrik yönteminden yararlanıldı [7].

Yüksek Performanslı Sıvı Kromatografisi (YPSK) Analizleri

Mantar örneklerin etanollü özütlerinin fitokimyasal içerikleri Yüksek Performanslı Sıvı Kromatografisi ile incelendi. Bu aşamada klorojenik asit, ferulik asit, kafeik asit, gallik asit, vanilik asit, kateşin, luteolin, apigenin, epikateşin, elajik asit, trans-sinnamik asit, o-kumarik asit, p-kumarik asit, mirsetin, kemferol ve kersetin gibi bazı fenolik asit ve flavonoitler standart biyoaktif bileşikler olarak kullanıldı.

Mantar Özütlerinin Antioksidan Aktivite Analizi

Bu çalışmada, mantarlardan hazırlanan etanollü özütlerin antioksidan aktivitelerinin analizi için, 2-difenil-1-pikrilhidrazil (DPPH) yöntemi kullanıldı [8].

Glutatyon-S-Transferaz (GST) Enzim Aktivite Analizi

Mantar örneklerinden hazırlanan etanollü özütlerin glutatyon-S-transferaz enzim aktivitesi üzerindeki etkilerini araştırmak amacıyla Habig tarafından geliştirilmiş yöntem kullanıldı [9]. Deney tüm mantar örnekleri için en az üçlü tekrarlar şeklinde gerçekleştirildi. Enzim aktiviteleri ise deneysel veriler kullanılarak aşağıdaki formülle hesaplandı.

$$EA(IU/ml) = (OD_{340}/dakika)\times(1/\varepsilon_{340})\times(seyreltme\ faktörü)$$

ε= Sönümleme Katsayısı CDNB için 9.60 $nM^{-1}.cm^{-1}$'dır.

Glutatyon Peroksidaz (GPx) Enzim Aktivite Analizi

Mantar özütlerinin GPx enzim aktivitesi üzerinde olan etkisi Paglia ve Valentine tarafından geliştirilmiş yöntem ile belirlendi. Enzim aktivitesi ise deneysel verilerin aşağıdaki formüle yerleştirilmesiyle hesaplandı [10].

$$EA\ (IU/ml) = (OD_{340}/dakika)\times(1/\ \varepsilon_{340})\times(seyreltme\ faktörü)$$

NADPH için 340 nm'deki sönümleme katsayısı (ε_{340}) 0.00622 $nM^{-1}.cm^{-1}$ olarak alınmıştır.

Katalaz (KAT) Enzim Aktivite Analizi

Mantar özütlerinin katalaz enzimi üzerinde etkilerini araştırmak için, bu enzimin hidrojen peroksiti suya dönüştüren reaksiyonun spektorofotometrik olarak 520 nm'de ölçüp, peroksit miktarındaki düşmeye bağlı olarak görülecek absorbsiyondaki düşüşün takibiyle gerçekleşti [11]. Enzim aktivitesi aşağıda sunulan formüle göre hesaplandı.

$$EA\ (IU/ml) = (\Delta H_2O_2/dakika)\times(seyreltme\ faktörü)/deney\ hacimi$$

SONUÇ VE TARTIŞMA

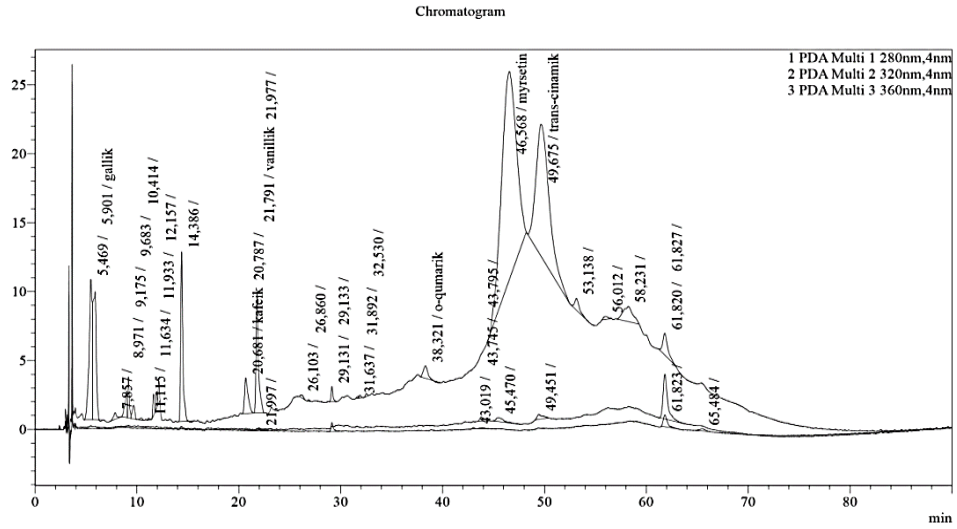
Biyoaktif Bileşiklerin Tayini

Elde edilen mantar özütlerin toplam fenolik madde içeriği, gallik asit grafiğinin standart eğrisinden elde edilen denklem kullanılarak hesaplandı ($y = 5.1718x - 0.0022$, $R^2 = 0.9983$). *L.*

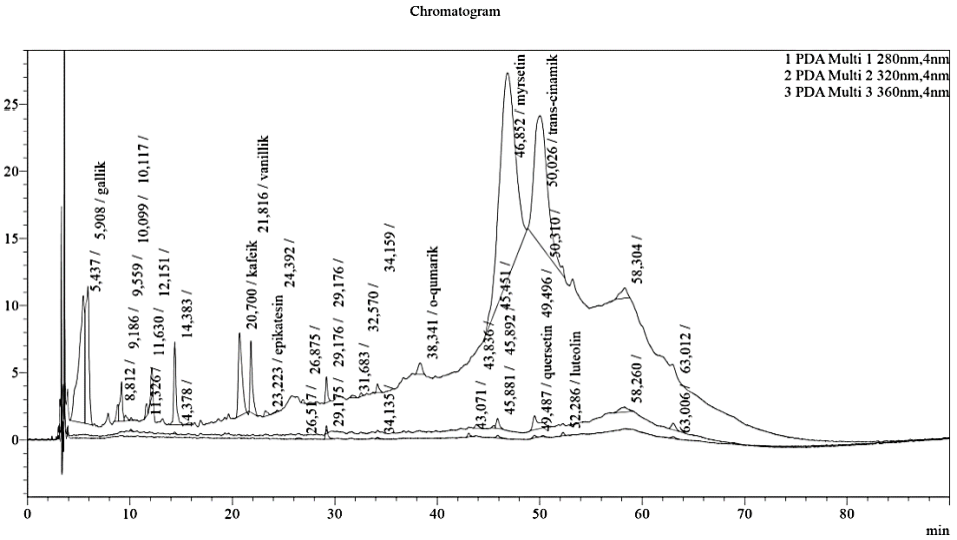
deliciosus ve *L. salmonicolor* özütlerinde bulunan toplam fenolik bileşik miktarları sırasıyla 6.281±0.0006 ve 8.615±0.0008 mg GAE/100 g kuru materyal olarak hesaplandı.

Total Flavonoit konsantrasyonu, kersetin grafiğinin standart eğrisinden elde edilen denklem kullanılarak hesaplandı (y= 4.24x+0.002, R^2 = 0.9997). *L. deliciosus* için toplam flavonoit miktarı 7.822±0.0041 mg QE/100 g kuru materyal ve *L. salmonicolor* için 2.303±0.0008 mg QE/100 g kuru materyal olarak hesaplandı.

Çalışma kapsamında mantar örneklerin etanollü özütlerinin fitokimyasal içerikleri YPSK yöntemi ile incelendi. Bu aşamada klorojenik asit, ferulik asit, kafeik asit, gallik asit, vanilik asit, kateşin, luteolin, apigenin, epikateşin, elajik asit, trans-sinnamik asit, *o*-kumarik asit, *p*-kumarik asit, mirsetin, kemferol ve kersetin standart biyoaktif bileşikler olarak kullanıldı. YPSK analiz sonucunda elde edilen mantar özütlerin fitokimyasal içerikleri Şekil 1 ve 2'de ve Tablo 1'de sunulmuştur. Bu analizler sonucunda aynı cinse ait iki farklı türün fenolik asit ve flavonoit profilleri belirlendi. Bu bağlamda, iki türün de baskın flavonoidinin mirsetin olduğu gözlemlendi. Ayrıca, aynı cinse ait olan her iki türde gallik asit, vanilik asit, *o*-kumarik asit, trans-sinamik asit, kafeik asit ve mirsetin bileşiklerinin benzer oranlarda bulunduğu gözlemlendi.



Şekil 1. *L. delicious*'un YPSK analiz kromatogramı



Şekil 2. *L. salmonicolor*'ın YPSK analiz kromatogramı

Tablo 1. Mantar özütlerin fitokimyasal içerikleri (*L. salmonicolor*, *L. delicious*)

mg/l	*L. salmonicolor*	*L. delicious*
Gallik asit	5.39	4.57
Vanilik asit	3.55	5.79
***o*-Kumarik asit**	0.55	0.58
Ferullik asit	-	-
Trans-sinamik asit	1.88	1.81
Elajik asit	-	-
***p*-Kumarik asit**	-	-
Klorojenik asit	-	-
Kafeik asit	4.07	2.26
Kateşin	-	-
Epikateşin	-	-
Mirsetin	12.08	11.36
Kuersetin	1.15	-
Kemferol	-	-
Apigenin	-	-
Luteolin	6.7	-

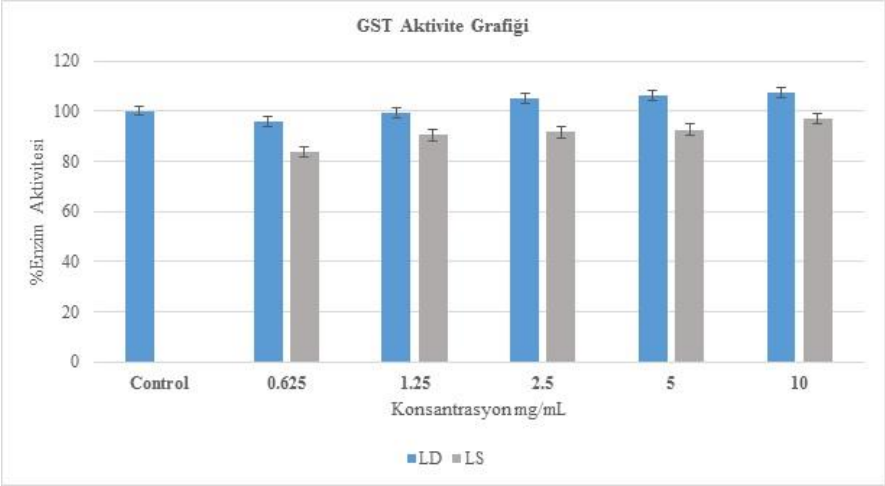
Antioksidan Aktivite

Bu çalışmada mantar özütlerinin serbest radikal temizleme kapasitelerini ölçmek amacıyla DPPH yöntemi kullanıldı. Öncelikle farklı konsantrasyonlarda hazırlanan özütlerin antioksidan etki gösteren yüzdesi hesaplandı ve DPPH konsantrasyonunu %50 oranında azaltan örnek miktarı mg/ml cinsinden belirlenerek, IC_{50} değeri analiz edildi. Pozitif kontrol olarak farklı konsantrasyonlardaki gallik asit ve kersetin çözeltisi standart olarak kullanıldı. Elde edilen sonuçlara göre, *L. delicious* ve *L. salmonicolor* özütleri serbest DPPH radikalini 10mg/ml dozda sırasıyla %78 ve %57 oranlarında temizledi. Ayrıca, her iki mantar türü için IC_{50} değeri sırasıyla, 0.906±0.0047 ve 1.088±0.0295 mg/ml olarak hesaplandı. Elde edilen verilere göre *L. delicious* mantarının antioksidan kapasitesinin *L. salmonicolor* mantarında göre daha yüksek olduğu görüldü.

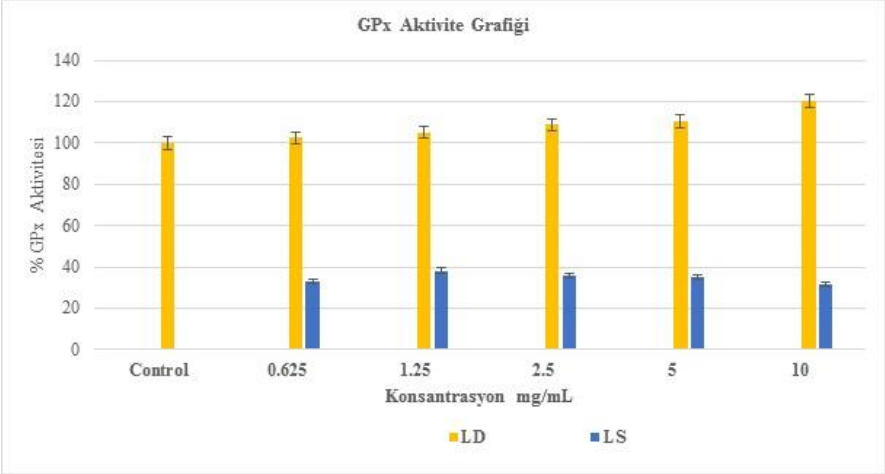
Mantarlardan elde edilen etanollü özütler sırasıyla, 0.625, 1.25, 2.5, 5 ve 10 mg/ml konsantrasyonlarında hazırlanarak GST enzimi üzerine etkileri araştırıldı. Elde edilen verilere göre, *L. salmonicolor*'ın etanollü özütünin tüm konsantrasyonlarda (0.625-10 mg/ml), GST enzim aktivitesini kontrole göre sırasıyla, %3 ile %17 oranında inhibe ettiği tespit edildi (Şekil 3). *L. delicious* mantar özütünün ise 2.5, 5 ve 10 mg/ml konsantrasyonlarda GST enzim aktivitesini kontrole göre sırasıyla, %4, %6 ve %7 oranında aktive ettiği gözlemlendi.

Mantarlardan elde edilen etanollü özütlerin konsantrasyonları sırasıyla 0.625, 1.25, 2.5, 5 ve 10 mg/ml olarak ayarlanarak, GPx enzimi üzerine etkileri araştırıldı. Elde edilen verilere göre *L. delicious*'den elde edilen etanollü özütünün tüm konsantrasyonlarda (0.625-10 mg/ml), GPx enzim aktivitesini kontrole göre %2 ile %20 arasında aktive ettiği tespit edildi. Bu çalışmada, *L. salmonicolor*'ın etanollü ekstresinin tüm konsantrasyonlarda GPx enzim aktivitesini kontrole göre % 65- %70 oranında inhibe ettiği görüldü (Şekil 4).

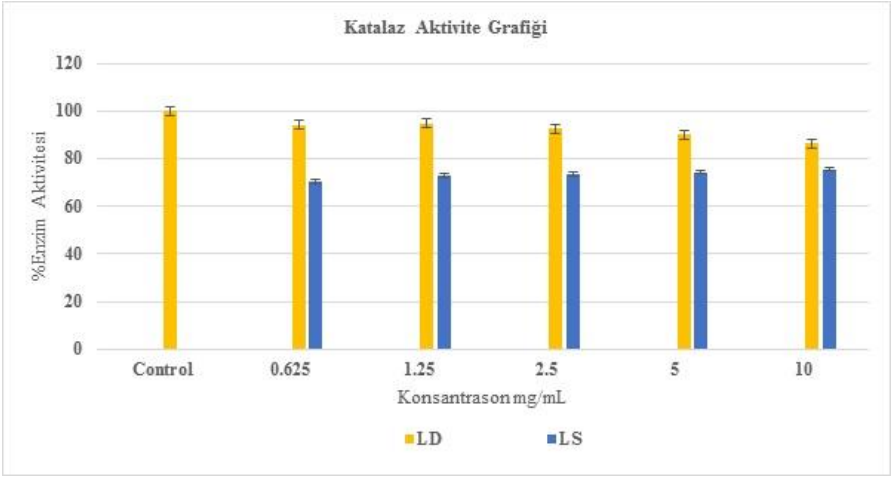
Ayrıca, etanollü mantar özütlerinin tüm konsantrasyonlarda (0.625-10 mg/ml) KAT enzimini inhibe ettiği gözlemlendi. *L. delicious*'un etanollü özütleri tüm konsantrasyonlarda katalaz enzimini %6 ile %14 arasında inhibe ederken, *L. salmonicolor*'ın özütlerinin tüm konsantrasyonlarda KAT enzimini % 30-% 25 oranında inhibe ettiği gözlemlendi (Şekil 5).



Şekil 3. Mantar özütlerinin glutatyon-S-transferaz enzim aktivitesi üzerine etkileri (*L.delicious* (mavi), *L. salmonicolor* (gri)



Şekil 4. Mantar özütlerinin glutatyon peroksidaz enzim aktivitesi üzerine olan etkisi (*L. delicious* (sarı), *L. salmonicolor* (mavi)



Şekil 5. Mantar özütlerinin katalaz enzim aktivitesi üzerine olan etkisi (*L. delicious* (sarı), *L. salmonicolor* (mavi)

Yapılan araştırmalarda fenolik bileşiklerin, özellikle de fenolik asitlerin, mantarların antioksidan aktivitelerinden sorumlu fitokimyasalları arasında ilk sırada yer aldığı konusunda fikir birliği bulunmaktadır [12]. Bunun nedenleri arasında bu yapıların bir veya daha fazla aromatik halka ile, bir veya daha fazla hidroksil (-OH) grubu içermesi yer almaktadır. Bu özellikleri sayesinde serbest radikalleri temizleme potansiyeline sahiptirler [13,14]. Bu çalışmada, YPSK analizinden elde edilen verilere göre *L. delicious* ve *L. salmonicolor* mantarların etanollü özütlerinin yüksek oranda mirsetin ve trans-sinnamik asit bileşiklerini içerdikleri gösterilmiştir. Ayrıca, bu iki bileşik dışında gallik asit, vanilik asit ve kafeik asit gibi fenolik asitleri de bünyelerinde bulundurduğu belirlenmiştir. Çalışmanın sonuçlarına bakıldığında *L. deliciosus* mantarının yüksek antioksidan aktivitesinin (%78), içerdiği bu fenolik bileşiklerin ve flavonoitlerinden kaynaklandığı düşünülmektedir. *L. delicious* mantarının etanollü özütü yüksek oranda mirsetin içermektedir. Mirsetin, bitki kaynaklı yaygın bir flavonoit olup nutrasötik değeri ile de tanınmaktadır. Bu madde çeşitli yiyecek ve içeceklerin temel bileşenlerinden biridir. Bilimsel çalışmalar bu bileşiğin, güçlü antioksidan, antikanser, antidiyabetik ve antienflamatuvar özelliğinin bulunduğunu göstermektedir [15]. Ayrıca, bu çalışmada elde edilen sonuçlar *L. delicious* mantarının etanollü özütünün yine fenolik asitler içerisnde yer alan gallik asit içerdiğini de ortaya koymaktadır. Bir çok bilimsel çalışmadan elde edilen sonuçlar, gallik asitin antibakteriyel, antifungal, antiviral, antienflamatuvar, antioksidan, antikanser ve antidiyabetik özelliği bulunduğunu de bildirmektedir [12].

Ökaryotik ve prokaryotik canlılarda, GST enziminin detoksifikasyon amacıyla indirgenmiş glutatyon (GSH) formunun ksenobiyotik substratlara konjugasyonunu katalize ettiği ve bu şekilde toksik maddelerin organizmadan uzaklaştırılmasını sağladığı (detoksifikasyon) bilinmektedir [16]. Enzim bu özelliği ile kemoterapi alan hastalarda çoğu zaman ilaç direncine sebep olmaktadır. Tüm bu bilgilere dayanarak bu çalışmada *L. delicious* mantarının GST enzim aktivitesinde artışa neden olduğu gösterilmiştir. Bu durumun tam tersi olarak, *L. salmonicolor* mantarının etanollü özütü GST enzim aktivitesini tüm dozlarda kontrole göre inhibe etmiştir. Dolayısıyla, normal şartlarda sağlıklı bir bireyin *L. delicious* mantarını tüketmesinin detoksifikasyon açısından enzim aktivitesine katkı sağlayabileceği düşünülmektedir. Ancak, *L. salmonicolor* mantarının ise GST enzimi üzerinde gösterdiği inhibisyon etkisi dolayısıyla kemoterapi alan hastalar tarafından gıda takviyesi olarak tüketilmesi, GST kaynaklı ilaç direncini ortadan kaldırması bakımından faydalı olabileceği düşünülmektedir.

GPx enzimi, redüklenmiş glutatyon (GSH) ile hidrojen peroksit molekülünün reaksiyonunu katalize edip, hidrojen peroksit molekülünün suya dönüşümünü sağlamaktadır. Bu durum, hücre membranı ve hücre organellerini oksidatif strese karşı korumaktadır. Bu çalışmada elde edilen sonuçlar, *L. delicious* mantarının etanollü özütünün tüm dozlarda GPx enzimini aktive ettiğini, dolayısıyla, *L. delicious* 'un etkili bir antioksidan molekül kaynağı olduğu düşüncesini desteklemektedir. Bu nedenle antioksidan savunma sistemini güçlendirmek amacıyla bu mantarın gıda takviyesi olarak kullanılmasının etkili olacağı düşünülmektedir.

Katalaz enzimi hidrojen peroksit molekülünden üretilen serbest radikallerin oluşumunu engeller. Bazı kanser tedavilerinde, kemoterapi ilaçları kanser tümörlerini hücre ölümüne götürebilmek için ortamda serbest radikal oluşumunu indükleyebilmektedir. Ancak antioksidan savunma sisteminde yer alan bazı enzimler, özellikle katalaz, oluşan bu serbest radikalleri temizleyerek ilaç direncine neden olabilmektedir [17]. Bu çalışmada elde edilen veriler, her iki mantar özütünün tüm dozlarda katalaz enzimini inhibe ettiğini göstermiştir. Dolayısıyla, kemoterapi alan hastaların bu mantarlarıgıda takviyesi olarak kullanımlarının ilaç direncine karşı faydalı olabileceğini akla getirmektedir.

Çalışmalar, *L. delicious*'un yüksek oranda stearik asit (doymuş yağ asidi), linoleik asit ve oleik asit (doymamış yağ asiti) içerdiğini göstermektedir. Ayrıca, *L. delicious*'un meyve kısımından elde edilen özütler yüksek oranda polifenolik bileşik ve mannitol içermektedir [18]. Kosaniç ve arkadaşları 2016 yılında [19], *L. delicious* mantarının metanollü özütünün içeriğini araştırmışlar ve yüksek oranda demir (Fe) ve çinko (Zn) metalleri içerdiğini ayrıca askorbik asite kıyasla daha güçlü antioksidan özelliği olduğunu göstermişler. Bununla birlikte yapılan diğer araştırmalarda, yüksek oranda kobalt (Co), kadmiyum (Cd), nikel (Ni) ve kurşun (Pb), ergosterol içerdiğini ve aynı zamanda çok güçlü serbest radikal temizleme özelliğine ve metal şelatlama potansiyeline sahip olduğunu göstermiştir [20,21]. Bir grup araştırmacı, halk arasında Kanlıca mantarı olarak bilinen *L. salmonicolor*'ın, yüksek oranda p-hidroksibenzoik asit içerdiğini tespit etmiştir ve antioksidan potansiyele sahip olduğunu belirtmiştir

[22]. Yaptığımız bu çalışmada, Karadeniz bölgesinden toplanmış olan *L. salmonicolor* mantarının yapılan yönteme göre etanollü özütünün ölçülebilen antioksidan aktivitesinin yaklaşık olarak %57 oranında olduğu hesaplanmıştır. Bu açıdan tıbbi değeri yüksek bir besin kaynağı olarak da değerlendirilebilir.

Gıda olarak tüketilen mantarların, zengin fitokimyasal içerikleri, düşük oranda şeker ve yağ içermeleri ve özellikle iyi birer diyet ürünü olmaları nedeni ile ideal bir gıda niteliği taşıdığı bilinmektedir. Ayrıca yenebilen mantarlar, protein ve mineral içeriğinden dolayı kalp ve damar hastalığı için iyi bir besin kaynağı olarak tanınmaktadır. Ayrıca, bu tür mantarlar antibakteriyal, antifungal, antiparazitik, detoksifikasyon ve antidiyabetik özellikleri nedeni ile önem arz etmektedir. Bu çalışmada, yenebilen mantar olarak bilinen *L. delicious* ve *L. salmonicolor*'ın kullanılan yönteme göre elde edilen etanollü özütlerinin biyolojik aktiviteleri ve fitokimyasal içerikleri incelenmiştir. Bulgular, her iki mantarın da yüksek seviyede biyolojik olarak aktif birçok molekül içerdiğini göstermiştir. Ayrıca, *L. delicious* mantarının GST ve GPx enzimlerini kontrol enzimine göre iyi derecede aktif olması ve yüksek oranda serbest radikal temizleme özelliği, bu mantarın güçlü antioksidan etki gösterme özelliğine sahip olduğunu açıklamaktadır. Dolayısıyla, bu mantarın tıbbi değeri yüksek besin kaynağı ya da gıda takviyesi olarak kullanılması antioksidan savunma sistemini destekleme açısından faydalı olabileceği düşünülmektedir.

TEŞEKKÜR

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Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTI-ARTHRITIC ACTIVITY OF *SANGUISORBA MINOR* SUBSP. *BALEARICA* AGAINST FREUND'S COMPLETE ADJUVANT-INDUCED ARTHRITIS IN RATS

SANGUISORBA MINOR SUBSP. BALEARICA'NIN FREUND'S COMPLETE ADJUVAN İLE OLUŞTURULMUŞ ARTRİTLİ RAT MODELİNDE ANTİ-ARTRİTİK AKTİVİTESİ

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ABSTRACT

Objective: *Therapeutic properties of Sanguisorba L. genus plants are supported by numerous in vivo and in vitro studies showing the anti-viral, anti-ulcerogenic, anti-cancer, anti-acetylcholinesterase, radioprotective, anti-allergic, and anti-inflammatory activities. The present study was designed to investigate anti-arthritic activity of the "Sanguisorba minor subsp. balearica (Bourg. ex Nyman) Muñoz Garm. & C.Navarro" (Smb), a subspecies of Sanguisorba L. genus, on Complete Freund's Adjuvant induced (CFA-induced) arthritic rat model.*
Material and Method: *The extract of aerial parts of the Smb was obtained by the consecutive steps of maceration process. The phytochemical content of the extract was analyzed by the High-Performance Liquid Chromatography (HPLC) method. Complete Freund's Adjuvant-induced arthritic rats were used to examine the anti-arthritic activity of the plant extract.*

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Result and Discussion: *HPLC analysis revealed a high amount of phenolic compounds in the Smb extract. The identified phenolics are ellagic acid, gallic acid, quercetin dehydrate and p-coumaric acid with the concentrations of 4.1288; 2.6342; 0.0871 and 0.0633 ppm, respectively. Injection of Smb extract caused a decrease in paw thickness in the CFA-induced arthritic animals. The decrease in paw thicknesses of animals treated by quercetin and Smb 70 mg/kg groups was higher than diclofenac sodium group. Smb 34 mg/kg group also showed* a *decrease in paw thickness, but it was lower compared to quercetin and Smb 70 mg/kg groups and higher than the diclofenac sodium group. According to the histopathological evaluations of the joint tissues, both 34 mg/kg and 70 mg/kg Smb extract treatment were improved the inflammatory deformations related to arthritis on the rats.*
Keywords: *Anti-arthritic activity, arthritis, Complete Freund's Adjuvant (CFA), histopathology, Sanguisorba minor subsp. balearica*

ÖZ

Amaç: *Sanguisorba L. cinsine ait bitkilerin tedavi edici özellikleri pek çok in vivo ve in vitro çalışma ile araştırılmış olup; anti-viral, anti-ülserojenik, anti-kanser, anti-asetilkolinesteraz, radyasyon koruyucu, anti-alerjik ve anti-enflamatuvar etkileri gösterilmiştir. Çalışmamız, Sanguisorba L. cinsine ait bir alt tür olan "Sanguisorba minor subsp. balearica (Bourg. ex Nyman) Muñoz Garm. & C.Navarro" (Smb) bitkisinin artrit modeli oluşturulmuş ratlarda anti-artritik etkisini araştırmayı amaçlamaktadır.*
Gereç ve Yöntem: *Smb'nin toprak üstü kısımlarının ekstresi birbirini takip eden aşamalardan oluşan maserasyon yöntemi ile elde edilmiştir. Ekstrenin fitokimyasal içeriği Yüksek Performanslı Sıvı Kromatografisi (YPSK) metodu kullanılarak tayin edilmiştir. Anti-artritik etkinliğin araştırılması için Freund's Complete Adjuvan (CFA) ile indüklenerek oluşturulmuş artritli ratlar kullanılmıştır.*
Sonuç ve Tartışma: *YPSK analiz sonuçları Smb ekstresinin yüksek miktardaki fenolik içeriğini ortaya koymuştur. Tanımlanan fenolikler şu şekildedir;* elajik asit , gallik asit, kuversetin dehidrat *ve p-kumarik asittir ve tespit edilen miktarları sırasıyla 4.1288;2.6342; 0.0871 and 0.0633 ppm'dir. Bitki ekstresi uygulanması CFA ile indüklenen artritli ratların ayak bölgelerindeki enflamasyonda bir azalmaya sebep olmuştur. Enflamasyon miktarındaki en yüksek baskılanma kuversetin ve Smb 70 mg/kg uygulanan grupta tespit edilmiştir ve bu etki diklofenak uygulanan gruptan daha yüksektir. Smb 34 mg/kg uygulanan grupta enflamasyon yine diklofenak uygulanan gruptan daha yüksek oranda baskılanmıştır. Ayak eklemlerinden alınan mikro kesitlere yapılan histopatolojik analizlere göre hem 34 mg. hem de 70 mg. Smb ekstresi artrite bağlı enflamatuvar deformasyonu iyileştirmiştir.*
Anahtar Kelimeler: *Anti-artritik aktivite, artrit, Complete Freund's Adjuvant (CFA), histopatoloji, Sanguisorba minor subsp. balearica*

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by symmetrical synovitis in large and small joints, leading a progressive functional destruction of synovial, cartilage and bone. The disease affects about 1-2% of the population and its incidence increases with age and gender, women being affected three times more than men [1,2]. Final outcome of uncontrolled rheumatoid arthritis is the impairment of articular cartilage, bone deformity, disability of joint function and pain. It has a negative impact on life quality of the patients and increase their morbidity rate [3,4]. The ultimate goal of RA treatment is to heal or minimize joint damage, relieve pain and maintain normal joint functions. A panel of drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease modifying anti-rheumatoid arthritic drugs (DMARDs) have been used to treat the symptoms of RA. However, all these drugs have several undesired effects. Recent researches aim to discover new therapeutical agents which have high safety profile and low adverse effects for the treatment of arthritis [5-7]. In this context, natural products have an increasing interest for therapeutical purposes. A well known medicinal plant from Turkey, *Sanguisorba minor* subsp. *balearica* was chosen for the current study with its therapeutic properties and biologically active phytoconstituents potential.

"Sanguisorba minor subsp. *balearica* (Bourg. ex Nyman) Muñoz Garm. & C.Navarro" is a subspecies of *Sanguisorba minor* genus and belonging to the Rosaceae family. It is an edible, perennial herb with pinnate leaves and it is famous with their reddish-green flowers. It is widely distributed throughout West Asia, North America and Europe [9,10]. In Turkey, it is known as a medicinal plant and intensively used for ethnobotanical purposes. Based on its location, it is known with many different names [11,12]. Ethnobotanical use and traditional names of the members of *Sanguisorba minor* genus plants are given in Table 1.

Table 1. Ethnobotanical use of *Sanguisorba minor* plants

Botanical name	Local name	Plant part used	Traditional use	Reference
Sanguisorba minor scop. subsp.muricata (spach)Briq.	Otukesme otu	Aerial parts	Skin diseases/eczema	[13]
Sanguisorba minor subsp. magnolii	Bostan güzeli	Aerial parts	Urinary system diseases	[14]
Sanguisorba minor subsp. minor	Küçük çayır düğmesi	Aerial parts	Constipation/Gastrointestinal disorders	[15]
Sanguisorba minor scop. muricata	Kelek ayağı	Leaves	Hypothyroidism/ Hyperthyroidism	[16]

Apart from its traditional use, *Sanguisorba* species were reported with their anti-viral [17,18], anti-ulcerogenic [19], anticancer [20,21], anti-acetylcholinesterase [22], radioprotective [23], immunomodulatory [24,25], anti-allergic [26], and anti-arthritic [10,27] properties. Among these therapeutic properties, ability to relieve inflammation is important. It was known that inflammation plays a central role in the pathogenesis of many diseases such as cancer, diabetes, obesity, cardiovascular diseases and rheumatoid arthritis. For this reason, inflammation and inflammation-based disorders are areas of interest and studied extensively. Limited number of studies were carried out on the anti-inflammatory effect of the *Sanguisorba* genus. In a case study, it was revealed that the ethanolic extract of *Sanguisorba officinalis* exerts inhibitory effects on Prostaglandin E (PGE) production and suggests a potent anti-inflammatory activity mediated by Nuclear factor kappa-B (NF-κB) and Activator protein 1 (AP-1) inhibitory properties [28]. The anti-inflammatory effect of ethanolic extract of *Sanguisorba officinalis* L. on skin disorders was tested in human keratinocyte HaCaT cells, and it was reported that ethanolic extract of *Sanguisorba officinalis* L. exerts anti-inflammatory effect by suppressing the expression of Tumour necrosis factor-alpha/ Interferon- γ (TNF-α/IFN-γ) stimulated chemokines and pro-inflammatory molecules in human keratinocyte HaCaT cell lines [29]. In view of its potent anti-inflammatory activity, the present study was designed to evaluate the anti-arthritic activity of "*Sanguisorba minor* subsp. *balearica" (Smb)*. Therefore, the present study was aimed to evaluate the therapeutic effect of *Smb* in rats with experimental arthritis model induced by Complete Freund's adjuvant (CFA). CFA-induced arthritis (AA) in rats has been used as an animal model for rheumatoid arthritis in the development of new therapeutic approaches. Because, rats exhibit a systemic inflammatory disease with similar bone and cartilage alterations to those observed in rheumatoid arthritis in human.

MATERIAL AND METHOD

Plant Material

Plant samples were collected from the locality of Kayseri, Pınarbaşı (Turkey), between "Eğrisöğüt village and Aşağı Beyçayır village Kumuk Ali Çeşmesi" at an altitude of 1750 meter in July 2017.

Voucher specimens are kept in AEF (Herbarium of Ankara University Faculty of Pharmacy) with the herbarium number of AEF 26985.

Preparation of The Plant Extracts

Aerial parts of the air-dried plant material (50 g) were powdered and subjected to maceration process in sterile distilled water for 24 hours by using a mechanical shaker (Heidolph Instruments) at 300 rpm at room temperature. Then, extract was filtered through Whatman filter paper, lyophilized (Christ Gamma 2-16 LSC), and weighed. Final yield of extraction was calculated as 15% for *Smb* weight/weight and plant extract was stored at -20ºC in the absence of oxygen.

HPLC Analysis

HPLC analysis were performed with Agilent 1200 LC series under the standardized conditions. Plant extract was filtered through 0.22 µm membrane filters and pass through a C18 HPLC column (4.6 mm x 25 cm and 5µm particle size). Flow rate was arranged as 0.3 ml/min with the 13 minutes gradual mobile phase flow, and the sample injection volume was 5 µl. Spectra were monitored between 200 – 500 nm.

Animal Model

Adult "Sprague- Dawley" male rats weighing 200-250 gram were used in this study. Animals were housed under standard conditions (12 hours light, 12-hour dark cycle; 27 ± 3℃ and 35-50 % humidity) and fed with a standard pellet diet. All animal experiments were carried out in accordance with the "National Institutes of Health Guide for Care and Use of Laboratory Animals" procedures. Complete Freund's Adjuvant (CFA) is applied as an immunization stimulant reagent and commonly used in many experimental models to mimic chronic inflammatory diseases. CFA-induced arthritis is a scientifically justified experimental model for rodents [30-34].

Induction of Arthritis and Treatment Protocol for Animals

Experimental animal groups were randomly assorted into six groups before the onset of "Complete Freund's Adjuvant" (CFA) injection. Groups were named according to their treated reagents as Group 1 (healthy control), Group 2 (negative control), Group 3 (diclofenac sodium treated), Group 4 (quercetin treated), Group 5 (*Smb* 34 mg/kg treated) and Group 6 (*Smb* 70 mg/kg treated) and each group comprised of six animals.

Except healthy control group, CFA reagent was injected on the left hind paw of the rats intraplantarly. After CFA injection, when the paw thicknesses were reached at the stable level in all experimental groups (on day 7) each group was treated with its specific reagent.

Dosages of the therapeutical reagents were adjusted by the reference to similar studies performed under the same experimental conditions. Diclofenac sodium is used as a member of non-selective COX inhibitor in this study for reducing the signs of the arthritis (positive control) [10,35-38]. The details of the animal treatments were given in Table 2.

Table 2. Experimental treatment protocol of animal groups

Group	Treatment
Group 1	Healthy control
Group 2	Negative control (CFA)
Group 3	CFA+Diclofenac* (5mg/kg)
Group 4	CFA+Quercetin* (25mg/kg)
Group 5	CFA+*Smb** (34mg/kg)
Group 6	CFA+*Smb** (70mg/kg)

*Reagents were administered daily orally from day 8 to 28.

Arthritic Score and Assessment of Paw Thickness

Basal paw volumes of all the animals were measured by plethysmometer just before CFA injection on day 0. After CFA injection, hind paw measurements were taken at different intervals till day 28. Morphological features of the arthritis were monitored by a set a visual scoring on a scale of 0-4 which refers to the clinical signs and symptoms of the arthritis, where 0: no change, 1: slight swelling and edema of the paw, 2: mild swelling and edema of the paw and/or limb, 3: severe swelling and edema of the paw and/or limb, 4: deformity and inability of paw and/or limb [35-37]. Inhibition degree of paw thickness as percentage was calculated as follows: "(1-T0/Tt) ×100, where T0 is the mean of paw thickness at day 0 and Tt is the mean of paw thickness at a particular time".

Histopathological Examinations of Joint Tissues

Lesioned hind paw tissues were cut at the metacarpal joint and fixed in 10% buffered formalin for 48-72 hours. Fixed tissues were decalcified for 72 hours in the decalcification solution. Then tissue samples were dehydrated through graded alcohol series. Finally, processed tissues were embedded in paraffin at 56°C- 58°C. Three sections of 4-5 µm thickness were taken from the prepared paraffin blocks, each of them was stained with haematoxylin-eosin (HE) and then evaluated histopathologically under the light microscope. Microphotographs were taken with focusing on the synovium, cartilage, and joint space.

Mean Histopathological Scores of Experimental Groups

Histopathologically examined and stained tissues were evaluated by a visual scoring system on a scale of 0-5 in terms of the severity of pathological damage of tissues [38,39]. Scoring were done according to four parameters which are; edema, inflammation, bleeding and necrosis.

Statistical Analysis

All analyzes were performed in triplicate and the average of measurements were calculated. Statistical analyses were calculated with GraphPad Prism version 9.1 program and One-Way ANOVA and Tukey post-hoc tests were used. The results obtained were averaged with "Standard Error of Means (SEM)" and the probability between $p<0.05$ and $p<0.005$ was considered statistically significant.

RESULT AND DISCUSSION

Total phenolic content of the plant extract were analyzed by HPLC method using a total of 7 reference standarts including coumarin, p-coumaric acid, gallic acid, kaempferol, catechin hydrate, ellagic acid and quercetin dihydrate. A standard curve was drawn based on the serial dilutions of reference standarts. Standart mixture includes 1 ppm of each phenolics. The HPLC chromatogram profile of the *Smb* extract were shown in Figure 1.

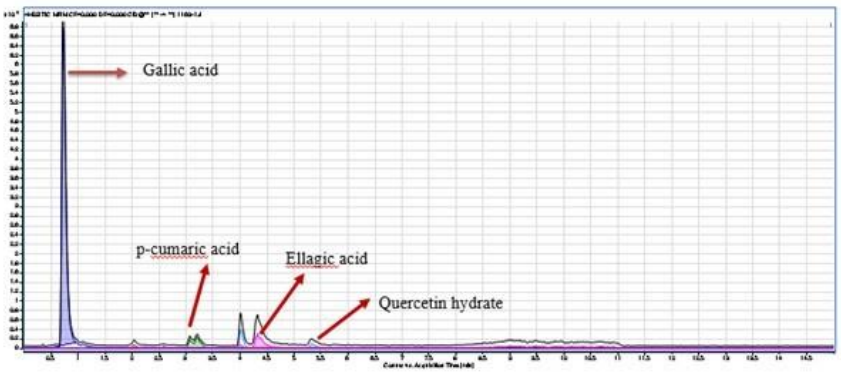

Figure 1. HPLC chromatogram profile of Smb extract. Solid line represents standard mixture. Purple area represents gallic acid, green area represents p-cumaric acid, pink area represents ellagic acid and blue area represents quercetin hydrate

The HPLC analysis of the plant extract showed that phenolic compounds were included 4.1288 ppm of ellagic acid, 2.6342 ppm of gallic acid, 0.0871 ppm of quercetin hydrate and 0.0633 ppm of p-coumaric acid. In addition to these phenolic compounds, low amount of catechin hydrate, coumarin and kaempferol (less than ≤ 0.005 ppm.). Quantitative results of the phenolic compounds of the *Smb* extract were given in Table 3.

Table 3. Quantitative phenolic compounds of *Smb* extract determined by HPLC

Plant Extract	Ellagic acid (ppm)	Gallic acid (ppm)	Quercetin hydrate (ppm)	p-coumaric acid (ppm)	Coumarin, catechin hydrate and kaempferol (ppm)
Sanguisorba minor subsp.balearica	4.1288	2.6342	0.0871	0.0633	≤ 0.005

Animal paw thicknesses were measured regularly by plethysmometer after CFA injection to obtain similar inflammation degree on the injection side. Measurements of the characteristic signs of rheumatoid arthritis on animal's paws is more accurate and well accepted method. It enables to express the effects of the treatments quantitatively on paw inflammation of the animals [41]. According to the measurements, paw thicknesses were reached at the stable level on day 7 in all experimental groups as shown in Figure 2.

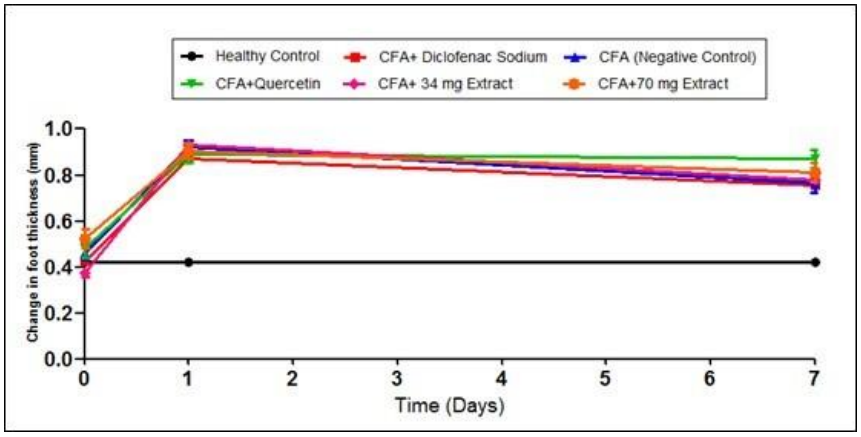

Figure 2. Animal paw thicknesses over a time period after CFA injection

Moreover, animal paw thicknesses were measured at regular intervals during the subacute and chronic phases between day 7 and day 28 of the arthritis. Treatment of animals with the plant extract and quercetin as well as diclofenac sodium caused a decrease in paw thicknesses. On day 10, 18, 24 and 28, the decrease in paw thicknesses of all the experimental groups diclofenac sodium, quercetin, "34 mg/kg *Smb"* and "70 mg/kg *Smb"* were significant when compared to negative control (CFA) group. In addition, "*Smb* 70 mg/kg" and quercetin groups had the highest decrease in paw thickness compared to diclofenac sodium and "*Smb* 34 mg/kg" groups as shown in Figure 3 and Table 4.

In order to express the differences between the groups more clearly, change in the paw thicknesses were calculated as " % decrease in paw thickness = (1-T0/Tt) ×100 ", where T0 is the mean of paw thickness at day 0 and Tt is the mean of paw thickness at a particular time. The highest decrease of paw thickness was calculated in quercetin and "*Smb* 70 mg/kg" groups as 70.08% and 61.23%, respectively, which showed higher decrease in paw thickness compared to the diclofenac sodium group having 49% of decrease. Moreover, "*Smb* 34 mg/kg" group had more decrease in paw thickness with 56% compared to the diclofenac sodium group. The inhibition degree of paw thickness in all experimental groups were given in Figure 4.

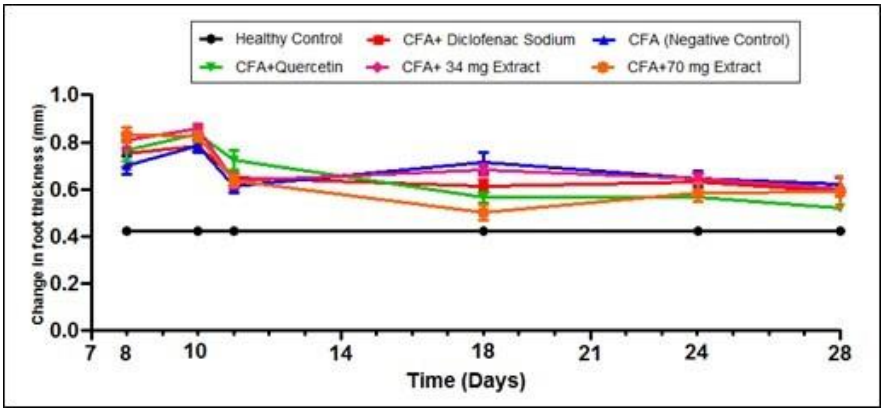

Figure 3. Change in paw thicknesses in subacute and chronic phases after the treatments

Table 4. Assessment of paw thicknesses after the treatment of animals

Groups	Treatment	Paw thickness (mm)				
		Day 7	Day 10	Day 18	Day 24	Day 28
1	Healthy Control	0.42±0.006	0.42±0.006	0.42±0.06	0.42±0.006	0.42±0.006
2	CFA Control	0.70±0.040	0.78±0.028	0.71±0.043	0.64±0.032	0.62±0.026
3	Diclofenac (5mg/kg)	0.75±0.055	0.78±0.026	0.61±0.026	0.63±0.033	0.59±0.019
4	Quercetin (25mg/kg)	0.76±0.049	0.83±0.033	0.56±0.021	0.56±0.018	0.52±0.017
5	*Smb* 34mg/kg	0.808±0.033	0.85±0.020	0.68±0.024	0.64±0.024	0.60±0.011
6	*Smb* 70mg/kg	0.832±0.033	0.82±0.019	0.50±0.034	0.58±0.039	0.58±0.066

*Values expressed as Mean ± SEM, n=6

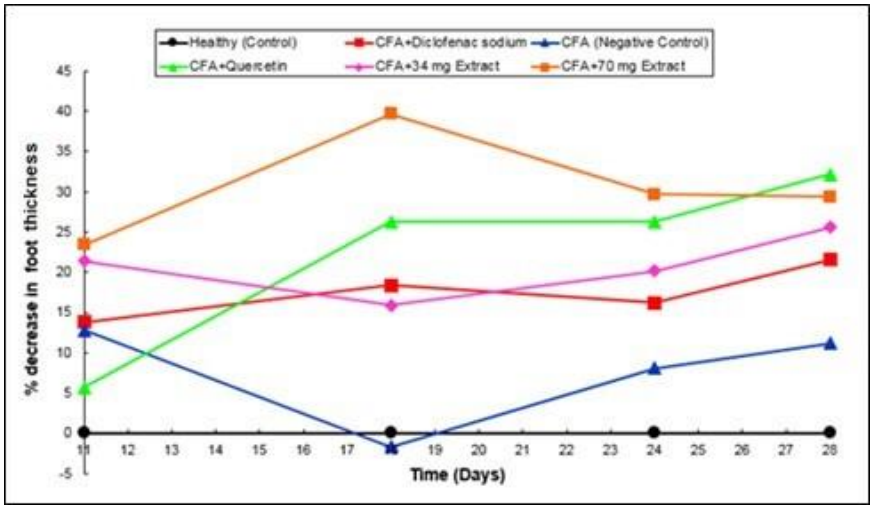

Figure 4. Change in paw thickness between the groups as percent compared to CFA control from day 11 till day 28.

Histopathological examination of joint tissues of animals enables the detection of prominent morphological disorders due to rheumatoid arthritis. Histopathological changes were evaluated basically on three parameters, including cartilage and/or bone destruction, inflammation, and appearance of the joint space. Microsections of joint tissues of the healthy control group animals had shown intact articular cartilage and normal joint space without inflammation. In CFA group (negative control), damage of the articular cartilage and narrowing of joint space with the severe inflammation were seen. Treatment of rats with diclofenac sodium has a slight recovery effect on arthritic signs. Multifocal bordered inflammatory areas were observed. There were varying degrees of decrease in inflammation and recovery between the joint spaces in animal groups treated with *Smb* at both "34 mg/kg and 70 mg/kg" doses. The improvement of histological appearance produced in *Smb* groups was better compared to the diclofenac group. Quercetin treatment of rats produced the most significant improvement in all arthritic parameters compared to all other groups (Figure 5 and Figure 6).

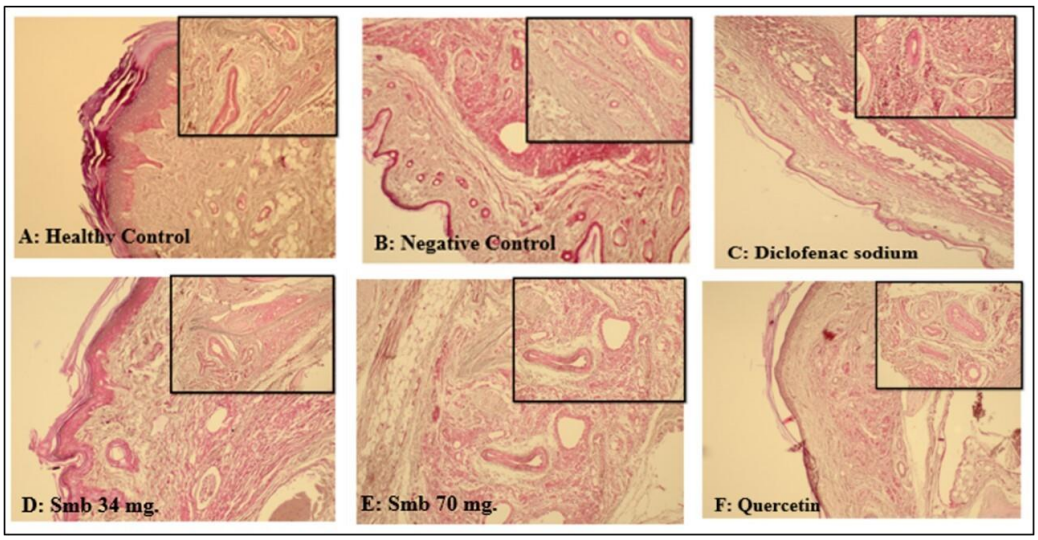

Figure 5. Microphotographs showing the histopathology in adjuvant-induced arthritic rats for six treatment groups (light microscopy 200X)

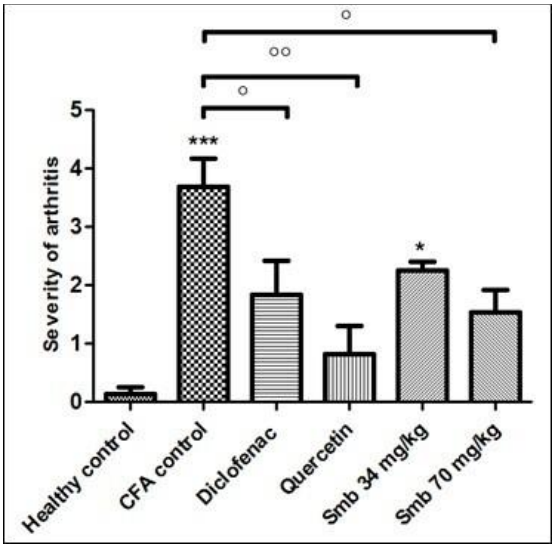

Figure 6. Mean histopathological scores of experimental groups. Values expressed as Mean ± SEM, n = 6, * p <0.05 34 mg *Smb* and *** p< 0.005 negative control compared to healthy control. ° p <0.05 positive control and 70 mg *Smb*, °° p <0.01 quercetin compared to the negative control

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects the joints and characterized by inflammation, swelling, deformity, pain and malfunction of the symmetrical joints. In the present study, CFA-induced arthritic rats were used as an experimental model which have close similarities to human RA disease for clinical and histopathological features. "*Sanguisorba minor* subsp. *balearica (Smb)"* belongs to the "*Sanguisorba"* genus in the family "Rosaceae", known as a medicinal plant and a number of studies have been carried out in the literature belonging to this genus. These studies demonstrated their high quantity of phytochemical content and also their therapeutical properties. *Sanguisorba minor* plant is mentioned as a "Promising Medicinal Plant" due to its high amount of bioactive compound [40]. More than 120 phytochemical compounds belonging to *Sanguisorba* genus plants, especially *S. officinalis* and *S. minor*, have been identified and major phytochemical compounds of plant extract have been described. These identified phytochemicals grouped as phenolics, flavonoids, neolignans and terpenoids [21,41-43]. It is emphasized that the therapeutical properties of the *Sanguisorba* species, were largely due to their polyphenolic and flavonoid content [26,42,44]. Ranfa et al., reported that *Sanguisorba minor* exhibited the highest total polyphenolic content among the other *Sanguisorba* genus [17]. It has also been shown that there is a relationship between flavonoid content of the plant and its anti-inflammatory effects [27,28,45,46]. Our HPLC results obtained from *Smb* water extract were in a correlation with the results from the literature. HPLC data revealed that, *Smb* extract composed of high amount of phenolic content (ellagic acid, gallic acid and coumaric acid) as well as flavonoid (quercetin) content. Hence, in this study we hypothesized that *Sanguisorba minor subsp. balearica*, which have high phenolic and flavonoid content, more likely to have anti-inflammatory effect on CFA- induced arthritic rats and we tested this possible effects both local and systemic aspects. Measurements of the characteristic signs of rheumatoid arthritis on animal's paws is more accurate and well accepted method. It enables to express the effects of the treatments quantitatively on paw inflammation of the animals [47]. Therefore, in the present study, paw thickness measurement of rats was used as an index that refers the anti-arthritic activity of *Smb* at the doses of "34 mg/kg and 70 mg/kg". The decrease in paw thicknesses in all experimental groups between day 10 to 28 were found to be significant when compared to negative control (CFA). "*Smb* 70 mg/kg" and quercetin groups have similar and the highest decrease in paw thickness compared to diclofenac and *Smb* 34 mg/kg groups. These results point out that, treatment with the *Smb* extract (both 34 mg/kg and 70 mg/kg) have an ameliorating effect on the paw inflammation in CFA-induced arthritic rats and this effect is better than the diclofenac sodium (member of non-selective COX inhibitor) treatment. All these results revealed that *Smb* extract with both the doses have a strong therapeutic effect compared to diclofenac sodium. Histopathological analysis provides important informations about the morphological changes and pathological signs of rheumatoid arthritis (RA) on the joint tissues. In the current study, histopathological analysis showed that *Smb* extract with both doses "34 mg/kg and 70 mg/kg" had a reducing effects on the severity of histopathological parameters including cartilage and/or bone destruction, inflammation, and appearance of the joint space as compared to negative control (CFA) group. This effect is also comparable with diclofenac and quercetin treated groups. Moreover, quercetin has the best-reducing effect in histopathological parameters among all treatment groups. All these results were found to be statistically significant.

In conclusion, supressing effect of *Sanguisorba minor subsp. balearica* extract on joint inflammation and destruction in CFA-induced arthritic rats were verified in the current study for the first time. Our data in paw thickness of animals and histopathological parameters provide important information for the anti-arthritic activity of *Smb* extract. The results that we have obtained are compatible with other studies [30-35,48]. RA have been treated with many different therapeutic approaches such as use of "prostaglandin inhibitors", "glucocorticoids", and "COX-2 inhibitors". Although these agents having good therapeutical effects, but they also have numerous side effects [49,50]. Current study showed at the first time therapeutic potential of *Smb* extract on arthritis. Therefore, *Smb* extract may serve as a source of natural anti-inflammatory plant but this needs further evaluation for use as an alternative treatment. Underlying mechanisms of this therapeutical effect of *Smb* extract on arthritis should be further evaluated by advanced biochemical and molecular studies.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

Animal studies were performed at "Gülhane Experimental Animal Production and Research Center" with the Ethical Committee Permission number of 17/13 on 28.03.2017. All animal experiments were carried out in accordance with the "National Institutes of Health Guide for Care and Use of Laboratory Animals."

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATING THE EFFECT OF pH AND ION STRENGTH ON LOADING AND RELEASE PROPERTIES OF DIFFERENT ION EXCHANGERS

pH VE İYON KUVVETİNİN İYON DEĞİŞTİRİCİLERİN YÜKLEME VE SALIM ÖZELLİKLERİ ÜZERİNE ETKİSİNİN İNCELENMESİ

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ABSTRACT

Objective: *Ion-exchangers are inert, water-insoluble polymers with ionizable functional groups on their surface. They can be used for purposes such as disintegrating or taste masking in orally disintegrating dosage forms, or they can provide a pH-dependent controlled release.*
Material and Method: *The loading and release properties of different cation exchangers were investigated by loading Atenolol to Amberlite CG50, Dowex 50W-X2 and Smopex 101 as weak or strong resin and strong fiber, respectively. The effect of the ionic strength of the medium on the loading capacities of these materials were investigated in water and pH 7.4 HEPES buffer using batch method and loading was monitored by pH, zeta potential, FTIR and SEM analysis. Loading capacity was calculated UV spectrophotometrically. The effect of pH and ionic strength on the atenolol release was investigated by the dialysis bag method in pH 1.2 HCl, pH 6.8 PBS and pH 6.8 HEPES media.*
Result and Discussion: *Due to its low molecular weight and high pKa atenolol was successfully loaded with a capacity over 93%. As the pH could be balanced a higher loading capacity was achieved in HEPES buffer. The decrease in zeta potential values proved that the complexes were successfully obtained and the ionic complex formation was also monitored with FTIR and SEM micrographs. Atenolol did not get released in pH 1.2 medium, contrarily to pH 6.8 in which the functional groups are ionized. The higher amount of counter ions in PBS buffer also affected the release. The highest release rate was obtained with Amberlite. All ion-exchangers provided a pH-dependent release fitting to Higuchi or Zero order kinetics that shows diffusion. Boyd equation results also showed that diffusion mechanism was particle controlled.*
Keywords: *Amberlite CG50, atenolol, cation-exchange materials, Dowex 50W, Smopex 101*

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ÖZ

Amaç: *İyon değiştiriciler, yüzeylerinde iyonize olabilen fonksiyonel gruplar bulunan suda çözünmeyen inert polimerlerdir. Ağızda dağılan dozaj formlarında dağıtıcı veya tat maskeleyici gibi amaçlar için kullanılabilirler veya pH'ya bağlı kontrollü salım sağlayabilirler.*

Gereç ve Yöntem: *Atenolol kullanılarak üç farklı katyon değiştiricinin yükleme ve salım özellikleri araştırılmıştır. Kullanılan katyon değiştiriciler zayıf veya kuvvetli reçine ve kuvvetli fiber olarak sırasıyla Amberlite CG50, Dowex 50W-X2 ve Smopex 101 olarak seçilmiştir. Ortamın iyonik kuvvetinin bu malzemelerin yükleme kapasitelerine etkisi, beç yöntemi kullanılarak su ve pH 7.4 HEPES tamponunda araştırılmış ve yükleme pH, zeta potansiyeli, FTIR ve SEM analizi ile izlenmiştir. Yükleme kapasitesi UV spektrofotometrik yöntemle hesaplanmıştır. Ortam pH değeri ve iyonik kuvvetinin salım üzerindeki etkisi diyaliz torba kullanılarak pH 1.2 HCl, pH 6.8 PBS ve pH 6.8 HEPES ortamlarında incelenmiştir.*

Sonuç ve Tartışma: *Atenolol düşük molekül ağırlığı ve yüksek pKa değeri nedeniyle % 93'ün üzerinde bir kapasite ile başarıyla yüklenmiştir. pH dengelenebildiğinden, HEPES tamponunda daha yüksek bir yükleme kapasitesi elde edilmiştir. Zeta potansiyel değerlerindeki düşüş, iyon değiştirici-etken madde komplekslerinin başarılı bir şekilde elde edildiğini kanıtlamıştır. İyonik kompleks oluşumu FTIR ve SEM mikrografları ile de görüntülenmiştir. Atenolol, fonksiyonel grupların iyonize olduğu pH 6.8'in aksine pH 1.2 ortamında salınmamıştır. İçerdiği karşıt iyonlar nedeniyle PBS tamponunda daha yüksek miktarda salım sağlanmıştır. En hızlı salım Amberlite ile elde edilmiştir. Tüm iyon değiştiriciler, difüzyonu gösteren Higuchi veya Sıfır derece kinetikle pH'a bağlı bir salım göstermiştir. Boyd eşitlik sonucuna göre salım aynı zamanda partikülden kontrolle gerçekleşmiştir.*

Anahtar Kelimeler: *Amberlite CG50, atenolol, Dowex 50W, katyon değiştiriciler, Smopex 101*

INTRODUCTION

Ion exchange mechanism has been used in several scientific areas related to pharmacy from the beginning of the 20th century; such as in water purification since 1930s, and in pharmaceutical and biomedical applications since 1950s [1-4]. Nowadays, ion exchangers are frequently used in fast disintegrating oral dosage forms as superdisintegrants or taste masking agents, especially in pediatric dosage forms [5-7]. Ion exchanger-drug complexes can also be effective in increasing storage stability of drugs. They can be used as pH-dependent carrier systems for ionic drugs and can be prepared in the form of oral dosage forms like films, tablets, capsules, or suspensions with sustained release properties [2, 8-10]. Moreover, ion-exchange resins and fibers are also under investigation since early 1990s as drug carrier systems in transdermal delivery alone or with the combination of iontophoresis and as wound dressing [11-14].

Classification of Ion Exchangers

The ion exchange resins and fibers consists a static polymer structure with functional groups on their surfaces. These groups can either be in acidic or basic form that can interchange their ions with the counter-ions of the medium. Due to the charge of the functional groups resins and fibers can be classified as cation or anion exchangers. The type of functional groups also affects the material properties being as strong or weak ion exchangers [1,4,15]. The main difference between resins and fibers comes from the cross-linkage of polymer skeleton; generally, the resins consist cross-linked polymer structure while the fibers do not [16-17].

Strong cation exchange resins generally consist of cross-linked polystyrene-divinyl benzene polymer structure with sulfonic acid (-SO_3H) groups, which have been prepared by the polymerization of polymer with sulfuric acid or chlorosulfonic acid. Dowex 50, Amberlite IR 120, and Amberlite IRP69 are some of the commercial examples of these resins. Weak cation exchange resins with carboxylic acid (-COOH) functional groups are prepared by polymerization of organic acids, such as acrylic or methacrylic acid in the presence of a cross-linking agent such as divinyl benzene to yield cross-linked networking. Some commercial examples are Amberlite IRC 50, Amberlite IRP64 and IRP88 [2,4].

In the case of anion exchange resins, cross-linked polystyrene polymers produced by chloromethylation of polystyrene beads with subsequent treatment with ammonia, primary, secondary,

or tertiary amines are used in production. Dowex 1, and Amberlite IR 400 are examples of strong anion exchange resins having quaternary ammonium groups, while Dowex 2 and Amberlite IR 4B are weak anion exchangers, which have predominantly tertiary amine substitutes [2,4].

The resins can also be classified due to their structure as gel-type (microporous) or macroporous type. Gel-type resins have lower cross-linked structure and a smoother surface while macroporous ones have a sponge-like structure with higher cross-linking [18].

Despite the resins, fibers consist of non-crosslinked hydrophobic polymer chains such as polypropylene or polyethylene as skeleton that carry a constant positive or negative electrical charge located on the surface of the fiber. Due to these negative or positive electric charges, they are called as cationic or anionic exchangers, respectively. Because of their larger surface area compared to resins, fibers provide a higher loading capacity. Besides they have good mechanical and thermal strength and chemically inert structure [16,17]. Smopex types (Smoptech Ltd., Turku, Finland) are one of the commercial examples of ion exchange fibers. Among them, a cation exchange type of Smopex 101 is composed of styrene sulfonic acid grafted polypropylene, whereas Smopex 102 is an acrylic acid grafted polypropylene fiber. Some other anion-exchange examples of Smopex are 103pe and 105pe types with uncrosslinked cellulose grafted polyethylene side chain skeleton and vinylpyridine ion exchange groups [16,19-20].

Ion Exchange Mechanism

The ion exchange mechanism of resins depends on the reversible interchange of the surface bounded ions with the ions of the same charge in the medium. This process also involves the competition of the counter-ions of the medium [1]. The ionic strength and pH of the medium, and the ionization, pKa, molecular weight properties of the drugs plays important role for both loading and release from the resins [4]. Unlike the resins, the ion exchange in fibers is occurred on a non-crosslinked and non-porous surface that affects the ionic reaction. The ion-exchange in resins and fibers are generally explained by Donnan principle and diffusion process [16,17].

In this study, the loading and release properties of different cation exchangers were compared by loading Atenolol (ATN) to Amberlite CG50, Dowex 50W-X2 and Smopex 101. Among the ion exchangers, Amberlite CG50 is a weak cation exchange resin with carboxcylic acid (–COOH) functional group containing macroporous methacrylate structure. Contrarily, strong cation exchange resin Dowex 50W-X2 contains sulfonic acid (-SO_3H) functional groups on gel type (microporous) divinyl benzene structure. Strong cation exchange fiber Smopex 101 also contains -SO_3H functional groups in non-crosslinked and non-porous polypropylene structure. The effect of pH and ionic strength of the medium on loading and release to these ion exchangers were investigated using water or pH 7.4 HEPES buffer as loading media and pH 1.2 HCl, pH 6.8 PBS or pH 6.8 HEPES as release media. ATN, which is a well-known β-blocker drug used for cardiovascular diseases, such as hypertension, angina pectoris, arrhythmias, and myocardial infarction was chosen due to its pKa of 9.5, good solubility and the cyclic structure of the molecule that causes a bitter taste [21].

MATERIAL AND METHOD

Materials

Atenolol (Abdi İbrahim; ATN), Dowex 50W-X2 (Dow Corning; DW), Amberlite CG50 H^+ type (Acros Chem; AMB), Smopex 101 (AlfaAesar; SMP), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES; Fluka), K_2HPO4 (Merck), NaOH (Merck), HCl (Merck), Dialysis tube MWCO 14000 (Sigma) were used. Water was used as 18.2 mΩ quality purified (Millipore) in all experiments.

Spectrophotometric Analysis of ATN

The UV spectrophotometric calibration of ATN was done in different media for drug loading and release studies. Briefly, water and pH 7.4 HEPES buffer media were used for loading studies and pH 1.2 HCl, pH 6.8 PBS, pH 6.8 HEPES media were used for release studies. The UV spectrophotometric calibration of ATN were separately done in all media studied. The calibration study parameters were as follows:

UV calibration of ATN in 18.2 mΩ water and pH 1.2 HCl media were done with the concentrations of 2.5, 5, 10, 12, 14, 16, 20 and 24 µg/ml obtained by diluting the 100 mg/ml stock solution. The calibration of ATN in pH 6.8 PBS, pH 6.8 and pH 7.4 HEPES media were done with the concentrations of 5, 10, 20, 30, 40, 60, 80, 100, 120, 140 160 µg/ml obtained by diluting the 500 µg/ml stock solution. The absorbance values of the solutions at these concentrations were read with a UV spectrophotometer (UVmini 1240, UV-Vis Spectrophotometer, Shimadzu) at 274 nm for all the buffers studied. The absorbance versus the concentrations were graphed and the calibration equations were obtained with the calibration line.

Production of Drug-ion Exchanger Complexes

Ion exchanger-drug complexes of ATN were prepared by batch method [22]. Briefly, the cation exchange resin (Dowex or Amberlite) and fiber (Smopex) particles were purified with absolute alcohol and 18.2 mΩ quality water (Merck Millipore) and dried before use. Cation exchangers were separately weighed in equal amount with ATN (1:1 ratio; 50 mg) and were mixed with the drug in 100 ml medium at 500 rpm using magnetic stirrer. The loading media were water and pH 7.4 HEPES buffer, respectively. The resulting complexes were washed with 18.2 mΩ water, filtered from 0.45 µm filter paper under vacuum and dried in an oven at 40°C overnight. The composition of the complexes and the media used in production are given in Table 1.

Table 1. Composition of drug-ion exchanger complexes and the media used in production

Ingredients/Code	F1	F2	F3	F4	F5	F6
Atenolol (mg)	50	50	50	50	50	50
Amberlite CG50 (mg)	50	-	-	50	-	-
Dowex 50-X2 (mg)	-	50	-	-	50	-
Smopex 101(mg)	-	-	50	-	-	50
Water (ml)	100	100	100	-	-	-
pH 7.4 HEPES Buffer (ml)	-	-	-	100	100	100

***In vitro* Characterization of Drug-ion Exchanger Complexes**

Drug loading capacity

Drug loading capacity was calculated from free drug remained in the medium (n=3). Briefly, 500 µl sample was taken from the 100 ml medium in 5, 15, 30, 45, 60, and 90th minutes and same amount of fresh buffer was added. Samples were centrifuged at 10.000 rpm for 2 min (Hettich ultrasantrifuge) and the aliquots were read spectrophotometrically at 274 nm. Percentage of drug loading was calculated from the amount remained in the medium with the following equation:

Loading capacity (%) = (C_0-C_t/C_0) x100

C_0 : Initial drug amount in the medium (mg)

C_t : Drug amount remained in the medium at predetermined time intervals (mg)

pH

The pH of the loading medium was investigated at certain time intervals using a pH-meter.

Zeta potential

In order to evaluate the zeta potential value of ion exchangers and the resultant complexes the dried particles were dispersed in 18.2mΩ water and immediately measured with Zetasizer (Nano-ZS, Malvern Instruments). All studies were conducted in triple.

Particle Size and Distribution

Laser diffraction (Mastersizer 3000; Malvern) analysis was carried out with a 1:10 (v/v) dilution of the ion exchange resins in water at 25°C. The detector was fixed at an angle of 90°. Data on particle size was characterized by Dv50 value that refers to the volume distribution at which 50% of the sample is smaller or larger. The size distribution was calculated by the equation of ´Span=(D90–D10)/D50´.

Fourier Transform Infrared (FTIR) Studies

The ion exchange mechanism was monitored on ion exchanger-drug complexes by scanning the existence or loss of the characteristic stretching bands of materials using Cary 630 FTIR (Agilent Technologies).

Scanning Electron Microscopy (SEM) Analysis

Surface morphology of the ion exchanger-drug complexes were examined using a scanning electron microscope (Quanto 200F, Fei) equipped with a digital camera at 5 kV accelerating voltage. Samples were sputter-coated with gold-palladium before use.

In Vitro Dissolution Studies

In vitro ATN release from ion exchanger-drug complex was studied by dialysis bag method in pH 1.2 and pH 6.8 media. Briefly, dialysis bags with MWCO 12000-14000 were saturated with appropriate buffer for overnight and then filled with the ion exchanger-drug complex equivalent to 10 mg of ATN. The dissolution studies were conducted for 2 hour in pH 1.2 HCl and then 6 hours in pH 6.8 PBS or pH 6.8 HEPES media. A thermostatic incubator was used at 37°C and 100 rpm (MaxQ 4450, Thermoscientific). 1 ml of sample was taken at 15, 30, 60, 90 and 120th min for pH 1.2 and 15, 30, 60, 120, 180, 240 and 360th min for pH 6.8, respectively. The analysis was done spectrophotometrically at 274 nm.

As the ion exchange mechanism can be explained by diffusion process, ATN release was evaluated using Korsmeyer-Peppas, Higuchi and Zero order kinetics. The Higuchi model was used to explain the Fickian diffusion using the drug release versus square root of time plot, while the n value in the Korsmeyer-Peppas $Mt/M\infty=Kt^n$ equation was used to investigate the type of release mechanism. The n values between 0.45 and 0.85 indicates the non-Fickian diffusion for swelling controlled transport whereas the values above 0.85 indicate zero order kinetics (case II transport mechanism) [7,8].

Boyd kinetics was also performed for evaluating the rate-limiting step. Here, for the released fraction (F) under 85 % the below equation is used:

$$F = Mt/M\infty = 1 - 6/\pi^2 \sum e^{-n^2Bt}/n^2$$

where B represented the rate constant, Mt and M∞ are the amounts of drug released after time and after infinite time. This equation can be simplified for the F values lower than 0.85 as;

$$Bt = 6.283 - 3.290F - 6.283(1 - 1.047\ F)^{1/2}$$

If the released fraction against time (Bt plot) is found linear in the equation, the rate limiting step for drug diffusion can be assumed as the resin matrix [22].

RESULT AND DISCUSSION

ATN Loading onto Different Cation Exchangers

The drug-ion exchanger complexes were produced by the batch method. In this method the ion exchanger is simply mixed in a known concentration and volume of drug solution in a beaker to provide an ion exchange between the drug and surface functional groups of the resin or fiber. The effective parameters on ion exchange mechanism and therefore drug loading are related with the properties of drug and resin/fiber and the loading conditions. The structure, miliequivalent (mEq) exchange capacity and cross-linking property of the resin or fiber; the molecular weight, existence of aromatic ring and

pKa of the drug; mixing rate or time, drug:ion exchanger ratio, pH and ionic strength of the media affect the drug loading with the batch method [2,4].

In this study, ATN was successfully loaded onto the ion exchangers in 1:1 ratio in both media and the optimum time for loading was chosen as 30 minutes as the pH value did not show dramatic change afterwards. Loading capacities in HEPES buffer were slightly higher than in water due to the existence of counter ions inside the buffer (Table 2).

Table 2. pH change during loading process and loading capacities obtained in different media, n=3

	pH values					
Medium	**Water**			**HEPES**		
Time (min)	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
0	9.68 ± 0.01	9.60 ± 0.02	9.65 ± 0.02	7.58 ± 0.02	7.56 ± 0.01	7.59 ± 0.01
5	8.20 ± 0.10	8.80 ± 0.08	9.11 ± 0.15	7.45 ± 0.06	7.43 ± 0.05	7.53 ± 0.04
15	6.96 ± 0.04	6.00 ± 0.01	8.90 ± 0.02	7.43 ± 0.06	7.42 ± 0.02	7.52 ± 0.03
30*	8.00 ± 0.06	6.77 ± 0.03	8.91 ± 0.03	7.46 ± 0.04	7.42 ± 0.02	7.54 ± 0.03
45	8.00 ± 0.03	6.70 ± 0.02	8.91 ± 0.05	-	-	-
60	-	-	-	7.46 ± 0.02	7.45 ± 0.03	7.46 ± 0.04
90	-	-	-	7.44 ± 0.04	7.42 ± 0.02	7.44 ± 0.07
*Loading capacity (%)	93.51 ± 0.350	98.37 ± 0.300	93.14 ± 0.350	99.90 ± 0.003	99.87 ± 0.027	99.90 ± 0.005

*****30 min was chosen as optimum loading time

One of the parameters that affects loading is the ionization characteristics of the ion exchangers. Among the chosen materials, Dowex resin with pKa value of 1-2, is strongly acidic, therefore the ion exchange and the drug loading or drug release was affected from the ionic strength of the medium rather than the pH change [23]. Contrarily, Amberlite shows weak acidic properties with pKa 4-6 and the ion exchange mechanism was affected from the pH of the medium. As both of the resins were in ionized state during loading process, ATN could be successfully loaded in water and HEPES media. Smopex fiber has been totaly ionized at pH 7.4 due to its lower pKa (pKa 0-1) [19]. Therefore, ATN could also be loaded onto fiber in both media. Loading ratio of ATN was slightly higher in pH controlled environment (Table 2).

Another property affecting ATN loading is the ion exchange capacity of the resins/fibers. Generally weak acidic resins have higher capacities than strong ones. In this study the weak acidic resin Amberlite CG50 was a bidendate –COOH resin with 10 mEq/g capacity, whereas Dowex 50W was a strong resin with $–SO_3H$ and 4-6 mEq/g capacity. Also strong acidic fiber Smopex 101 had $–SO_3H$ and 4.1 mEq/g capacity. All ion exchangers were regenerated before use for providing a total loading capacity, as they were in H^+ form commercially. However, ATN loading did not get affected from the total ion exchange capacity as the drug molecule did not have high molecular weight. Besides, the high pKa and aromatic structure of ATN had advantages on loading. It is reported that the aromatic ring existence of drugs highly affects the ion exchanger-drug interaction as the counter-ions of aromatic rings show higher affinity to the ionized functional groups of resins and fibers [17].

pH Values

Measurement of pH change is a way to monitor the complex formation, especially when the column process is used in production [22]. Returning to initial pH value or a balanced pH can be considered as the ionic complexation.

In this study, initial pH value of ATN stock solution in water was measured as pH 9.6. Addition of acidic resins decreased this pH value down to 6.0 in the presence of Dowex; pH 6.96 in the presence of Amberlite despite to Smopex fiber, in which significant pH change was not observed (Table 2). Contrarily, ATN solution in HEPES buffer provided a controlled pH value between 7.56-7.59 initially, which slightly dropped with the addition of ion exchanger and remained more balanced than water medium throughout the experiment. Therefore, ATN was able to be loaded by 99.9% under conditions with constant pH and the addition of counter-ions that do not compete with the drug. However, maximum loading was determined as 93% for Amberlite and Smopex in the water medium. Reasonably,

this could be due to the absence of counter-ions in water medium that causes ATN to be released back for maintaining the ionic balance.

Zeta Potential Values

Controlling the zeta potential value can be a diagnostic tool in the prediction of complexation between the ion exchanger and the drug. A significant change in zeta potential value can be interpreted as the existence of ionic complexation.

The ionized functional groups of the cation exchangers provide a negatively charged zeta potential in alkaline solutions because of the hydroxyl ion attack to the surface. In case of strong cation exchangers, a negatively higher zeta potential value can be expected due to the protonozation of strong acidic functional groups such as $-SO_3H$. Besides, the cross-linkage can also be effective on the zeta potential value of ion exchangers. It is reported that, due to the swelling the increased adsorption of water can cause lower zeta potential value in low cross-linked resins [24].

As it can be seen from Table 3, zeta values of unloaded resins measured in deionized water were found negatively charged which means that the functional groups existing on the resin surface are ionized in water. Drug free Dowex resin gave a relatively higher zeta potential value due to its sulfonic acid groups. After complexation the negative zeta values decreased significantly. In case of strong acidic fiber Smopex, the zeta potential value could not be measured reproducibly due to its rod shape with theoretically 250 µm length.

Table 3. Particle size and zeta potential characteristics of ion exchangers and drug complexes, n=3

Ion exchanger	Drug free			Drug loaded [a]		
	Dv50(µm)	**Span**	**Zeta (mV)**	**Dv50 (µm)**	**Span**	**Zeta (mV)**
Amberlite CG50 4 % cross-linkage, 75-150 µm dry size	171 ± 0.45	0.700	-23.7 ± 2.81	196 ± 2.17	0.647	-15.1 ± 2.75
Dowex 50W-X2 2% cross-linkage, 50-75 µm dry size	130 ± 1.14	0.749	-31.0 ± 5.01	138 ± 1.58	0.976	-14.9 ± 4.87

[a] F4-F6 complexes were used.

Particle Size and Distribution

The particle size and cross-linking properties are effective on the ion exchange mechanism and therefore drug loading and release. In case of resins, the decrease in cross-linking capacity causes an increase in the swelling of the particle [2,4]. Therefore, the swelling of the resins were investigated via measuring the wetted particle size of the resins using laser diffraction method with Mastersizer (Model 3000; Malvern). The results (Table 3) showed that the highest swelling was seen with Dowex resin, which also provided highest drug loading (98.37 %) in water media. This resin has a lower cross-linking capacity (2%) than Amberlite (4 %). Besides, dry resin particles of Dowex were smaller (50-75 µm) than Amberlite resin (75-150 µm) in size. Smopex, being rod shaped with high surface area and noncross-linked, non-porous structure, is reported to show less swelling than the resins [25]. However, the size of this ion exchanger was not measurable with light scattering method due to its rod shape. Its non-porous structure and high surface area was later observed with SEM studies.

It is reported that sulfonic acid functional groups are the other reason of high swelling in strong cationic resins due to the increased hydration caused by the sulfonic acid group. Therefore a higher wet size and swelling occurs. This high swelling due to the increased amount of sulfonic acid group not only affects the loading but also causes a sustained/prolonged release [26].

Ion Exchanger-drug Complex Characterization by FTIR

FTIR is a useful tool for proving the ionic complexation. If the complexation occurs the characteristic peaks related with the functional groups of both ion exchanger and the drug can either

shift or dissappear due to bonding. Therefore, the drug free and drug loaded particles investigated with FTIR.

As it can be seen from FTIR spectra (Figure 1), ATN was successfully complexed with the ionic exchangers. ATN shows an -NH stretching band due to its -NH_2 functional group, which were found at 3347 and 3158 cm^{-1} in our study (Figure 1a). Among the resins, Dowex with a divinyl benzene structure and -SO_3H functional group, generally gives characteristic strong stretching band near 3390 cm^{-1}. This band was found at 3334 cm^{-1} in our study and was disappeared in ATN-DW after ionic complexation occurred (Figure 1b and 1c). The bands at 2985-2931cm^{-1} are O-H stretching and 1686 cm^{-1} C=O stretching bands of –COOH for Amberlite (Figure 1d). The characteristic 1686 cm^{-1} C=O stretching band shifted to 1669 cm^{-1} by decreasing for Amberlite (Figure 1e). Similarly, the characteristic band related with the sulfonic acid functional group of Smopex 101 near 3436 cm^{-1} was also shifted to 3447 cm^{-1} after complexation (Figure 1f and 1g). The absence of any characteristic bands for ATN in the ion exchange complexes also revealed the ionic interaction [12].

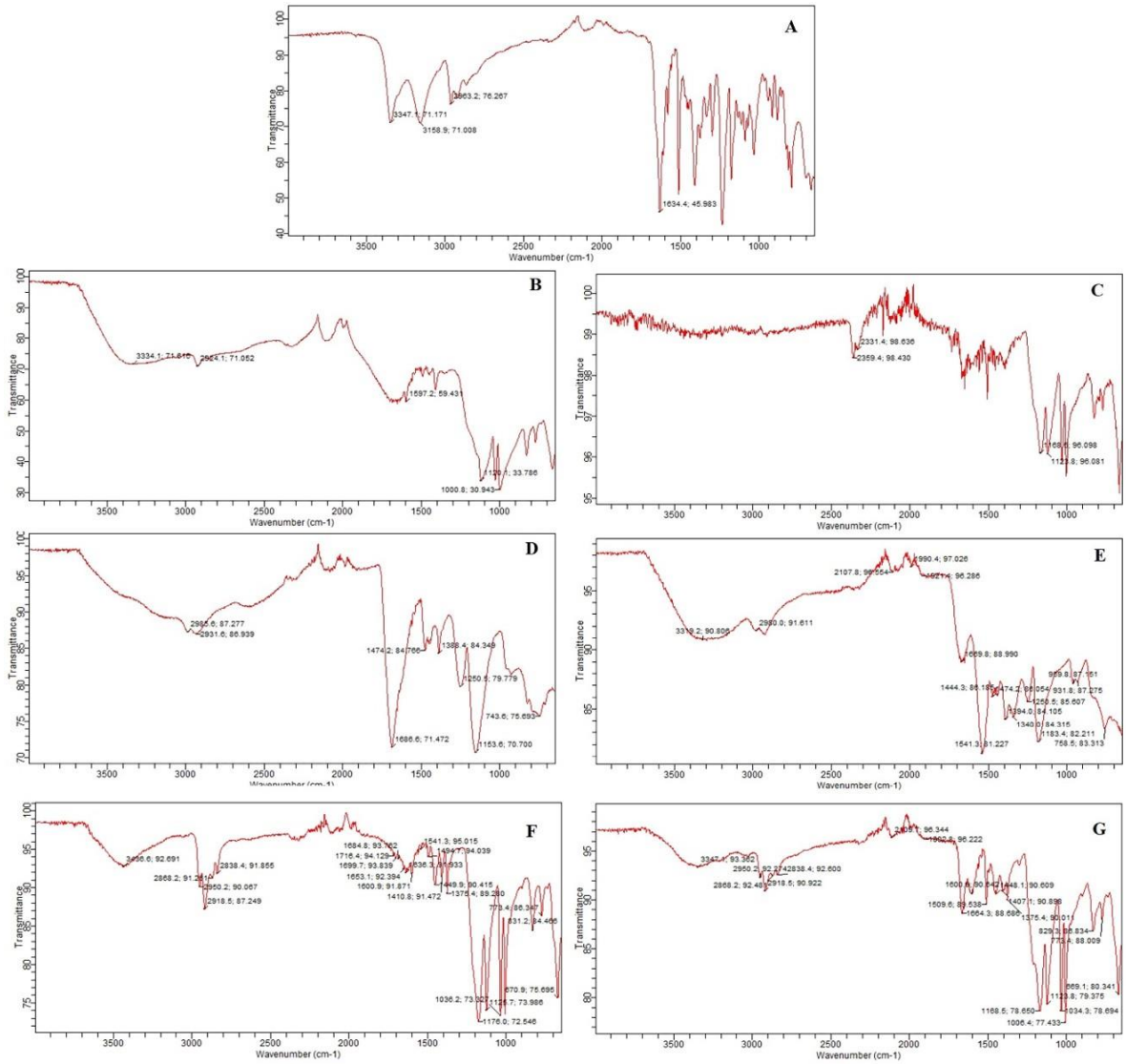

Figure 1. FTIR spectrums of; a) Atenolol, b) DOWEX resin, c) ATN-DW complex, d) AMBERLITE resin, e) ATN-AMB complex, f) SMOPEX fiber, g) ATN-SMP complex

Morphological Characterization of by SEM

Drug loaded and drug free ion exchangers were scanned using SEM. Among the chosen ion exchangers, Amberlite has a macroporous methacrylic type matrix with relatively higher crosslinked (X4) structure and bidentate –COOH groups inside the matrix. The macroporous structure of Amberlite can be seen in Figures 2A to 2C.

Sulfonated divinyl benzene resin Dowex have a spherical shape with smooth surface (Figure 2D). Since the crosslinking of this resin is low (X2) it shows more tendency to swell and take water inside. Therefore some fractures could occur on the hollow parts of the resin surfaces (Figure 2E). It is reported that these fractures has no effect on the ion-exchange mechanism [22,26]. Figure 2F shows the surface of ATN-DW complex. ATN provided an amorphous film surrounding the surface and the absence of drug crystals can be interpreted together with FTIR peaks as the ionic interaction occurred.

As it can be seen in SEM micrographs, Smopex fiber has a rod shaped non-porous structure with high surface area (Figure 2G). Its size is reported as 250 µm in length and 12 µm in diameter. Due to its non-crosslinking property, the water intake and therefore swelling is less than the resins and the drug can only be in contact with the surface of the fiber [12,17,24]. As shown in Figures 2H and 2I, ATN was loaded on the surface of this fiber and was in amorphous state.

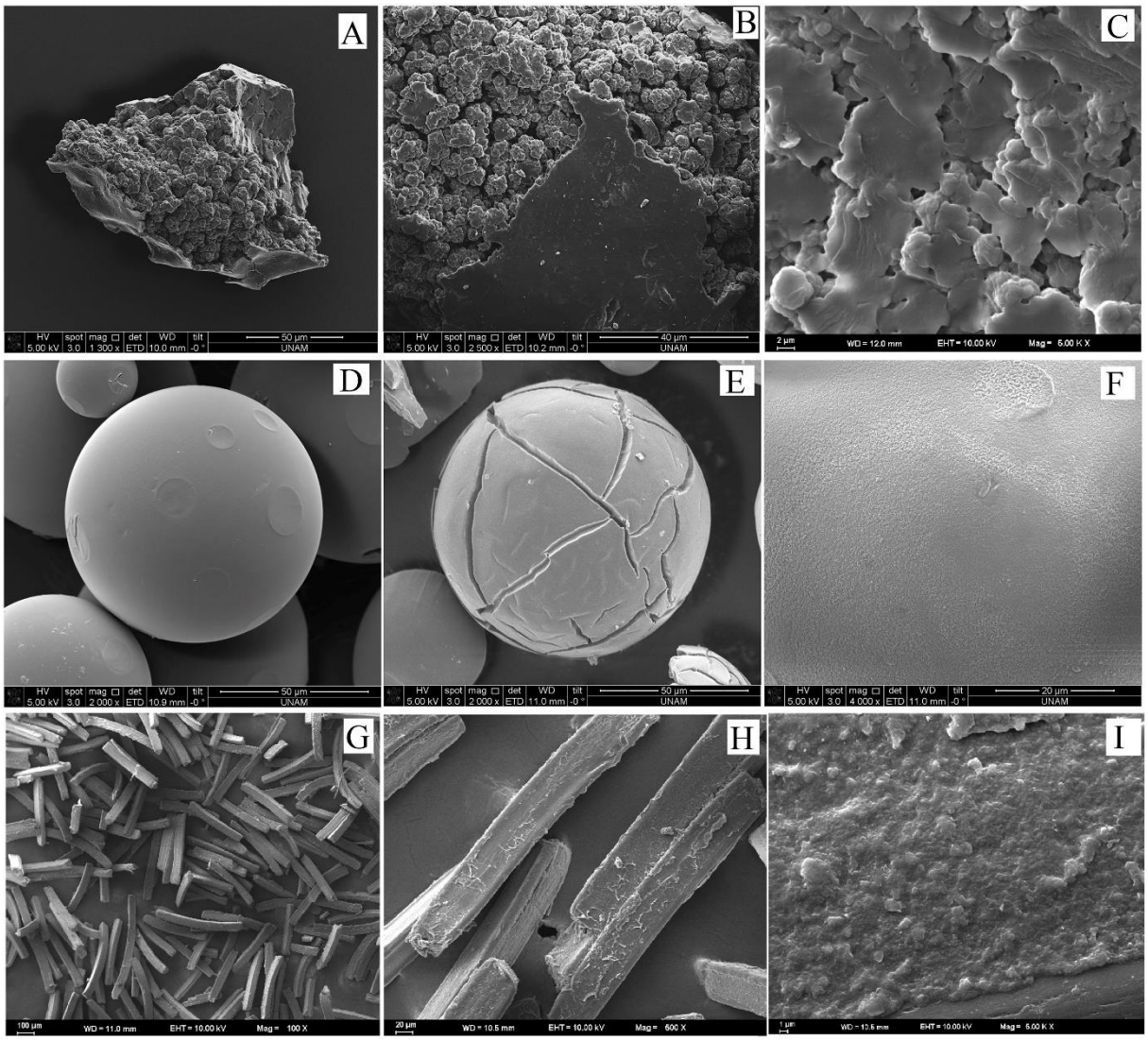

Figure 2. SEM micrographs of A) AMB, B-C) ATN-AMB (F4), D) DW, E-F) ATN-DW (F5), G) SMP, H-I) ATM-SMP (F6)

In Vitro Dissolution Studies

In vitro ATN release from ion exchanger-drug complexes was studied by dialysis bag method in pH 1.2 HCl for 2 h and pH 6.8 PBS or HEPES media for 6 h, respectively. According to the results given in Figure 3, ATN was released at pH 6.8 media while there was only neglected amount of drug release (< 1%) at pH 1.2 HCl. Even the ionization of functional groups in strong cation exchangers began from pKa 1-2, the seconder amine group of atenolol is not in ionized form in acidic pH values.

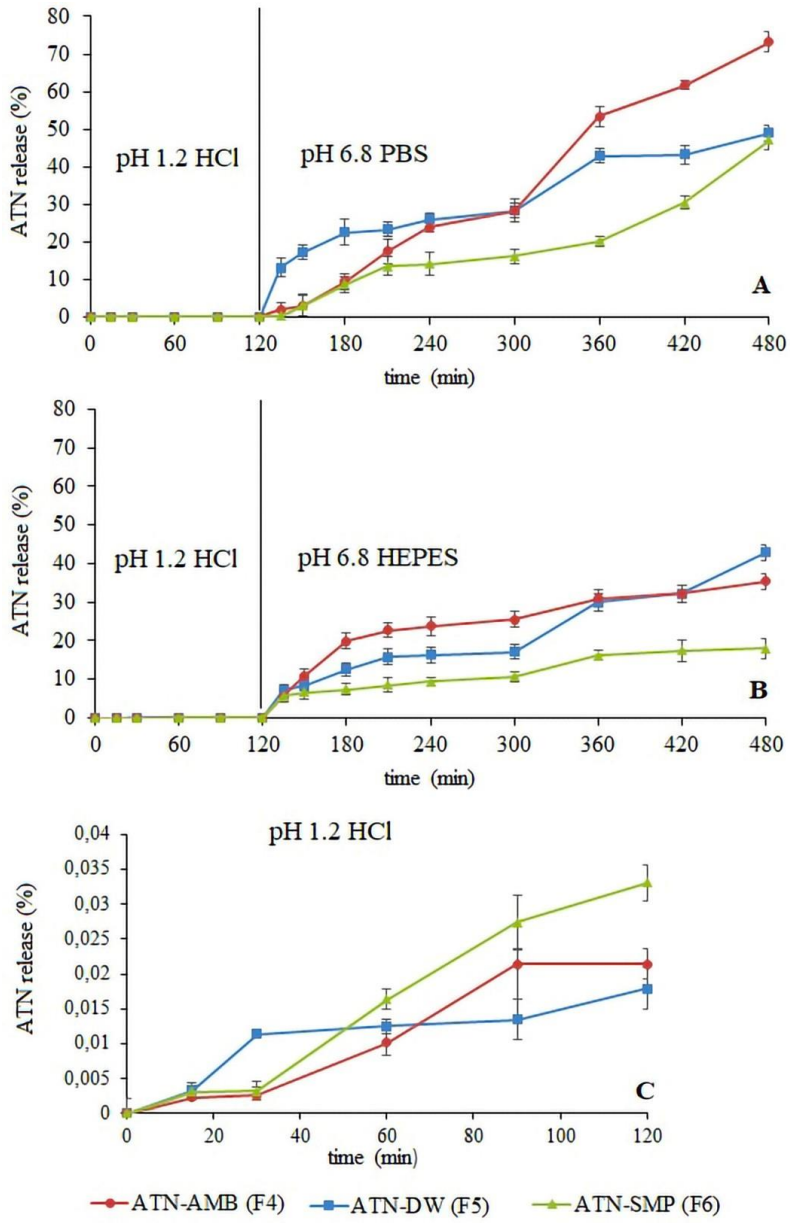

Figure 3. Atenolol release from ATN-AMB (F4), ATN-DW (F5) and ATN-SMP (F6) complexes in, a) HCl and PBS, b) HCl and HEPES and c) HCl media

ATN released at pH 6.8 media in different rates due to the ionic properties of the buffers, structure of ion exchangers used and ionization for both resins/fiber and drug. Among the buffers studied as release media, HEPES is a well-known buffer material consisting sulfonic acid, which provides a weak ionic strength that do not compete with drug ions inside. However, PBS consisting monovalent ions

such as K, Na and Cl more readily compete with the ionized ATN and is preferably bound onto the ion exchangers. Therefore the released amounts of ATN in PBS buffer were found to be relatively higher than in HEPES buffer for all type of ion exchangers.

When the release profiles were evaluated from the point of ion exchanger properties, the slowest release was achieved with the strong type Smopex fiber in both media. Being strong exchanger type, Dowex resin also provided slower release than Amberlite resin. Drug release from strong cationic resins with sulfonic acid groups are reported to be more dependent to ionic strength of the medium than the pH. The amount of sulfonic acid groups in strong cationic resins is also effective in providing a sustained or prolonged release from the complexes. The increasing amount of sulfonic acid groups results with a high swelling capacity and larger wet size, which also causes to lengthen the diffusional path [25].

In case of Amberlite resin with weak acidic macroporous structure, not only the higher capacity of ionic substituents but also the higher cross-linking percentage and particle size than the Dowex resin affected the ion exchange (Table 3). All these properties are effective not only on the loading capacity but also on the release rate. Generally a faster drug release can be expected from the resins with larger particles and higher cross-linking. Here, the degree of cross-linking is the parameter that controls the porosity of the resin structure and therefore the internal diffusion is reported to be influenced from the macroporous structure of the resin [2,27].

Release Kinetics

The effect of counter ions and ion strength on the release kinetics of drug-ion exchanger complexes were investigated by calculating zero order, Higuchi, Korsmayer-Peppas and Boyd kinetics in pH 6.8 media. Results are given in Table 4.

Table 4. Release kinetics results for pH 6.8 release media

		F4 (ATN-AMB)		F5 (ATN-DW)		F6 (ATN-SMP)	
Medium		**HEPES**	**PBS**	**HEPES**	**PBS**	**HEPES**	**PBS**
Zero order	r^2	0.8217	0.9860	0.9522	0.9020	0.9109	0.9304
	k	0.0846	0.2102	0.1011	0.1139	0.0436	0.1120
	F	36.872	564.01	159.53	73.663	81.745	106.94
Higuchi equation	r^2	0.9597	0.8940	0.9167	0.9643	0.9559	0.8418
	k	1.8720	4.0960	2.0310	2.4100	0.9147	2.1800
	F	190.52	67.444	88.530	215.83	173.59	42.564
Korsmeyer-Peppas	r^2	0.9257	0.9856	0.9283	0.9481	0.9065	0.8961
	n	0.5214	1.1870	0.5390	0.3992	0.3822	1.2980
	log k	1.7560	3.1460	1.8480	1.3710	1.7610	3.6260
	F	87.218	479.09	90.630	127.87	67.873	60.381
Boyd kinetics	r^2	0.9709	0.8618	0.8579	0.9469	0.9518	0.6937
	k	0.0004	0.0021	0.0005	0.0008	8.9×10^{-5}	0.0005
	F	266.66	49.901	48.314	142.67	157.81	18.120

Drug release from resin or fiber particles are generally explained by Donnan principle which depends on the diffusion of ions between the medium and the resin or fiber particle. This ion exchange in turn occurs by diffusion from the particle and the thin film surrounding the particle; which means that drug release is controlled from either the resin/fiber matrix or the ionic film layer surrounded by the particle, respectively. The slower release is generally considered to be the rate-limiting step in Boyd kinetics, which explains this phenomenon. Since drug release from resins and fibers occurs by diffusion, Korsmeyer-Peppas and Higuchi kinetics are also useful in explaining the drug release mechanism. Accordingly, the release can occur by non-fickian or fickian diffusion. Generally, the first case describes diffusion through a swelling matrix, while the second provides a matrix-controlled mechanism [8,22,28].

As ion exchange resins are structured from a polymer matrix, it is obvious to expect a matrix-controlled release. Therefore, Higuchi equation, which describes the release of drugs as a square root of time-based on fickian diffusion, was also applied to the pH 6.8 release data. Generally, release

mechanism from ion exchange resin complexes highly fitted to Higuchi kinetics in HEPES medium in which a slower release was achieved (Figure 3, Table 4). The results obtained in HEPES medium also fitted to Boyd kinetics that proves the particle controlled release mechanism.

The release of the drug from complexes was also fitted to Korsmeyer-Peppas equation which evaluates the fraction of drug released at time t. According to n data obtained from Korsmeyer-Peppas equation, the drug release from the ion exchangers were fitted to either fickian or non-fickian diffusion. The latter profiles also fitted to zero order release kinetics.

As a conclusion, drug loading and release was affected from the pKa of both ion exchanger and ATN, pH of the medium and the structure of the resins and fiber. Due to the existence of aromatic ring in ATN structure, the counter-ions of aromatic ring showed higher affinity to the ionized functional groups of resins. Therefore, all the ion exchangers show high affinity to ATN especially in HEPES medium. Even this affinity caused a high loading capacity in HEPES medium, contrarily it resulted with a slower release. This could be not only the aromatic rings reported to slow down the release of ionic species from the ion exchangers, but also the result of the counter ions inside the medium. The release from all ion exchangers were fitted to either Fickian or non-Fickian diffusion by fitting to Higuchi or zero order release kinetics and the main parameter controlling the release was found as the ion exchanger, especially in pH 6.8 HEPES media.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

TOPLUM ECZACILARININ TAKVİYE EDİCİ GIDA VE BİTKİSEL İÇERİKLİ ÜRÜN PAZARLAMA VE SATIŞINDA SOSYAL MEDYA FİKİR LİDERLERİ (INFLUENCER) KULLANIMINA BAKIŞLARI

THE PERCEPTIONS OF COMMUNITY PHARMACISTS ON THE USE OF SOCIAL MEDIA INFLUENCERS IN MARKETING AND SALES OF FOOD SUPPLEMETS AND HERBAL PRODUCTS

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ÖZ

Amaç: *Bu çalışma ile Türkiye'de toplum eczacısı olarak faaliyet gösteren eczacıların sosyal medya platformlarında fikir liderleri kullanımı yoluyla takviye edici gıda ve bitkisel içerikli ürün tanıtımına ve pazarlanmasına bakışlarının derinlemesine anlaşılması amaçlanmaktadır.*
Gereç ve Yöntem: *Bu araştırma nitel araştırma deseninde durum çalışması olarak planlanmıştır. Araştırmada veri toplamak için yarı yapılandırılmış görüşme tekniği kullanılmıştır. Veriler ham şekilde kâğıda döküldükten sonra tematik analiz yoluyla çözümlenerek kodlanmıştır. Veriler arasında bağlantı kurabilmek amacıyla temalar ve alt temalar oluşturulmuştur.*
Sonuç ve Tartışma: *Çalışmaya 12 toplum eczacısı katılmıştır. Yapılan tematik analiz sonucunda araştırma bulguları üç ana tema altında toplanmıştır. Bunlar "pazarlamada fikir lideri kullanımına ilişkin tutumlar", "fikir liderlerinin özellikleri" ve "fikir liderliği rolünü benimseme"dir. Bu çalışmada bazı eczacılar fikir liderliği rolü konusunda ılımlı görüş bildirirken, diğerleri de eczacıların fikir liderliği rolüne karşıt görüş bildirmişlerdir. Bu çalışmada eczacılar, gelecekte eczacıların sosyal medyada fikir lideri rolü benimseyebilmesi için çeşitli yasal düzenlemelerin yapılması gerektiği ve bir sağlık profesyoneli olarak profesyonel anlamda bir sosyal medya hesabını yönetebilmek için yetkinliğin önemini vurgulamışlardır.*
Anahtar Kelimeler: *Bitkisel ürün, dijital pazarlama, fikir liderliği, sosyal medya, takviye edici ürün*

ABSTRACT

Objective: *The objective of this study is to gain an in-depth understanding of Turkish community pharmacists' perception on the promotion of food supplements and herbal products by social media*

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influencers.
Material and Method: *This research was planned as a case study in a qualitative research design. Semi-structured interview was used to collect data. After the data were transcribed verbatim, the data were analyzed and coded through thematic analysis by developing themes and sub-themes to make the connection.*
Result and Discussion: *Twelve community pharmacists participated in the study. Three main themes developed which are "attitudes towards phenomenon marketing", "characteristics of phenomena" and "adopting the role of phenomenon". In this study, some pharmacists expressed a moderate view on the use of influencers in promoting food supplements and herbal products, while others expressed an oppositional view to the opinion leadership role of pharmacists. Various legal arrangements should be made for pharmacists to adopt the role of influencers in social media in the future of pharmacy practice. Gaining competence to manage a social media account professionally as a healthcare professional to convey credible messages about food supplements and herbal medicines to consumers is essential.*
Keywords: *Food supplements, herbal medicines, influencer marketing, opinion leadership, social media*

GİRİŞ

Günümüzde pek çok sosyal medya platformu, insanların günlük hayatta karşılaştığı sosyal, kültürel, ekonomik sorunları başkalarıyla paylaşabilmelerine olanak tanımakla birlikte insanların herhangi bir ürün alırken karar aşamasında bilgi toplamak üzere kullandığı destekçisi olmaktadır. Sosyal medya platformlarında başkaları üzerinde etkileyici gücü olan ve insanları belirli bir yönde etkileyen sosyal medya fikir liderleri (influencer) tüketicilerin satın alım kararında etkili olan kişilerdir [1].

Geleneksel pazarlama yöntemlerinde firmalar, ünlü ve tanınmış kişilerin ürün tanıtımında kullanımına ağırlık verirken [2,3], sosyal medya çağı ile birlikte sıradan, herhangi bir tanınırlığı olmayan herkesin başkaları üzerinde sözel, görsel ve yazılı içerikler yaratarak etkileme potansiyeli olduğu keşfedilmiş ve bu kişiler dijital ortamlarda özellikle sosyal medyada ürün tanıtımlarında kullanılmaya başlanmıştır [3,4]. Ayakkabıdan kozmetiğe pek çok ürün hakkında sosyal medya fikir liderleri yoluyla tüketiciyi bilgilendirmek, bu ürünlere ilişkin tanıtım yapmak ve kullanıcı deneyimlerini sunmak vasıtasıyla ürün alım kararı oluşturmak için kullanılan bu tekniğe "influencer pazarlama" denilmektedir. [5,6] Türkçe'ye "hatırlı pazarlama, dijital pazarlama, tavsiye pazarlama, fenomen pazarlama, nüfuz pazarlaması, etkileşimli pazarlama, dijital kanaat önderliği vb." şeklinde aktarılmaya çalışılmıştır [3].

Sosyal medya fikir liderleri belirlediği pazarlama mesajını hedef kitlesinin ilk halkasını oluşturan alıcılara göndererek, zincirleme olarak yayılmasını beklemektedir [7]. Hedef kitleyi oluşturan zincirin tamamına ulaşmaya gerek yoktur. Çünkü bir halkanın kendisinden sonra gelen halkası potansiyel müşteridir [8]. Böylelikle firmaların ürün ve hizmetlerini geleneksel reklam anlayışıyla ulaşamayacağı, çok daha geniş kitlelere taşıması olanaklı hale gelmektedir.

Literatür incelendiğinde fenomen pazarlama yöntemiyle tanıtılan ürünler arasında güzellik ve kişisel bakım ürünlerinin [9], gıda ürünlerinin [10,11], moda ve giyim ürünlerinin [12-14] olduğu görülmektedir. Yeni trendler, stiller, markalar ve hatta mekanların önerildiği bu yöntem giderek tüketicilerin satın alım kararı verirken kullandığı en etkili yöntemlerden birisi olmaktadır. Sosyal medya fikir liderlerinin sağlığa ilişkin ürünlerin tanıtımında kullanımını konu almış bir tek çalışmaya rastlanmıştır [15]. Bu çalışmada fikir liderliğinin sağlığa ilişkin ürün tanıtımında kullanılmasının olası risklerinden bahsedilmiştir. Ancak eczacıların sosyal medya üzerinden takviye edici gıda ve bitkisel içerikli ürün tanıtımı ve pazarlanması noktasında fikir liderliği kullanımına bakışlarını ve böyle bir rolü benimsemek isteyip istemeyeceklerine dair yapılmış herhangi bir çalışmaya rastlanmamıştır.

Türkiye'de takviye edici gıda ve bitkisel içerikli ürünlerin Sağlık Bakanlığı'ndan ruhsat alma zorunluluğu olmadığı için internet ortamlarında satışı ve reklamı önünde herhangi bir engel bulunmamaktadır [16]. Dolayısıyla son zamanlarda sosyal medya platformlarında fikir liderleri kullanılarak pazarlanan ve tanıtılan ürünler arasında takviye edici gıda ve bitkisel içerikli ürünlerin bulunduğu görülmektedir [15]. Özellikle COVID-19 sonrasında ilgili ürünlerin immün sistemi destekleyici olduğu öne sürülerek çoğunlukla sağlıkla ilişkisi bulunmayan sosyal medya fikir liderleri tarafından pazarlanması durumuna sıklıkla tanık olunmaktadır [15,17]. Bu durum bir sağlık profesyoneli

kontrolünde kullanılmazsa takviye edici gıda ve bitkisel içerikli ürünlere ilişkin halk sağlığı sorunlarını beraberinde getirebilmektedir [18]. Nitekim literatür incelendiğinde bu ürünlerin gerek içerik gerek üretim teknolojisi gerekse de halka reklam ve tanıtımlarının yapılması sebebiyle akılcı olmayan kullanımı nedeniyle halk sağlığını tehdit ettiğini gösteren pek çok çalışma ile karşılaşılmaktadır [19,20].

Toplum eczacıları kolay ulaşılabilir olmaları ve birinci basamak sağlık hizmeti sunumunda görevli olmaları dolayısıyla takviye edici gıda ve bitkisel içerikli ürünlerin güvenli ve etkin kullanımı konusunda kritik öneme sahiptir [21]. Yapılan pek çok çalışmada eczacılar, takviye edici gıda ve bitkisel ürünlerin eczacılık hizmetlerinin birer parçası olduğunu ve bu ürünlerin halka ulaştırılmasında eğitici ve bilgilendirici olma sorumluluğunu üstlenmeleri gerektiğini ifade etmektedirler [20-22]. Yapılan bir sistematik derlemede eczacıların takviye edici gıda ve bitkisel içerikli ürünlerin güvenli kullanımı, yan etkilerinin raporlanması, hastaların eğitilmesi ve bilinçlendirilmesi ve bu ürünlerle ilgili diğer sağlık profesyonelleri ile iş birliği kurulması noktasında görev ve sorumluluk üstlenmesi gerektiği bildirilmiştir [23].

Bu çalışma ile Türkiye'de toplum eczacısı olarak faaliyet gösteren eczacıların sosyal medya platformlarında takviye edici gıda ve bitkisel içerikli ürün tanıtımında ve pazarlanmasında fikir liderlerinin kullanımına bakışlarının derinlemesine anlaşılması amaçlanmaktadır. Bu çalışma, eczacıların, bu ürünlerin sosyal medyada pazarlanması yoluyla tanıtımı sonucu oluşacak tıbbi sorunların ortadan kaldırılabilmesi açısından, ileride olası bir sosyal medya fikir liderliği rolünü üstlenip üstelenmemek noktasında görüşlerinin ortaya çıkarılması açısından önemli görülmektedir.

GEREÇ VE YÖNTEM

Araştırmanın Modeli

Bu araştırma nitel araştırma deseninde durum çalışması olarak planlanmıştır. Nitel araştırmalarda durum çalışmaları herhangi bir olgu ya da olayın derinlemesine anlaşılmasına olanak vermektedir [24]. Dolayısıyla bu çalışmada toplum eczacılarının takviye edici gıda ve bitkisel içerikli ürünlerin sosyal medyada fikir liderleri yoluyla pazarlanmasına ilişkin görüş ve deneyimlerinin kendi bağlamı içerisinde derinlemesine incelenmesi amaçlandığından, araştırmada sözü edilen yöntem ve model kullanılmıştır.

Katılımcı Grubu

Bu çalışmaya Ankara ilinde toplum eczacılığı yapan eczacılar dahil edilmiştir. Katılımcı grubunun belirlenmesinde amaçlı örnekleme yöntemlerinden ölçüt örnekleme kullanılmıştır. Katılımcıların belirlenmesinde dikkat edilen ölçüt, sosyal medya hesabı olmak ve sosyal medyada aktif zaman geçiriyor olmaktır (günde en az yarım saat). Araştırmaya katılım ölçütü olarak belirlenen sosyal medya hesabı olmak ve günde en az yarım saat bu hesaplarda zaman geçirmek ile ilgili bilgilere potansiyel katılımcıların beyanları sonucu erişilmiş olup, her iki ölçütü de karşılayan katılımcılar onam vermeleri sonucunda araştırmaya dahil edilmişlerdir. Ölçüt örneklemenin yanında kar topu yönteminden de yararlanılmıştır. Araştırmaya katılımcı olmayı kabul etsin etmesin ulaşılan toplum eczacılarından araştırma için potansiyel katılımcı olabilecek en az bir isim talep edilmiştir. Katılımcıların önerdiği her eczacıyla kar topu yöntemi dahiline iletişim kurulmuştur.

Verilerin Toplanması

Araştırmada veri toplamak için yarı yapılandırılmış görüşme tekniği kullanılmıştır. Bu doğrultuda literatürden faydalanarak bir görüşme formu oluşturulmuştur. Görüşme formu oluşturulurken üç farklı uzmanın içerik, dil ve anlatım yönünden görüşleri alınmıştır. Bu uzmanların görüşleri doğrultusunda görüşme formuna bir yeni soru ilave edilmiş ve gerekli düzenlemeler yapılmıştır. Soruların işlerliği ve akıcılığının test edilmesi için bir pilot çalışma yapılmıştır. Görüşme formundaki sorular iki toplum eczacısına yöneltilmiş elde edilen veriler araştırmaya dahil edilmemiştir. Bu pilot çalışma sonrası iki soruda düzenlemeye gidilmiş ve görüşme formu son şeklini almıştır.

Nitel görüşmeler gönüllü mahremiyetinin korunacağı bir mekânda yüz yüze yapılmıştır. Görüşmeler sırasında gönüllünün onay vermesi halinde ses kaydı alınmıştır. Yüz yüze görüşmelere başlamadan önce katılımcılar bilgilendirilmiş olur formunu imzalamışlardır. Görüşmeler yaklaşık 20-50 dakika arasında sürmüştür. Görüşmelerden elde edilen veriler birbirini tekrar etmeye başladığında

veri satürasyonuna ulaşılması dolayısıyla veri toplama sonlandırılmıştır. Yüz yüze görüşmeler Haziran-Ekim 2022 tarihleri arasında gerçekleştirilmiştir.

Verilerin Analizi

Tamamlanan görüşmeleri takiben görüşmede esnasında tutulan kısa notlar ve görüşmenin ses kaydı katılımcının kimliğini anonimleştirerek (katılımcılar K1, K5 vb. sembolize edilmiştir) transkripsiyon yoluyla yazıya dökülmüştür. Veriler ham şekilde kâğıda döküldükten sonra tematik analiz yoluyla çözümlenerek kodlanmıştır. Veriler arasında bağlantı kurabilmek amacıyla temalar ve alt temalar oluşturulmuştur.

SONUÇ VE TARTIŞMA

Çalışmaya 12 toplum eczacısı katılmış olup, katılımcılara ait demografik bilgiler Tablo 1'de gösterilmiştir. Yapılan tematik analiz sonucunda araştırma bulguları üç ana tema altında toplanmıştır.

Tablo 1. Katılımcılara ait demografik bilgiler

Katılımcı	Cinsiyet	Eğitim Durumu	Eczane Lokasyonu	Takviye Edici Gıda ve Bitkisel Ürünlerin Cirodaki Dağılımı (%)	Sahip Olunan Sosyal Medya Hesapları	Günlük Sosyal Medyada Geçirilen Tahmini Süre (saat)
K1	K	Y.Lisans	Semt	35	Instagram Facebook Snapchat Linkedin	1
K2	K	Lisans	Cadde	35	Instagram Twitter	1
K3	K	Lisans	Semt	40	Instagram	2-3
K4	E	Doktora	Hastane karşısı	5	Instagram Facebook	1
K5	E	Lisans	Semt	40	Instagram Facebook	1-1.5
K6	E	Y.Lisans	Hastane karşısı	10	Instagram Facebook	2
K7	K	Lisans	AVM	65	Instagram Facebook	2-2.5
K8	E	Lisans	Cadde	45	Instagram Twitter	1
K9	E	Y.Lisans	Semt	30	Instagram Facebook Twitter	3
K10	K	Y.Lisans	Semt	35	Instagram	2
K11	E	Lisans	Cadde	35	Instagram	1
K12	K	Doktora	Semt	20	Instagram Facebook Twitter	1

Bunlar "pazarlamada fikir lideri kullanımına ilişkin tutumlar", "fikir liderlerinin özellikleri" ve "fikir liderliği rolünü benimseme"dir. Her bir temaya ilişkin alt tema ve alt temaları temsil eden kodlamalar katılımcılarla yapılan görüşmelerden aynen alıntılama yaparak sunulmuştur. Çalışmaya ait tema ve alt temalar Tablo 2'de verilmiştir.

Tablo 2. Ana tema ve alt temalar

Ana Tema	Alt Tema	Katkı Sunan Katılımcılar
Pazarlamada fikir lideri kullanımına ilişkin tutumlar	Ilımlı görüşler: Kaçınılmaz gerçek	K1, K3, K7
	Karşıt görüş: Halk sağlığı tehdidi	K1, K3, K5, K6, K7
Fikir liderlerinin özellikleri	Duygusal bağlılık	K1, K8
	İmrenme	K9, K10
	Takipçi sayısı	K1, K3, K6, K7, K12
	Fiziksel özellikler	K1, K2, K3, K8, K9, K10, K11
	Bilinçli paylaşım	K1, K2, K3, K4, K6, K10
Fikir liderliği rolünü benimsemenin önündeki engeller	Mevzuat değişikliği	K4 haricinde hepsi
	Yetkin kullanım	K7, K12

Pazarlamada Fikir Lideri Kullanımına İlişkin Tutumlar

Ilımlı Görüşler: Kaçınılmaz Gerçek

Çalışmaya katılan toplum eczacıların bir kısmı dijital çağın gelmesiyle birlikte takviye edici gıda ve bitkisel ürünlere ilişkin pazarlama faaliyetlerinin sosyal medya platformlarında yapılmaya başlanmasının kaçınılmaz olduğunu ifade etmişlerdir.

> *"… sosyal medya büyük bir kirlilik, aynı zamanda da geleceğin gittiği yer. Geleceğin şekillendiği yer. Bence çok yakın gelecekte mağazalardan, eczanelerden ürün anlatımı danışmanlık bunların hiçbiri kalmayacak. Her şey online'a dönecek.*" K1, 70-72

Bu konuya ılımlı bakan eczacıların bu tanıtımı yapan kişilerin satış odaklı değil yalnızca bilgilendirme amaçlı yapmaları gerektiğini belirtmişlerdir.

> *"Yani hani evet yapılabilir ama çok genel bilgiler verilmeli … bunu mutlaka kullanın gibi değil de örnek veriyorum uyku probleminiz varsa pasiflora diyebilir, melisa diyebilir ama mutlaka eczacınıza danışın şeklinde bir kısım olmalı paylamışında…" K2, 140-143*

Katılımcıların bazıları sosyal medya platformlarında takviye edici gıda ve bitkisel ürünlerin tanıtımlarının kapsamına ilişkin görüş bildirmişlerdir. Bu ürünler hakkında bilgilendirmenin mutlaka sağlık profesyonelleri yoluyla yapılması gerektiğini savunmakla birlikte bu ürünler hakkında bilgilendirme dışında satış odaklı pazarlama faaliyetlerine karşı oldukları anlaşılmıştır:

> *"Pazarlanmasına karşıyım. Çünkü meslek etiğimize aykırı buluyorum. Ama doğru bilgiyi verecekse yine eczacılar vermeli" K6, 92-93*

Günümüzde sosyal medyanın kullanımının hızla artması, bu mecraları çeşitli ticari ürünlerin tanıtılması ve pazarlanması için elverişli kılmıştır. Bu noktada popülerlik kazanan sosyal medya fikir liderleri yeni bir iş kolu olarak karşımıza çıkmaktadır. Takviye edici gıda ve bitkisel ürün tanıtılmasında ve pazarlanmasında fikir lideri kullanımı önemli bir araç olarak görülmektedirler. Eczacıların bu ürünlerin tanıtımı ve bilgilendirilmesi noktasında sosyal medya platformlarında aktif rol alıp alamayacağı günümüz gelişen teknolojileri göz önüne alındığında irdelenmesi gereken bir durumdur. Bu çalışmada bazı eczacılar bu konuda ılımlı görüş bildirirken, diğerleri de eczacıların fikir liderliği rolüne karşıt görüş bildirmişlerdir. Eczacıların sosyal medyada fikir liderliği rolü hakkında yapılan tartışmalarda eczacıların bu konuda benzer şekilde çekimser olduğu görülmektedir [25].

Karşıt Görüş: Halk Sağlığı Tehditi

Katılımcıların çoğunluğu bu ürünlerin sosyal medyada sağlık eğitimi almamış fikir liderleri yoluyla pazarlanmasının ciddi bir halk sağlığı tehditi yaratacağını savunmuşlardır:

> *"Şu andaki gözlemlediklerim genelde satış yapmıyor, yönlendiriyor. İşte Trendyol... büyük satış sitelerine yönlendiriyor… fakat yapılmalı mı? Kesinlikle internetten yapılmamalı. Çünkü*

mesela bununla ilgili birçok benzer şey var … Amerika'da çok ilaç zehirlenmesi yaşanıyor… bu ayakkabı ya da işte eldiven gibi değil ki rengini beğeneyim o da bana uysun. Böyle bir şey değil… İnsan sağlığı için gerçekten kötü olur bence." K3, 120-127

"… az önce dediğim gibi hastalık yok hasta var… kişiye özel olması gerekiyor. Çünkü biz o kişinin … diyetini bilmiyoruz … kronik hastalıklarını, kullandığı ilaçlarını bilmiyoruz. Bilgilendirsek de o karşıya ne kadar geçiyor … Özellikle mesela zayıflama ürünlerinde direkt insanlar sadece zayıflatır mı zayıflatmaz mı? Ona odaklanıyor. Siz bir zayıflatma ürününü diyelim sosyal medyada tanıttığınızda bunun yaralarını, zararlarını, ilaç etkileşimlerini her şeyini anlattığınızı eminim o yine sadece kullanıcı yararlarına odaklanıp 'ya bana bir şey olmaz' deyip onu kullanmak isteyebilir." K5, 78-86

Katılımcıların bazıları ise fikir lideri sağlık profesyoneli olsa dahi sosyal medya üzerinden takviye edici gıda ve bitkisel ürünlerin pazarlama faaliyetlerinin ve tanıtımlarının bilgilendirme amaçlı bile olsa yapılmaması gerektiğini ifade etmişlerdir.

"Bir eczacının, bir doktorun kendi sayfasında ürün tanıtımı yapması olacak şey değil! Mesleğin doğasına aykırı! Biz ürün tanıtımı yapmalı mıyız? Kesinlikle hayır!" K8, 98-99

Fikir Liderlerinin Özellikleri

Çalışmaya katılan eczacılar tüketicilerin takviye edici gıda ve bitkisel ürünler hakkında alım niyeti oluştururken takip ettikleri fikir liderlerinde belirli faktörlerin etkili olduğunu belirtmişlerdir. Fikir liderlerinin sahip olduğu farklı özelliklerin ve sosyal medya hesaplarını yönetme biçimlerinin kullanıcıların dikkatini çektiğini, fikir liderlerini algılayışlarındaki çeşitli faktörlerin bu kişileri takip etmelerinde belirleyici olduğunu bildirmişlerdir. Dolayısıyla katılımcı eczacılar tüketicilerin takip ettikleri fikir liderlerinin paylaşımlarını bu sebeplerle dikkate aldıklarını savunmuşlardır. Fikir lideriyle kurulan duygusal bağlılık, fikir liderine imrenme, fikir liderinin takipçi sayısı, fikir liderinin fiziksel özellikleri ve yaptığı bilinçli paylaşımlar fenomenlerin takip edilmesi ve pazarlama stratejilerinin dikkate alınmasındaki unsurlar olarak ortaya çıkmıştır.

Duygusal Bağlılık

Katılımcılar sosyal medyada takip edilen fikir liderleri ile duygusal bir bağlılık kurulduğunu ve bu sebeple fikir liderlerinin önerdiklerinin ciddiye alınabileceğini ifade etmişlerdir:

Bence Türk insanı daha çok duygusal bağ kuruyor. Ne kadar güvenilir olduğuna baktığını zannetmiyorum ya da güvenilirliği neye göre ölçüyor bilmiyorum.' K1, 96-97

İmrenme

Çalışmaya katılan eczacılar sosyal medyada tüketicilerin takip ettikleri fikir liderlerinin hayatlarına imrendiğini ve kendisini onun yerine koyduğunu düşünmektedirler. Böyle durumlarda özenilen fikir liderinin tavsiyelerinin ve önerdiklerinin etkili olma potansiyelinin daha yüksek olduğunu ifade etmişlerdir. Dolayısıyla tüketicilerin fikir liderini örnek alarak onun kullandığı veya önerdiği takviye edici gıda ve bitkisel ürünleri alma eğiliminin fazla olabileceği ifade edilmiştir.

"Sanırım insanların … yaşadığı o bizim yapamadığımız şeyleri yapmasını seviyoruz ve karşıdaki insanla kendimizi bağdaştırdığımız için onu izlediğimizde bizde sanki aynı şeyleri yapıyormuş gibi hissediyoruz. O yüzden bir süre sonra onun kullandığı ürünleri de kullanırsak onun gibi olabileceğimizi düşünüyoruz belki bilinçaltında." K10, 57-60

Takipçi Sayısı

Katılımcıların bazıları fikir liderlerinin takipçi sayısının takviye edici gıda ve bitkisel ürün arayışında olan tüketicileri etkileyebileceğini vurgulamışlardır:

"Çok takipçisi varsa … çok kişi buna demek ki inanmış diyor." K1, 97-101

Fiziksel Görünüş

Katılımcılar fikir liderlerinin fiziksel görünüşlerinin veya çekiciliklerinin sosyal medyada takip edilmek ve önerdiklerinin dikkate alınması için önemli unsurlar olduğunu bildirmişlerdir:

"Hani bunlar … çok güzel, genelde kadınlar çok bakımlı … bütün kadınların olmak istediği gibi hani olmak istediği bir görünüme sahip gür saçlar işte pasparlak yüz! Tabii bunlara dayanarak da bunu kullanarak da … birtakım şeylerin reklamını yapıyorlar… gördüğü zaman insan aa diyor bak 'saçı ne kadar güzel' işte 'ne kadar çekici bir kadın' işte … bilmiyorum onun yüzünden herhalde. Başka bir şey aklıma gelmiyor yani. Bilgi değil çünkü." K2, 93-99

Bilinçli Paylaşım

Katılımcıların bazıları takip edilen fikir liderlerinin takviye edici gıda ve bitkisel ürün önerirken yaptığı paylaşımlarda bir uzmana yönlendirme yaklaşımını benimsiyorsa tüketicilerin bu fikir liderlerini daha sıklıkla takip ettiği ve paylaşımlarını daha çok ciddiye aldığını savunmuşlardır:

"İşte yani bazıları da gerçekten hani iyi influencerlar… bir ürünü öneriyor ama altına şey yazıyor. Doktora ya da eczacınıza danışın muhakkak diyen birçok insan var. 'Bunu ben kullandım evet. Bana uygundu. Ama siz kullanırken muhakkak bir bilene danışın' diyen de oluyor. Bunlara da saygılıyız. Sonuçta biz de takip ediyoruz." K3, 74-77

Bu çalışmada eczacılar, tüketicilerin sosyal medya hesaplarında takip ettikleri fikir liderlerinde aradıkları özelliklere ilişkin çeşitli görüşler bildirmişlerdir. Eczacılara göre tüketicilerin takviye edici gıda ve bitkisel ürün konusunda bir fikir liderinin aktardıklarını dikkate alabilmesi için onunla duygusal bağ kurması gerekmektedir. Yapılan bir çalışmada duygular ve duygusal durumlar kişilerin motivasyonunu, muhakemesini ve satın alma kararını etkilediği gösterilmiştir [26]. Bu çalışmada insanların özendiği ve yaşam şekline imrendiği fikir liderlerinin aktardıklarını ciddiye alabilecekleri sonucu ortaya çıkmıştır. Yapılan bir çalışmada tüketicilerin satın alım davranışlarındaki artışın, Instagram'daki fikir liderleriyle kendileri arasında kişisel ilgi ve meraklarıyla doğru orantılı olarak kurdukları bağın fazla olduğu zamanlarda gerçekleştiğini göstermektedir [1]. Fikir liderlerinin takipçi sayısının tüketicileri etkileyebilecek bir diğer unsur olduğu görülmüştür. Yapılan bir çalışmada fikir liderlerinin birden fazla kişiye ulaşması ve etkileme yeteneğine sahip olması, takipçileriyle sürekli iletişim halinde olmaları ve tanıttıkları ürün veya hizmetlerde uzman olmaları dolayısıyla takip edildiklerinden bahsedilmektedir [27]. Bu çalışmada eczacılar, fikir liderlerinin önerdikleri ürünlere ilişkin yaptıkları paylaşımlarda mutlaka bir sağlık profesyoneline yönlendirme yapmasını bilinçli paylaşım olarak nitelendirmiştir. Böyle bir yaklaşımı benimseyen fikir liderlerinin tüketiciler tarafından daha güvenilir algılanabileceği ortaya konmuştur. Tüketicilerin bu tür ürünlerin etkililiği ve güvenliği konusunda farkındalıklarının düşük olduğu ve hatta bitkisel içerikleri dolayısıyla güvenli olduğu algısının yaygın olduğunu gösteren çalışmalar mevcuttur [18,28]. Dolayısıyla halkın farkındalığının düşük olduğu ve sağlığı tehdit etme potansiyeli olan bu ürünlerle ilgili yapılan fikir liderlerinin paylaşımlarında eczacıların bilinçli paylaşım noktasındaki beklentilerinin haklı olduğu söylenebilir. Bu çalışmada fikir liderlerine dair tüketicileri etkileyebileceği düşünülen bir başka unsur ise fikir liderinin fiziksel görünüşü olmuştur. Benzer olarak sosyal medya kullanıcılarının fikir liderlerini takip etme sebebi olarak çekiciliğin gösterildiği pek çok çalışma mevcuttur [4,29].

Fikir Liderliği Rolünü Benimsemenin Önündeki Engeller

Çalışmaya katılan eczacılar, sosyal medya ve dijital platformlar üzerinden takviye edici gıda ve bitkisel ürünlere ilişkin bilgilendirme bağlamında sağlık profesyonellerinin fikir liderliği rolünü üstlenmesi gerektiğini belirtmişlerdir. Ancak bu rolü benimseyebilmek için birtakım şartların yerine getirilmesi gerekmektedir.

Mevzuat Değişikliği

Eczacıların bu rolü Türkiye'deki eczacılık hizmetleri bağlamında üstlenebilmesi için Türk eczacılık mevzuatının değiştirilmesi gerektiği öne sürülmüştür:

"*… teknolojinin gittiği noktada kimse artık mağazalardan alışveriş etmiyor yani bir sürü dünya zinciri dünya devi marka bile mağazalarını kapatıyor. İnsanların alışveriş eğilimleri değişti. Dolayısıyla zamana ve çağa adapte olmak gerekir. Dolayısıyla ben gerekli yasal zemin oluşturulduktan sonra eczaneler eliyle pazarlama faaliyetlerinin online'da yapılması gerektiğini düşünüyorum. Çünkü biz yapmazsak başkaları bunu bir şekilde yapıyor.'* K1, 147-152

Yetkin Kullanım

Çalışmaya katılan bir grup eczacı ise fikir liderliği rolüne her eczacının uyum sağlayamayacağını dijital ortamları daha yetkin kullanabilen eczacıların bu rolü üstlenebileceğini vurgulamıştır:

> *"Benim yaşım 48. Benim bile internet ve teknoloji kullanımıyla ilgili problemlerim oluyor… onlardan (genç nesli kastediyor) bir tık daha gerideyiz biz. Daha Z kuşağından kişilerin bu işe girmesi lazım."* K12, 154-155

Bu çalışmada katılımcılar, gelecekte eczacıların sosyal medyada fikir liderliği rolü benimseyebilmesinin önündeki engellerden ilkinin yasal düzenlemeler olduğunu belirtmişlerdir. Türkiye'de eczacıların sağlık ve ilaca dair herhangi bir ilaç veya ürünün satış ve tanıtımını eczane dışında yapması yasaktır [30]. Ancak dijital çağın gerekleri göz önüne alındığında, sağlık ürünleri de dahil pek çok ürünün internet ortamında pazarlandığı günümüzde, eczacılara bu konuda yetki verecek gerekli mevzuat düzenlemelerinin yapılması için çalışmalara başlanması gerektiği görülmüştür. Böylelikle halk sağlığı açısından kritik olabilecek bu ürünlerin sosyal medya ortamlarında denetimsiz bir şekilde işin ehli olmayan kişiler tarafından değil de konunun uzmanı eczacılar tarafından daha güvenli bir şekilde yapılabileceği ön görülmektedir. Aynı zamanda başta eczacılar olmak üzere sağlık profesyonellerinin sosyal medya üzerinde fikir liderliği rolü üstlenebilmesi için dijital ortamların farklı dinamiklerine hâkim olmak gerektiği ve bu konuda bir yetkinliğin olması gerektiği ortaya çıkmıştır.

Bu bağlamda, bu çalışmada elde edilen sonuçların gelişen teknolojinin etkisiyle tüm dünyada dönüşmeye başlayan eczacılık pratiğini yansıtacak şekilde yapılandırılacak mevzuat değişikliklerine ve ileriki dönemde bu doğrultuda yapılacak eczacılık eğitim müfredatına ilişkin düzenlemelere ışık tutabileceği düşünülmektedir.

TEŞEKKÜR

Çalışmaya katılarak görüşlerini sunan eczacılara teşekkür ederim.

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Bu makale için gerçek, potansiyel veya algılanan çıkar çatışması bulunmamaktadır.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

FORMULATION AND *IN VITRO - IN VIVO* EVALUATION OF TETANUS TOXOID-MANNITOL DRY POWDER INHALATION FOR PULMONARY DELIVERY

PULMONER VERİLİŞE YÖNELİK TETANOZ TOKSOİD-MANNİTOL KURU TOZ İNHALASYON FORMÜLASYONU VE İN VİTRO - İN VİVO DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *As the conventional vaccines were accompanied by the limitations of pain, cold chain storage and sterility issues, a mucosal vaccine which is administered through pulmonary route was fabricated. A dry powder inhalation of tetanus toxoid (TT) and mannitol was prepared and evaluated for stability and immunogenicity in comparison to the conventional TT vaccine.*
Material and Method: *TT and mannitol dry powder inhalation was prepared and evaluated for particle size analysis, scanning electron microscopy, FTIR, flow properties, TT content estimation, flocculation, and in vitro drug vaccine release studies. Immunological studies of the formulation were performed on BALB/c mice.*

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Result and Discussion: *The powder blend of tetanus toxoid and mannitol remained stable under the process conditions and after storage. The result was confirmed through a flocculation test. The FTIR analysis indictated no interactions between the components. The homogenization process yielded a powder with a geometrical particle size diameter of 1312 ± 1310.9 nm which was found suitable for pulmonary administration. The zeta potential and polydispersity index {PDI} were found to be -22.6 ± 0.16 mV and 0.499 ± 0.015, respectively. The diffusion studies indicated immediate release of the TT with 82.4 ± 6.7% of drug released within 2 h following the diffusion mechanism and zero order kinetics and it was found that mannitol didn't retard the release of tetanus toxoid. Additionally, the flow properties of the dry powder inhalation were reported to have good flow properties. More importantly, the immunological studies inferred the induction of high systemic and mucosal immunity over conventional vaccines.*
Keywords: *Dry powder inhalation, mucosal immunity, Tetanus toxoid, pulmonary administration, vaccine*

ÖZ

Amaç: *Konvansiyonel aşılara ağrı sınırlamaları, soğuk zincir depolama ve sterilite sorunları eşlik ettiğinden, pulmoner yolla uygulanan mukozal bir aşı üretimiştir. Tetanoz toksoidi (TT) ve mannitolün kuru toz inhalasyonu hazırlanmış ve konvansiyonel tetanoz toksoid aşısına kıyasla stabilite ve immünojenisite açısından değerlendirilmiştir.*
Gereç ve Yöntem: *TT ve mannitol kuru toz inhalasyonu hazırlandı ve partikül boyutu analizi, FTIR, akış özellikleri, kapsülleme etkinliği, flokülasyon ve in vitro ilaç aşısı salım çalışmaları açısından değerlendirildi. Formülasyonun immünolojik çalışmaları, BALB/c fareleri üzerinde gerçekleştirildi.*
Sonuç ve Tartışma: *TT ve mannitolün toz karışımı, işlem koşulları altında ve depolama sonrasında stabil kalmıştır. Sonuç, bir flokülasyon testi ile doğrulanmıştır. FTIR analizine göre hiçbir etkileşim tespit edilmemiştir. Homojenizasyon işlemi ile, 1312 ± 1310.9 nm'lik geometrik partikül boyutuna sahip, pulmoner uygulama için uygun bulunan bir toz elde edilmiştir. Zeta potansiyeli ve polidispersite indeksi (PDI) sırasıyla -22.6 ± 0.16 mV ve 0.499 ± 0.015 olarak bulunmuştur. Difüzyon çalışmaları, difüzyon mekanizması ve sıfır derece kinetiği takiben 2 saat içinde açığa çıkan etkin maddenin %82.4 ± %6.7'si ile TT'nin derhal salındığını göstermiş ve mannitolün tetanoz toksoidinin salınımını geciktirmediği bulunmuştur. Ek olarak, kuru toz inhalasyonunun akış özelliklerinin iyi akış özelliklerine sahip olduğu rapor edilmiştir. Daha da önemlisi, immünolojik çalışmalar, geleneksel aşılara göre yüksek sistemik ve mukozal bağışıklığın indüklendiğini ortaya çıkarmıştır.*
Anahtar Kelimeler: *Aşı, kuru toz inhalasyonu, mukozal bağışıklık, pulmoner uygulama, Tetanoz toksoidi*

INTRODUCTION

Tetanus is a deadly sickness that produces convulsions (seizures) and severe muscle spasms, which can result in spinal bone fractures. In 30% to 40% of instances, tetanus results in mortality. Tetanus vaccination is advised for all newborns 6 to 8 weeks of age and older, as well as all children and adults [1, 2]. It has been an integral part of maternal care for the prevention of neonatal tetanus [3]. A series of three to four shots is given during maternal care. It is advised during minor cuts and surgeries. Tetanus toxoid (TT) vaccination is known to induce short-lived immunity in patients [2]. As it is apparent, the introduction of the vaccination programmes was found to be effective in controlling the disease [2]. Despite the benefits of the intra muscular tetanus toxoid vaccine, it is also accompanied by limitations of sterility issues, pain, cold chain storage, and minimal induction of mucosal immunity. Mucosal linings are exposed to a wide variety of microorganisms and are naturally provided with immune components to restrict microbial entry [4-9]. It indicates the potential for the administration of vaccines through mucosal ports. In this regard, mucosal vaccines are available on the market, which are also known for their non-invasive approach. Oral, nasal, sublingual, vaginal, and pulmonary vaccine delivery routes have been investigated and reported for conferring mucosal and systemic immunity [10-13]. Mucosal immune responses are increasingly being recognised as critical for disease protection [14]. Vaccines administered through mucosal ports produce mucosal immune

responses most effectively, whereas vaccines injected are generally poor inducers of mucosal immunity and hence less effective against infection at mucosal surfaces [14]. Nonetheless, clinical vaccination development has relied heavily on antigen injection, and the majority of vaccines currently in use are given intramuscularly or subcutaneously. Several observations have implied a paradigm shift for mucosal vaccine delivery [15]. When compared to other delivery routes, pulmonary delivery has a number of advantages, including rapid drug uptake, a wide surface area for solute transport, and good bioavailability, as well as its non-invasive nature, rich vasculature, and minimum proteinase activity [16]. Additionally, pulmonary routes have evidenced the induction of mucosal antibodies in the bronchial associated lymphoid tissue (BAL) fluids [17]. Although the benefits of the pulmonary tract are appealing, product deposition in the alveoli appears difficult. It is to be borne in mind that 2-5 μ particles are reported for efficient deposition in the lower respiratory tract [18,19]. In earlier studies, the micronization method was found to have a good chance of making content that could be used for deep lung delivery [20].

Previous studies reported the fabrication of particulates to micron size through homogenization [21]. Pulmonary products available on the market are metered dose inhalers (MDIs), soft mist inhalers (SMIs), nebulisers, and dry powder inhalers (DPIs) [22]. Metered dose inhalers have long been popular due to their simple design and low cost, but they are reported for their inefficient drug delivery and use of propellants [23]. On the other hand, soft mist inhalers are expensive [24]. Although nebulisers are often used in hospitals, they require repeated heating of the product, which might damage the heat sensitive contents [25]. Among different inhalation products, dry powder inhalation is sought because of the benefits of drug administration efficiency, avoidance of propellants, and availability of the product form as a solid, further contributing to its stability [11]. Owing to its advantages, DPI is believed to be the right option for the administration of vaccine constituents. The DPI formulations are required to possess suitable parameters for effective deposition of the particles in the lungs. Changes in the shape, size, surface energy, and surface content of the drug particles can have a considerable impact on the aerosol performance of dry powder inhalation. In order to improve powder aerosolization, the physico-chemical properties of the powders can be optimised by adopting suitable techniques and formulation compositions [26]. It was observed that homogenisation of the admixture of powders yielded micron-sized particles [26]. Mannitol is known for its wide use in DPI's and has also been reported for its use in market formulations [27-30]. Due to its established toxicity profile and blends of DPI's with sufficient fine particle fraction, it is selected for the given study for the preparation of DPI's of mannitol and TT. Additionally, mannitol has been reported for its promising features for its use in dry powder inhalation. Its non-hygroscopic nature makes it a suitable carrier for the administration of proteins. Mannitol's sweet aftertaste serves as an indicator of dose deposition. Because conventional vaccines had disadvantages such as discomfort, cold chain storage, and sterility, a mucosal vaccine that is delivered via the pulmonary route was developed. In comparison to the standard TT vaccination, a dry powder inhalation of tetanus toxoid and mannitol was developed and tested for stability and immunogenicity.

MATERIAL AND METHOD

Mannitol was purchased from Sigma Aldrich chemicals, Bengaluru, India. Dano Vaccines and Biologicals Pvt. Ltd., Hyderabad, India, generously donated 1500 Lf/ml tetanus toxoid and commercial tetanus toxoid (5 ml vials each containing 5-25 Lf/ml, Batch No. 1923). Premium Serums and Vaccines Pvt. Ltd., Mumbai, India, provided Tetanus Antitoxin I.P. (3000 I.U./ml, Batch No. 141016). Bradford reagent was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Mannitol was procured from Sigma Aldrich, Mumbai, India. The ELISA kit was purchased from Elabscience, Houston, USA (Catalog No: E-EL-M0692). The remaining chemicals were all of analytical quality.

Formulation of Mannitol-TT Microparticles

Based on a previous report on DPI formulation, the quantity of mannitol was selected [31]. An accurate quantity (7.5 g) of mannitol, 300 limit of flocculation (Lf) of TT was added to 50 ml of

distilled water and stirred by homogeniser for a period of 15 min. The preparation was subjected to cool centrifugation for 30 min at 1000rpm. The supernatant was separated and the pellet was dried in a desiccator to obtain a dry powder of the formulation [21]. Three repetitions were procedure was repeted three times.

Particle Size Analysis

The TT formulation was dispersed in de-ionized water to achieve a 1% concentration and agitated at 100 rpm at 37°C. Using a 532 nm laser and a Zetasizer Nano Instrument (Malvern Instruments, Nano ZS, ZEN3600, UK), the particle size, zeta potential value, and polydispersity index of the DPI formulation were detected [32,33]. Samples are taken in triplicate.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy [33] was used to investigate the size and surface morphology of nanoparticles. The images were captured using a 5.0 kV field emission scanning electron microscope (Philips CM12, Eindhoven, Netherlands) at a 9.7 mm working distance. Tetanus toxoid nanoparticles were dispersed in *n*-hexane and sonicated for 20 min at a concentration of 50 µg/ml and placed on a specially polished sample grid for sample preparation. Before microscopical analysis, the samples were vacuum-dried for 18 h and then sputter-coated with a 20 nm gold layer.

Fourier Transform Infrared Spectroscopic Analysis (FTIR)

A Shimadzu 435 U-04 IR spectrophotometer, Japan, was used to analyse FTIR spectra for pure tetanus toxoid and the formulation as KBr disc. The characteristic peaks obtained in the optimised formulation and the pure samples were recorded [33].

Determination of Flow Properties

The powder flow properties were determined as per the standards of United States Pharmacopoeia and mentioned by Lau. [34]. All the tests were performed in triplicate.

Bulk density is a parameter that indicates the degree of lightness of a powder. DPI requires being light and having a low bulk density. When the particles are loosely packed, it is characterised by high bulk volume and low bulk density. Powders with low bulk density are designated as "light powders". The cylinder is filled with a powdered substance. Tapping was done 50 times. The bulk volume was measured from the initial and final volumes after tapping. The measurements were substituted in the formula [34].

$$Bulk\ density = weight\ of\ powder/bulkvolume$$

When voids or air gaps between particles are removed, tapped density is often expressed in terms of mass per unit volume. Tapped density is an important parameter which indicates the flow properties. To determine the same, the powder was tapped 1250 times and the volume tapped was measured. The measurements determined are substituted in the formula [34].

$$Tapped\ density = Weight\ of\ powder/Tapped\ volume$$

The flow property of the DPI was determined by the angle of repose. The frictional forces determine the flow properties. The frictional forces are quantified by the angle of repose. It is the angle formed by the powder heap and the horizontal plane. For the determination of the same, an appropriate amount of the powder blend is allowed to form a pile on the horizontal plane by passing the mixture through a funnel fixed with a clamp to a stand. The height and radius of the pile were measured and the angle of repose was determined by the given formula. [34].

$$Tan\ \theta = h/r$$

Where h is the height (cm) of the pile, and R is the radius (cm) of the pile.

The Hausner ratio is a measure of interparticulate friction, and it is used to rate the flow character. A powder with a lower Hausner ratio has better flow characteristics. A Hausner ratio of > 1.25 indicates ‘poor’ flow. The Hausner ratio is calculated by the following formula.

$$H = \rho T / \rho B$$

Where, ρB is the freely settled bulk density of the powder, and ρT is the tapped bulk density of the powder.

TT Content Estimation in Microparticles

The amount of tetanus toxoid in micropartiles was measured by decanting the supernatant after centrifugation and quantifying the TT using the Bradford reagent UV-Visible spectrophotometric technique [35]. The binding of protein molecules to Coomassie dye under acidic circumstances resulted in a colour change from brown to blue in this experiment.

An appropriate amount of the given powder sample was transferred into a mortar and distilled water was added and rendered at 1 mg/ml and triturated. From the mixture, 30 µl of sample was mixed with 1.5 ml of Bradford reagent and assayed by a UV-Visible spectrophotometer at 295 nm [35,36].

Flocculation Test

The flocculation test was performed in triplicate to determine the loaded TT's structural integrity. The TT-mannitol formulation equivalent to 50 Lf was placed into a series of 6 flocculation tubes to conduct the test. Antitoxin was added to these flocculation tubes in graduated doses that differed by 10%, and the mixtures were incubated at 50 ± 1°C in a contanst water bath (Joanlab, Huzhou, China). The flocculation duration and antitoxin concentration at which the toxoid's initial flocculation was seen during incubation determine the toxoid's Lf value. The length of the flocculation (Kf) was recorded. This can be used to assess the toxin's effectiveness. The antigen quality is good if the Kf is low, and vice versa [37,38].

***In Vitro* TT Release Studies**

To simulate the conditions in the pulmonary region, the optimised nanoparticle formulations were put on a filter paper between the donar and receiver compartments of a Franz diffusion cell apparatus [13] containing pH 7.4 phosphate buffer. The apparatus was put on a magnetic stirrer and swirled continuously for 4 h, after which the samples were extracted at regular intervals and analysed using UV-Visible spectrophotometry at 295 nm with Bradford reagent.

Stability Studies

Stability studies were conducted to see how storage conditions and shelf life affected the prepared product. These experiments were carried out for a year at 4°C/0% RH and 25°C/60% RH, and flocculation tests were conducted to determine the stability [39].

***In Vivo* Efficacy Studies**

BALB/C mice weighing 18 to 25 g were chosen for this investigation and maintained in a HEPA-filtered environment with a 12 h light/12 h dark cycle and a constant temperature of 22°C. A normal diet was provided, as well as unrestricted access to water. Animals were separated into three groups (each with eight animals) and vaccinated with a dry powder mucosal vaccine inhalation formulation of 1 mg, equivalent to 0.5 Lf, and a traditional intramuscular immunization. Mice are initially anaesthetized with diethyl ether. The animals were quickly secured on the platform's 45° slant. Intubation was used to administer a tetanus toxoid dry powder formulation to anaesthetized animals. Each animal's tongue was gently taken out using blunt plastic-tipped forceps, and an otoscope was used to find the tracheal aperture in order to enter the cannula tube with the dry powder inhalation. A plastic syringe was filled with 1 ml of air and carefully attached to the top of the cannula tube to blow out the loaded dry particle inhalation. DPIs containing TT were breathed into the lungs of the animals [40]. The negative control group received saline solution intraperitoneally, while the standard group received the market formulation by intraperitoneal immunization. The animals received the original dose during the first week and the booster dose during the fourth week. Blood samples were obtained before immunisation and after 4, 6, 8, and 10 weeks. The sample tubes were allowed to clot at room temperature for 1 h before being centrifuged at 1000 rpm for ten minutes.

Serum samples were isolated and maintained at -20°C for the ELISA test to determine immunoglobulin G (IgG) titers [41].

ELISA Analysis

The total IgG titres in serum samples were determined using the Elabscience Mouse IgG ELISA kit and analysed using the sandwich method. The diluted standard, blank, and sample each had their own set of wells. In each well, 100 µl of standard dilution, blank, serum, and Bronchoalveolar lavage (BAL) samples were introduced to the respective wells. The plate was then incubated at 37°C for 90 min after the sealer was placed on it. After removing the liquid, 100 µl of Biotinylated Detection Ab working solution was added to the well, which was then incubated at 37°C for 60 min. The solution was decanted from each well, and 350 µl of wash buffer was added. The solution was allowed to sit on the plate for 1 min before being aspirated three times. After filling each well with a 100 l Horseradish peroxidase (HRP) conjugate working solution, the plate was capped and incubated at 37°C for 30 minutes. The liquid was decanted, rinsed, and aspirated five times. After that, a new sealer was applied to the plate, and 90 µl of substrate reagent was added to each well. The plates were kept dark and the wells were incubated at 37°C for around 15 min. After 50 µl of stop solution was added to each well, the plates were read at 450 nm. The standard was used to determine the concentrations of IgG in the samples.

RESULT AND DISCUSSION

DPIs are believed to be the right option for delivering vaccine constituents to the lungs. This is due to their stability, ease of use, and non-invasive administration [11]. In the present research, tetanus toxoid-mannitol microparticles were successfully prepared.

Particle Size Analysis Results

The geometric particle size distribution's volume weighted median, d(0.5), was 776.4 ± 4.2 nm. The size of a 10% volume of particles with a diameter less than d(0.1) was 348.9 ± 6.3 nm, while a 90% volume of particles with a diameter less than d(0.9) was 3099.4 ± 9.4 nm. Polydispersity index (PDI) is a measure of the variance in the molecular chain lengths and weights in a polymer. The PDI obtained was 0.499 ± 0.02, which indicate that monomer units of TT were arranged in chains of different length which will influence its antigenicity. This could be the reason why the commercial formulations of TT are available in 5-25 Lf/ml.

It was observed that the particles lying in the micron range find effective deposition for deep lung delivery. To achieve peripheral drug penetration in the event of pulmonary drug delivery for systemic absorption, fine particle size aerosols would be necessary. Particles smaller than 3 µ have an approximate 80% chance of reaching the lower airways, with 50–60% landing in the alveoli [42]. Smaller sizes below the micron range possess a high amount of free energy and form aggregates. They are characterised by poor flowability and aerosolization performance, and tend to stick to the inhaler. The negative charge of the particulates is believed to prevent the formation of aggregates due to the existence of repulsive forces. In terms of aerosol quality and efficiency, particle size distribution is critical [43]. It is frequently assessed using PDI. A higher PDI value for the carrier (mannitol) suggests a wider particle size distribution, resulting in a more heterogeneous drug combination. This may result in more variability in drug deposition in the lungs after inhalation [44]. The PDI obtained was 0.499 ± 0.02, which indicates partially homogenous dispersion of the particles.

Scanning Electron Microscopy (SEM) Results

The SEM images of pure TT showed descrete crystalline forms, whereas the TT-mannitol formulation showed the crystalline TT particles (white color) uniformly dispersed in mannitol crystals (grey color) as shown in Figure 1.

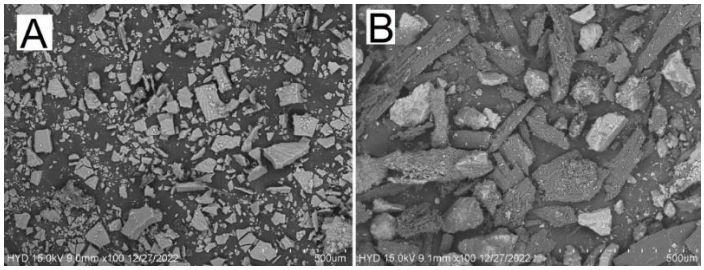

Figure 1. SEM images of (A) tetanus toxoid and (B) TT-mannitol formulation

Fourier Transform Infrared Spectroscopy (FTIR) Results

The FTIR spectra of pure TT and TT formulation were given in Figure 2, and the peaks were observed at 3416, 1646.2, and 629.6 cm^{-1}, while the formulation resulted in peaks at 3400 and 1600 cm^{-1}. The results of FTIR analysis suggest that tetanus toxoid remained intact during the preparation. The additional peaks indicate the molecular dispersion of mannitol and tetanus toxoid.

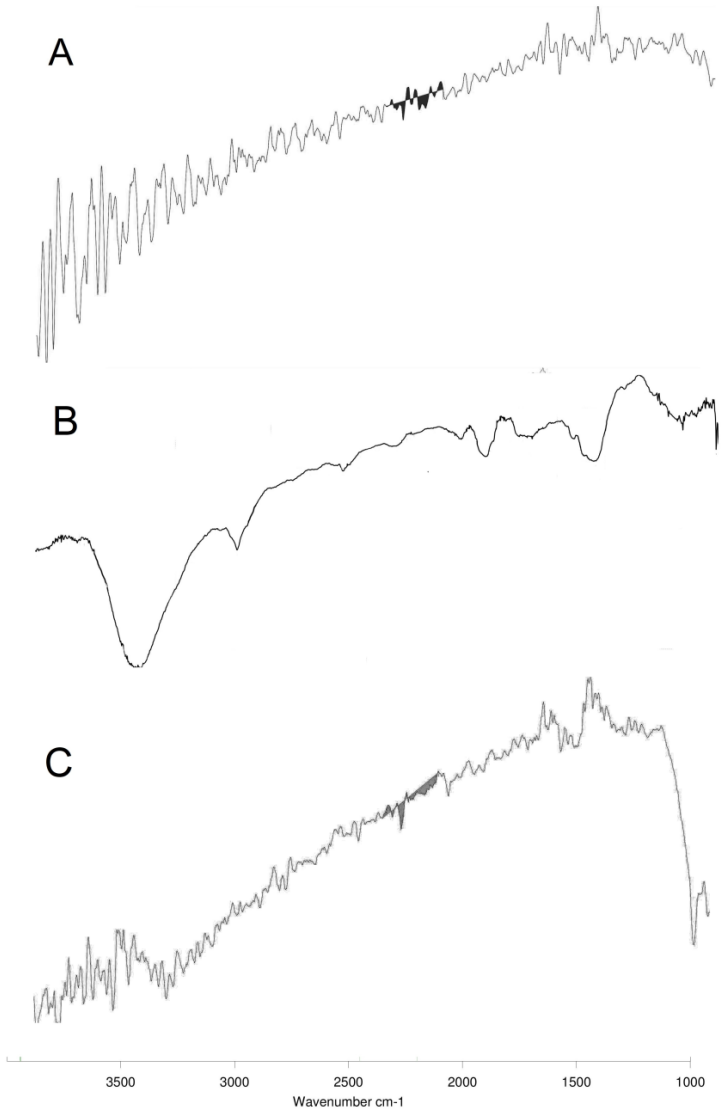

Figure 2. FTIR spectra of (A) mannitol, (B) tetanus toxoid and (C) TT-mannitol formulation

Flow Properties and TT Content Estimation Results

The flow properties of the powder blend are characterised by bulk density, tapped density, and angle of repose. The parameters should report less value for efficient flow. The dry powder inhalations should possess good flow properties to deagglomerate and disperse during breath actuation. The formulation of mannitol and tetanus toxoid dispersion has reported good flow properties as shown in Table 1. The TT content of the given formulation was found to be 95.3 ± 3.1 % and the drug content of the sample indicated 94 ± 1.8 %. Furthermore, non-hygroscopic nature of mannitol contributes to the free flowing properties of the TT-mannitol formulation.

Table 1. Determination of flow properties

Parameter	Formulation
Angle of repose (θ)	18.1 ± 1.6
Bulk density (gm/cm^3)	0.51 ± 0.01
Tapped density (gm/cm^3)	0.65 ± 0.01
Hausner ratio	1.27 ± 0.01

All values are average of three determinations

Flocculation Test Results

Limes flocculation means, when the concentration of toxin, or toxoid, is held constant while the concentration of antitoxin is altered in constant volume mixtures, the mixture that flocculates first includes the most nearly equivalent quantities of toxin, or toxoid, and antitoxin [44]. The antigenicity of tetanus toxoid was confirmed by the development of flocculation with antitoxin, as shown in Figure 3. The presence of flocculation was found after 14 ± 0.5 min. The World Health Organization's Expert Committee on Biological Standardization determined that flocculation tests using reference toxoids are extremely reliable and reproducible [37]. The flocculation test determines the sample's stability and antigenicity. Moreover, the observed Kf indicates the toxoid's purity and stability in the formulation. Furthermore, the flocculation test was carried out after the material had been kept at room temperature. As a result, the tetanus toxoid dry powder inhalation can be stored at ambient temperature without the need for cold chain storage. The presence of flocculation found after 14 ± 0.5 min indicated that the sample was stable. Similar results were obtained for tetanus toxoid vaccine formulation with chitosan microspheres in earlier study [45].

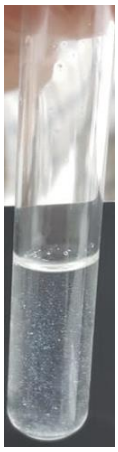

Figure 3. Flocculation test of Mannitol-TT formulation

In Vitro TT Release Studies Results

In vitro diffusion studies were conducted using the Franz diffusion cell apparatus. As observed

in Figure 4, there was immediate release of TT and 82.4 ± 6.7% of the drug was released within 2 h. The zero-order kinetics and Higuchi plot showed r^2 of 0.869 and 0.958, respectively. Similar release of pure TT was observed with and 88.3 ± 4.15% of the drug was released within 2 h. The zero-order kinetics and Higuchi plot showed r^2 of 0.84 and 0.952, respectively. Student t-test was performed on the drug release patterns, in whicih which the *t*-value obtained was -0.16155 and the *p*-value as 0.43684. Therefore the TT release from pure TT and formulation was *not* significant at $p < 0.05$.

The results of *in vitro* TT release studies indicated that mannitol didn't significantly interfere in the release of TT. From the above results, the release was found to follow zero-order kinetics according to the r^2 values. Also, the Higuchi plot's r^2 values showed that the way TT was released from the formulation was through diffusion.

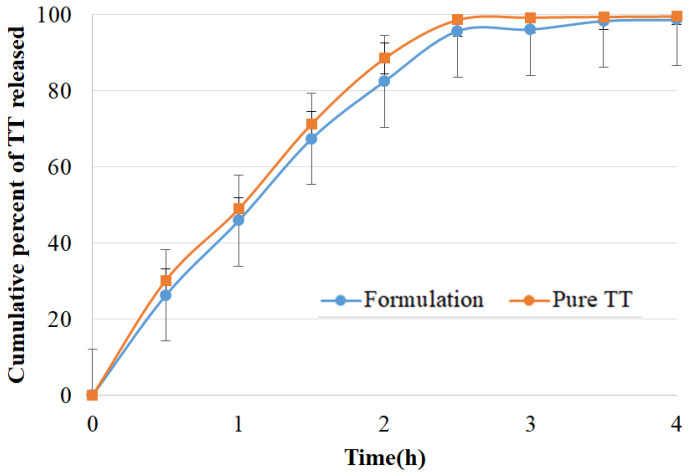

Figure 4. Diffusion analysis of mannitol-TT formulation

Stability Studies Results

The flocculation test was used to determine the stability of the TT-mannitol formulation for dry powder inhalation. The flocculation was observed at 14 ± 0.5, 14.5 ± 0.25, and 15.5 ± 0.5 min respectively at 3, 6 and 12 month period. This means as long as the Lf is maintained, the stability of vaccine is assured. The vaccine was shown to be stable at 25ºC and 60% RH for 12 months without the need for refrigeration. The product's results remained unchanged when stored at 4°C with 0% RH. The TT DPI formulation, stored at 25ºC and 60% RH for 12 months, showed stability using a flocculation test. This indicates that, the dry powder inhalation of tetanus toxoid can be stored at ambient temperature without any need for cold chain storage. Furthermore, the data points to the dry powder vaccine's commercial viability.

In Vivo Efficacy Studies Results

BALB/c mice were used to compare the dry powder inhalation of the formulation with the traditional vaccination. The existing tetanus toxoid vaccine was given via intramuscular injection. An ELISA test was used to determine the vaccine formulation's immunogenicity. The total IgG concentration in the mice's serum and BAL fluids was calculated. The results showed that antibodies were present before the vaccine was given and that the antibody level had decreased by week four. In addition, Table 2 and Figures 5 and 6 showed an increase in antibody levels in the tenth week. The negative and positive control groups both demonstrated a gradual decline in serum IgG titers after 10 weeks of the trial. The serum IgG level was marginally enhanced to 14.4 ± 2.8 ng in those mice that received the booster dosage. The developed TT formulation, on the other hand, showed a nearly two-fold increase in IgG titer, going from 48.4 ± 7.4 to 105.5 ± 8.1ng. In BAL fluids, a similar trend was seen for IgG titer. Table 2 shows a two-fold increase in IgG titers before and after the booster dosage.

According to previous research, conventional vaccines cause systemic immunity and poor mucosal immunity. Because infections enter the body through mucosal ports, developing a mucosal vaccination that provides both systemic and mucosal immunity is necessitated. For this reason, mucosal routes such as the oral and nasal have been investigated, with encouraging results [13,46]. The pulmonary pathway was investigated in this study to see if it might be used to induce systemic and mucosal immunity. The pulmonary route's near closeness to the blood vascular system suggests that it could be preferred over other non-invasive ways.

Table 2. Determination of IgG concentration in serum and BAL fluids

Group	IgG in serum (ng/ml)						IgG in BAL fluids (ng/ml)	
	0 w	4th w	8 weeks (ng/ml)		10 weeks (ng/ml)		BAL fluids (ng/ml)	
			8th w		10th w		10th w	
			BB	AB	BB	AB	BB	AB
Negative control	36.2 ± 6.9	2.25 ± 1.4	0.6 ± 0.1	0.8 ± 0.2	1.12 ± 0.4	-	34.4 ± 5.2	-
Positive control	54.6 ± 7.8	5.43 ± 0.87	7.23 ± 1.2	9.6 ± 2.1	10.8 ± 2.4	14.4 ± 2.8	38.1 ± 4.7	42.5 ± 4.8
Formulation	48.4 ± 7.4	8.1 ± 1.5	16.3 ± 2.4	29.52 ± 4.4	45.4 ± 5.6	105.5 ± 8.1	60.2 ± 5.1	102.5 ± 8.4

All values are average of three determinations. BB = before booster dose, AB = after booster dose, w = week

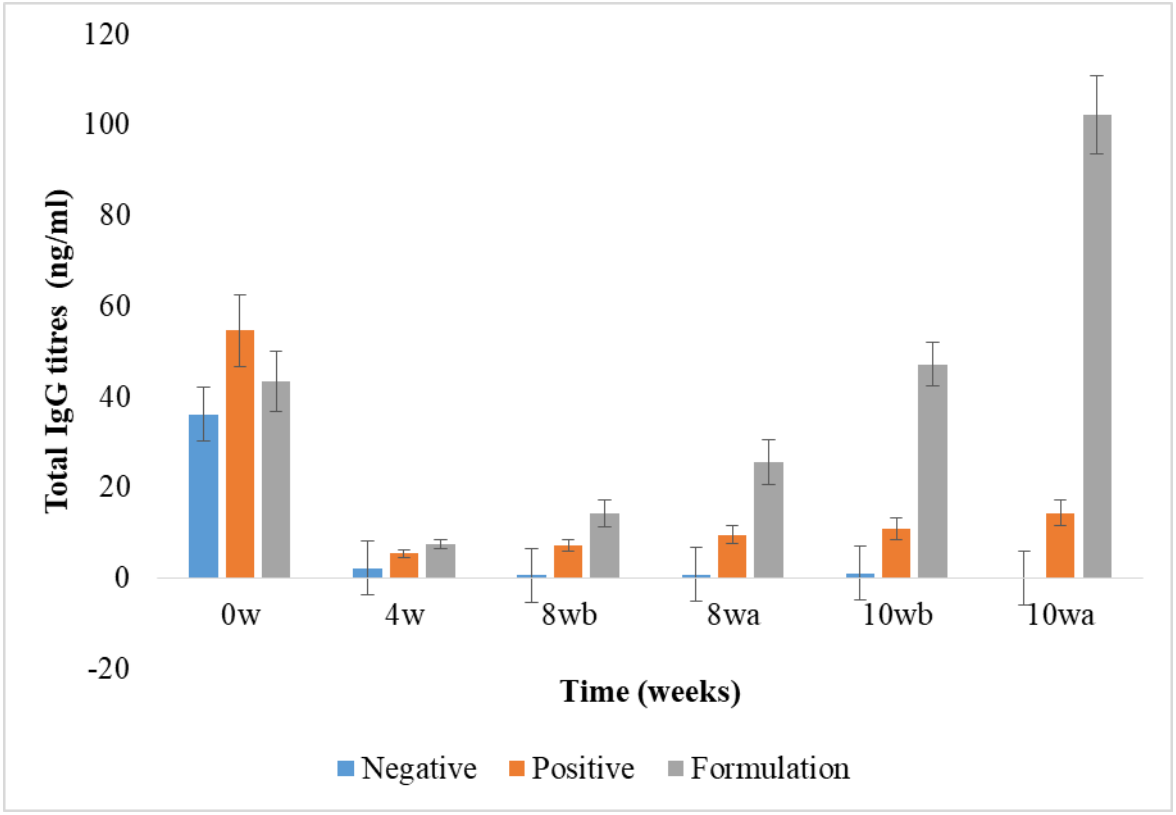

Figure 5. Serum immunity studies

Neg-Negative; Pos-Positive; 0w:0 week; 4w:4th week; 8wb:8th week before booster; 8wa:8th week after booster; 10wb:10th week before booster; 10wa:10th week after booster

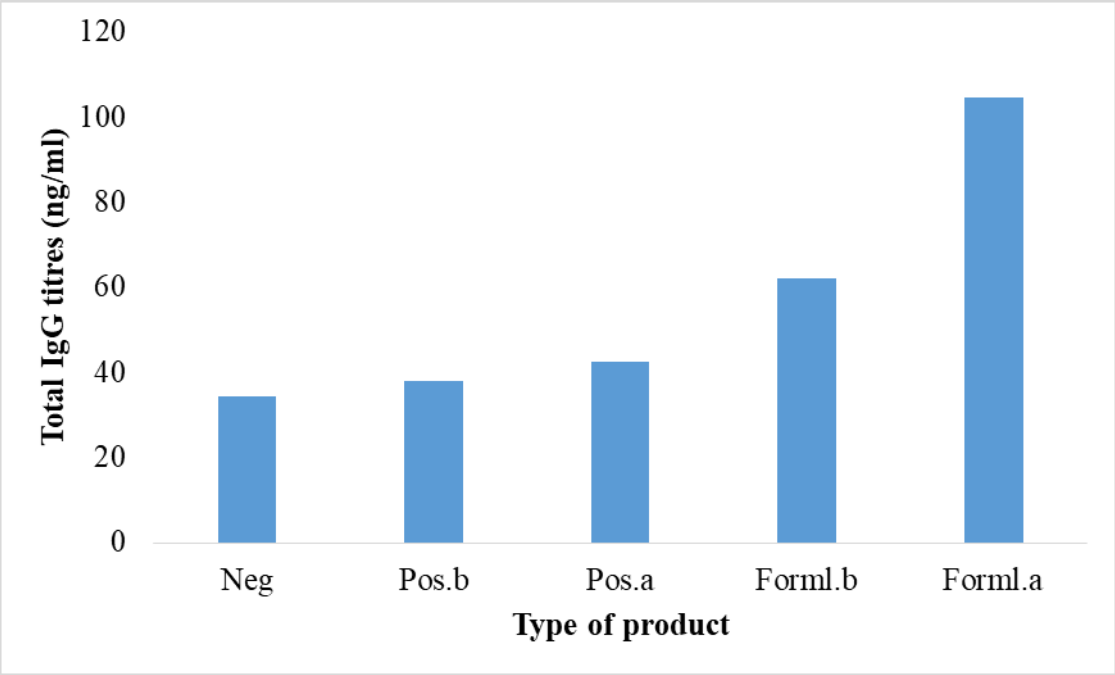

Figure 6. Mucosal (BAL) immunity studies
Neg: Negative control; Pos.b: Positive group before booster dose; Pos.a: Positive group after booster; Formln.b: Formulation before booster dose; Formln.a: Formulation after booster dose

The results of the ELISA test showed that antibodies were present before the vaccine was given, and that the antibody level had decreased by week four. Table 2 shows a two-fold increase in IgG titers before and after the booster dosage, indicating that the formulation was capable of producing mucosal antigenicity in the mice. The dry powder vaccination group imparted a considerable boost in systemic and mucosal immunity as compared to the positive control group, which had received regular conventional immunization. Furthermore, mucosal immunity was much stronger than systemic immunity, indicating that vaccinations may be delivered to fight respiratory diseases. It was shown that the pulmonary mucosal vaccination proved effective.

Owing to its suitable particle size and good flow properties, the TT-mannitol formulation was found to be an efficient carrier to be used as a dry powder inhalation for pulmonary administration. The geometrical particle size of the particles was within the micron range, which indicates that they can be well absorbed by alveoli. Further, the non-hygroscopic nature of mannitol contributes to the free flowing properties of the TT-mannitol formulation. The immunogenicity of the solid formulation infers the potential of the pulmonary TT-mannitol formulation. Future detailed *in vivo* studies with other animal models can show that this formulation works for both systemic and mucosal immunity.

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AUTHOR CONTRIBUTIONS

Concept: M.M.A., P.K., S.K.A.; Design: M.M.A., S.M., P.C.; Control: P.K., S.K.A.; Sources: N.V.K.A., S.I., S.M.; Materials: J.D.V., N.V.K.A., S.I.; Data Collection and/or Processing: M.M.A., J.D.V., S.M., P.C.; Analysis and/or Interpretation: M.M.A., P.K., S.K.A., P.C.; Literature Review:

M.M.A., P.K., S.I.; Manuscript Writing: M.M.A, N.V.K.A., J.D.V., S.M.; Critical Review: M.M.A., P.K.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

The authors declare that this work involved animal research, and the protocol for this investigation was approved by the Institutional Animal Ethics Committee in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals' disciplinary principles and guidelines CPCSEA proposal number RB-9/5/2021, dated March 28, 2021.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

UTILITY OF QUALITY BY DESIGN APPROACH IN RP-HPLC METHOD DEVELOPMENT FOR QUANTIFICATION OF LAMIVUDINE AND EFFAVIRENZ IN COMBINATION FORMULATION

RP-HPLC KULLANILARAK TASARIM YAKLAŞIMI YOLUYLA KOMBİNE FORMÜLASYONDA BULUNAN LAMİVUDİN VE EFFAVİRENZ'İN TAYİNİNE YÖNELİK YÖNTEM GELİŞTİRİLMESİ

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ABSTRACT

Objective: *For the measurement of lamivudine(LAM) and effavirenz (EVZ) in combination formulation, an uncomplicated and reliable liquid chromatographic approach has been proposed.*
Material and Method: *The design of experiments (DoE) was used to set up the experimental conditions for the multivariate optimization of the RP-HPLC method. The crucial method parameters were determined by a risk assessment. The mathematical models were created using three independent variables: percentage of acetonitrile, percentage of methanol, and buffer pH. The*

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impacts of these independent elements were thoroughly investigated using the central composite design (CCD), which was utilized to analyze the response surface methodology.
Result and Discussion: *The LAM and EVZ retention time and resolution were both concurrently optimized using the desirability function. Acetonitrile, methanol, and phosphate buffer (pH 7.0) in the proportions of 40:20:40 v/v each were used in the optimized and anticipated data from the contour diagram, with detection occurring at a wavelength of 215 nm. Baseline separation of both pharmaceuticals with high resolution and a run time of under 6 minutes was accomplished under these ideal conditions. The validated test parameters followed ICH recommendations. As a consequence, the findings demonstrated that the Quality by Design methodology could be successfully used to optimize the RP-HPLC technique for the concurrent quantification of LAM and EVZ.*
Keywords: *Central composite design, effavirenz, lamivudine, quality by design, RP-HPLC*

ÖZ

Amaç: *Kombinasyon formülasyonunda lamivudin (LAM) ve effavirenzin (EVZ) ölçümü için karmaşık olmayan ve güvenilir bir sıvı kromatografik yaklaşım önerilmiştir*.
Gereç ve Yöntem: *Deney tasarımı (DoE), RP-HPLC yönteminin çok değişkenli optimizasyonu için deneysel koşulları ayarlamak üzere kullanıldı. RP-HPLC yönteminin çok değişkenli optimizasyonu, deneysel koşullar, deney tasarımı (DoE) kullanılarak gerçekleştirildi. Kritik yöntem parametreleri bir risk değerlendirmesi ile belirlendi. Matematiksel modeller üç bağımsız değişken kullanılarak oluşturulmuştur: asetonitril yüzdesi, metanol yüzdesi ve tampon pH'ı. Bu bağımsız öğelerin etkileri, yanıt yüzeyi metodolojisini analiz etmek için kullanılan merkezi bileşik tasarım (CCD) kullanılarak ayrıntılı bir şekilde incelenmiştir.*
Sonuç ve Tartışma: *LAM ve EVZ tutma süresi ve çözünürlüğü, arzu edilebilirlik işlevi kullanılarak aynı anda optimize edildi. Her biri 40:20:40 v/v oranlarındaki asetonitril, metanol ve fosfat tamponu (pH 7.0), 215 nm'lik bir dalga boyunda meydana gelen algılama ile kontur diyagramından optimize edilmiş ve beklenen verilerde kullanılmıştır. Her iki farmasötik maddenin yüksek çözünürlükte ve 6 dakikanın altında çalışma süresiyle temel ayrımı, bu ideal koşullar altında gerçekleştirildi. Doğrulanmış test parametreleri, ICH önerilerini izledi. Sonuç olarak, bulgular Tasarıma Göre Kalite metodolojisinin LAM ve EVZ'nin eş zamanlı ölçümü için RP-HPLC tekniğini optimize etmek üzere başarılı bir şekilde kullanılabileceğini göstermiştir.*
Anahtar Kelimeler: *Effavirenz, lamivudin, merkezi kompozit tasarım, RP-HPLC, tasarıma göre kalite*

INTRODUCTION

Lamivudine (LAM) is an inhibitor of the reverse transcriptase enzyme that has been shown to be effective against the "HIV-1, HIV-2, and hepatitis B viruses". LAM, chemically "4-amino-1-[(2*R*, 5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one". Lamivudine triphosphate is the active 5'-triphosphate metabolite of lamivudine, a synthetic nucleoside analogue that is phosphorylated intracellularly (L-TP). Viruses' DNA chains are broken when enzymes like HIV reverse transcriptase and HBV polymerase incorporate this nucleoside analogue. Chemically EVZ, "(4*S*)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1*H*-3,1-benzoxazin-2-one", acts as a competitive inhibitor of DNA polymerase and is an inhibitor of reverse transcriptase that does not rely on nucleosides. Figure 1 illustrates the structure of both drugs. In the management of hepatitis B and HIV illnesses, the combination of two or more antiviral medicines represents a significant achievement. Patients are more compliant with multidrug combination treatment due to the lower medication load each day. The combination of lamivudine and effavirenz available with tenofovir disoproxil fumarate, zidovudine ,stavudine, didanosine etc. In these FDC the dosage of lamivudine and effavirenz available as 150/300 mg and 300/600 mg respectively.

Several HPLC techniques have been published in the literature for the determination of lamivudine alone [1-4], effavirenz alone [5-7], lamivudine and effavirenz with other drugs, in pharmaceutical products and biological samples [8-11]. There is no published QbD-based RP-HPLC technique for the concurrent measurement of lamivudine and effavirenz. The use of "Quality by Design" (QbD) or "Design of Experiments" (DoE) is advocated to provide robustness throughout the statistical

quality control monitoring of analytical technique validation and the research of variables that have a negative influence on the quality of pharmaceutical analysis. The conventional way of developing a method consists of trial and error and adjusting one parameter at a time. Due to considerations such as the restricted access of chromatographic supplies such the column, solvents, and chemicals , as well as the crucial physicochemical features of the analyte, this method frequently has difficulty setting robust chromatographic conditions. Recently, FDA has approved multiple NDAs using the QbD approach to analytical methods like HPLC and UV spectrophotometery. These NDAs have regulatory flexibility for movement within the stated method operational design region (MODR). Quality by Design (QbD) has been an integral part of developing new pharmaceuticals since its deployment by the FDA, influencing its robustness. A current treatment of the HPLC technique's robustness using QbD necessitates the evaluation of all elements that have the greatest effect on the method's outcomes. It is impracticable, challenging, and more expensive to experimentally verify several factors at once. The reversed-phase technique [12-15] is the method of preference in analysis due to its ease of use, adaptability, and diversity of applications, which include handling compounds with a variety of polarities and molecular masses.

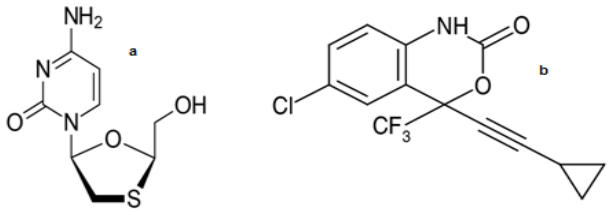

Figure 1. Structure of Lamivudine (a) and Effavirenz (b)

MATERIAL AND METHOD

Chemicals and Reagents

Both of these drug standards brought from the pharmaceutical industry. Merck India Ltd., Mumbai, supplied LC-grade methanol, water, and LC-grade acetonitrile. SD Fine chemicals, Mumbai supplied potassium dihydrogen phosphate and orthophosporic acid. Odivir kit (Lamivudine 300 mg, Effavirenz 600 mg, Cipla Limited) was obtained from a local pharmacy.

HPLC Instrumental Condition

The technique was created using a Waters Acquity (HPLC) system (Milford, MA, USA) with a PDA detector (model #2996) and Empower-2 software. The two analytes were separated by using methanol : acetonitrile: phosphate buffer pH 7 (20:40:40, v/v) with an isocratic flow rate of 1 ml/min, at room temperature. Separation and analysis were executed on an Xterra C18 column (250 mm x 4.6 mm ; 5 µm) in a temperature-controlled laboratory (25°C was maintained for all chromatographic runs). At 215 nm, the PDA detector was utilized to monitor the two drugs. The solvents were degassed in an ultrasonic bath after being filtered through a 0.22 µm membrane filter. As a diluent, mobile phase was utilized.

Software

Utilizing Design-Expert version 12, calculations for the desirability function, experimental design (CCD), and data analysis were made.

Preparation of Buffer Solutions

Potassium dihydrogen orthophosphate weighing 1.38 grams was added to a 1000 ml volumetric flask, dissolved with HPLC water and diluted to 1000 ml. Ortho-phosphoric acid was used to bring the pH down to 7.

Preparation of Standard Solutions

A sample containing 25 mg of each drug is weighed before being moved to a volumetric flask measuring 25 ml. After adding 15 ml of acetonitrile, the solution is sonicated for 15 minutes. To create a stock solution containing 1000µg/ml, the volume is brought up to the required level using acetonitrile. Working standard solutions of 100 µg/ml are created by withdrawing 2.5 ml from the standard stock solutions, transferring it to 25 ml volumetric flasks, and topping off the volume with diluent.

Preparation of Pharmaceutical Samples

"Odivir-kits (Tablets)" each tablet with labelled claim content "300 mg lamivudine" and "600 mg efavirenz" were weighed, the mean weight was recorded, and the pulverised LAM and EVZ were carefully weighed and placed into a volumetric flask of capacity 50 ml which is previously cleaned and dried. The flask's contents were mixed in diluent, subjected to a 30-minute sonication, diluted to the desired volume, and designated as a sample stock solution on the label. PVDF 0.45 µm filters were used to filter the sample stock solution. Filtered sample stock solution in the quantity of one milliliter was placed in a ten milliliter volumetric flask and adjusted to the necessary strength.

Software Aided Method Optimization

According to the literature, several design methodologies were offered to test the robustness of the procedure. They were utilized when screening techniques needed to be optimized for separation and while assessing robustness and optimizing formulations, products, or methods. The significant chromatographic variables in the present research were chosen based on preliminary studies and existing study of literature. Because of its effectiveness in terms of the number of runs needed, the CCD screening design may be a useful option for examining the robustness of a limited number of variables (three or less). Several variables, including the fraction of acetonitrile and methanol in the mobile phase and the buffer's pH, were addressed during method development. As a result, CCD was used to assess the impact of three distinct parameters in chromatography on three identified critical response characteristics. 20 test runs were incorporated into the design and aided in factor screening by assessing each component's major influence to get study results. A 3 x 2 factorial design indicates the presence of two levels and three factors. These were a "low (-1)" and a "high (+1)" level, factors on the other were (X_1) fraction of methanol in mobile phase (20 % and 40 %), (X_2) proportion of acetonitrile in mobile phase (20 % and 40 %), and (X_3) pH of buffer (5 and 7). Table 1 displays the retention times for LAM (Y_1), EFZ (Y_2), and resolution (Y_3), which were employed as the experimental design's responses. The data obtained were incorporated into Design-Expert version 12. In order to explore, examine response behavior around optimal values of the variables, and get the greatest system performance, response surface quadratic approach was utilized. To assess the model's significance, analysis of variance (ANOVA) was used. Conditions were chosen from this optimized method, and their performance in terms of accuracy, precision (< 2% RSD), and robustness as a targeted response, was verified. Twenty experiments were conducted using the conditions and outcomes reported in Table 2.

Method Validation

"International Conference on Harmonization (ICH) (2005) Q2R(1)"[16] criteria were used to validate the optimized chromatographic technique for "system suitability, linearity, limit of detection, limit of quantitation, precision, accuracy, specificity, and robustness".

System Suitability Test

LAM and EFZ concentrations of 15µg/ml and 30µg/ml, respectively, in six replicates of standard solutions were injected prior to the sample analysis to test the system appropriateness characteristics of Resolution, tailing factor, and theoretical plate count. The % RSD should always be less than 2.0%. In each chromatogram, the acceptance requirements for system suitability standards were determined.

Table 1. Experimental plan of CCD showing factors with levels

Factor	Code	Range levels	
		Low (-1)	High (+1)
% Methanol composition in mobile phase	X_1	20	40
% Acetonitrile composition in mobile phase	X_2	20	40
pH of Buffer	X_3	5	7
Responses			
Retention time LAM	Y_1	-	-
Retention time EVZ	Y_2	-	-
Resolution factor	Y_3	-	-

Table 2. Coded values for factor level and observed responses in CCD for 20 analytical trials

Exp. (Run)	Type	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	Center	30	30	6	3.184	5.384	4.48
2	Axial	30	30	4.31	2.121	4.212	4.21
3	Axial	46.81	30	6	3.981	5.123	4.09
4	Axial	30	46.81	6	1.567	3.982	3.91
5	Axial	30	30	7.68	2.01	3.674	3.1
6	Center	30	30	6	3.184	5.384	4.48
7	Factorial	20	20	5	4.512	5.687	2.15
8	Factorial	20	20	7	2.412	3.998	3.45
9	Center	30	30	6	3.184	5.384	4.48
10	Factorial	20	40	7	1.689	3.921	4.555
11	Factorial	40	40	7	3.119	4.12	2.04
12	Center	30	30	6	3.184	5.384	4.48
13	Factorial	20	40	5	2.512	5.287	4.32
14	Factorial	40	40	5	3.376	5.981	3.98
15	Axial	30	13.18	6	4.982	4.772	0.54
16	Axial	13.18	30	6	3.763	6.021	6.98
17	Factorial	40	20	5	4.265	5.391	2.13
18	Center	30	30	6	3.184	5.384	4.48
19	Center	30	30	6	3.184	5.384	4.48
20	Factorial	40	20	7	2.982	6.102	5.85

Linearity

The suggested approach's linearity was established at five levels over the 5-25µg/ml for LAM and 10-50µg/ml for EFZ ranges. Each linearity solution was administered in triplicate using the appropriate sample concentrations. By utilizing linear regression analysis to plot the peak area versus the concentration, the calibration curve was created.

Accuracy and Precision

The accuracy was tested by spiking a predetermined quantity of standard to the tablet solution in triplicate at levels of 50, 100, and 150%, and then performing the optimized technique on the samples. Then, percentage recoveries for both medications were determined. For acceptance, the target concentrations' mean recovery was set at 100 ± 2%. By examining the intermediate precision and repeatability, the precision of the optimized technique was ascertained. Intermediate precision is expressed through changes in laboratories, such as various days, analysts, equipment, etc. The phrase

"inter-assay precision" also applies to intermediate precision. Repeatability describes the accuracy over a brief period of time while using the same operating variables. To evaluate the method's precision, six homogeneous LAM and EFZ samples were analyzed.

Limit of Detection(LOD) and Limit of Quantitation(LOQ)

Using the standard deviation approach, LOD and LOQ of LAM and EVZ were assessed. Based on the response's standard deviation (σ) and calibration curve's slope (S), LOD was determined at 3.3 σ /S and LOQ at 10 σ /S.

Robustness

The robustness of the technique is the capacity to withstand modest and intentional changes in the method parameters. By infusing the system suitability solution with slight purposeful modifications to the chromatography's parameters (mobile phase ratio, flow rate, and analytical wavelength), the robustness of the optimized technique was examined. It was calculated using the percentage of the RSD.

RESULT AND DISCUSSION

Preliminary Studies and Factor Selection

An initial investigation was conducted to find a simple, robust, and inexpensive RP-HPLC technique for estimating LAM and EFZ in mixtures. Using data from preliminary studies and other published works, the key chromatographic parameters were chosen. Studies aimed at selecting the factor levels for use in screening and optimization studies highlighted the requirement to improve mobile phase conditions in order to separate LAM and EFZ in a small space of time. The simultaneous estimate of both drugs was shown to be more suited for the mobile phase composition of phosphate buffer pH, volume of methanol and acetonitrile, which led to a significant impact in retention time. As a result, it is regarded as one of the essential criteria for method development.

QbD Assisted Method Development

The current work on the optimization of the analytical approach used CCD design. It is an effective and extensive DoE centered on meticulous exploration of three essential elements of the RP-HPLC technique (percentage of methanol, acetonitrile, and pH of buffer).

To assess method robustness, a “multivariate approach DoE with CCD” was used in the RP-HPLC technique to examine the concurrent modifications of the parameters on selected responses, includes LAM retention time(Y_1), EVZ retention time(Y_2), and resolution(Y_3). Based on an analysis of this data and the influence of three factors on responses, it was possible to develop mathematical models to determine the link between the factors and the examined responses. After analyzing the data, we found that the response surface quadratic model provided the greatest match for CCD. ANOVA was used in Design Expert to further validate the model. The retention time adjusted R-squared values for LAM(Y_1) and EVZ(Y_2) were in reasonable agreement with the predicted values, with a deviation of less than 0.2. The current model may not be the best predictor of the response, as indicated by the negative predicted R-squared for resolution(Y_3). Signal-to-noise ratio is measured with adequate precision. The obtained responses for the Y_1, Y_2, and Y_3 were 9.1254, 5.811, and 7.1743, respectively, which suggests an adequate precision; a ratio of larger than 4 is preferred. Navigation of the design space is done using the quadratic model. The model is considered significant since the responses' model F-values for the retention times of LAM (Y_1), EVZ (Y_2), and resolution (Y_3) were 6.48, 4.13, and 3.53 respectively. There is only a 0.36 percent probability for Y_1 and 0.19 percent chance for Y_2, and a 3.10 percent probability of resolution (Y_3) thanan F-value, suggesting that this might be caused by noise. Consequently, the p value of the significant responses was less than 0.05, indicating that the terms of the model are significant. The high adjusted R-square value and low standard deviation show that the fitted models and experimental data are well correlated. The results are presented in Table 3. Predictions regarding the responses for specific levels of each factor may be made using the equations in terms of coded factors. This equation can be used to figure out how important each factor is by comparing the factor coefficients. Here are the final equations for Y_1, Y_2, and Y_3:

LAM (Y_1) = +3.18 + 0.2185X_1 - 0.6750X_2 – 0.3405X_3 + 0.2464X_1X_2 + 0.1729X_1X_3 + 0.2879X_2X_3 + 0.2527X_1^2 - 0.0414X_2^2 – 0.3860X_3^2;

EVZ: (Y_2) = +5.37 + 0.0872X_1 - 0.2341X_2 – 0.3742X_3 - 0.1144X_1X_2 + 0.2381X_1X_3 - 0.2811X_2X_3 + 0.1669X_1^2 - 0.2556X_2^2 - 0.4090X_3^2;

Resolution: (Y_3) = +4.49 - 0.3907X_1 + 0.5113X_2 + 0.1060X_3 -0.6544X_1X_2 + 0.0306X_1X_3 - 0.8406X_2X_3 + 0.3298X_1^2 - 0.8405X_2^2 -0.3349X_3^2.

Table 3. ANOVA regression analysis for models and responses

Response	Mean	SD[a]	%CV[b]	Press value	R^{2c}	Adjusted R^2	Predicted R^2	Adequate Precision	SS[d]	d[e]	MS[f]	F[g]	P
$R_T$of LAM(Y_1)	3.12	0.4768	15.28	17.28	0.8537	0.7221	0.6121	9.1254	13.27	9	1.47	6.48	0.0036
R_T of EVZ(Y_2)	5.03	0.5853	11.64	27.63	0.6947	0.4199	0.4629	5.811	7.79	9	0.8661	4.13	0.0082
Resolution (Y_3)	3.91	0.9515	24.34	72.92	0.7607	0.5454	-0.6273	7.1743	28.78	9	3.20	3.53	0.0031

R_T: Retention time, a: Standard deviation, b: Coefficient of variations, c: Coefficient of Regression, d: Sum of squares, e: Degrees of freedom, f: Mean sum of squares, g: Fischer's ratio

It is evident from the coefficient values in the aforementioned equations and their corresponding that factors like methanol volume (X_1) has positive impact on retention time of LAM and EVZ, Y_1 and Y_2. Acetonitrile volume (X_2), and pH of buffer (X_3), had a negative impact on retention time of LAM and EVZ, Y_1 and Y_2. The pH of buffer (X_3) had a positive impact on resolution (Y_3), whereas methanol volume (X_1) has a negative effect and acetonitrile volume (X_2) had positive effects. The interactions between X_1 and X_2 had a positive impact on Y_1 and a negative impact on Y_2 and Y_3, whereas the interactions between X_2 and X_3 had a positive impact on Y1 and a negative impact on Y_2 and Y_3 and a positive impact on Y_1, Y_2, and Y3. All chromatographic responses were positively impacted by the squares of factor $X1^2$ whereas X_2^2, and X_3^2, had negative impact on all responses of the model.

In order to understand how the factors and their interactions affected the response, response surface and contour plots were examined. The contour plots had curvature, indicating a nonlinear relationship between the variables and the responses. Figures 2 and 3 illustrate contour plots in 2D (A) and 3D (B) demonstrating the impact of methanol volume (X_1) and acetonitrile volume (X_2) on retention time of LAM (Y_1) and EVZ (Y_2). A curved rising trend was noticed for the methanol volume (X_1) that demonstrated higher retention time of LAM (Y_1), as well as EVZ (Y_2) and there was a curved downward trend was observed for acetonitrile volume (X_2), which showed lower retention time of LAM (Y_1), as well as EVZ (Y_2). Therefore, lower levels of X_1 and high levels of X_2 were suggested to achieve retention time of LAM (Y_1) and EVZ (Y_2). Figure 4 depicts the curvature impacts of the methanol volume (X_1) and acetonitrile volume (X_2) on resolution using 3D and 2D contour plots. Both X_1 and X_2 showed a rising curvature trend, indicating greater resolution at higher levels. To achieve resolution, the optimal values of X_1 and X_2 were chosen.

Consistent with the goals and boundaries of each response, a composite desirability was used to find the ideal combination of conditions. The entire experimental region was investigated for the compositions, whereby constraints established were satisfied to the highest extent possible, i.e., unity, as demonstrated in Figure 5 and Figure 6. The accomplishment of planned goals within the established limits was demonstrated by the desirability function "R," which is equal to unity. After determining the optimal RP-HPLC chromatographic conditions, we decided as volume of methanol (X_1) , volume of acetonitrile (X_2) and buffer pH 7.0 in ratio (20 : 40 : 40 % v/v) and retention time as a result in which LAM (Y_1) 1.729±0.0162, retention time of EVZ (Y_2) 3.85±0.013, and resolution (Y_1) 4.52±0.021 min, respectively, as shown in Figure 7.

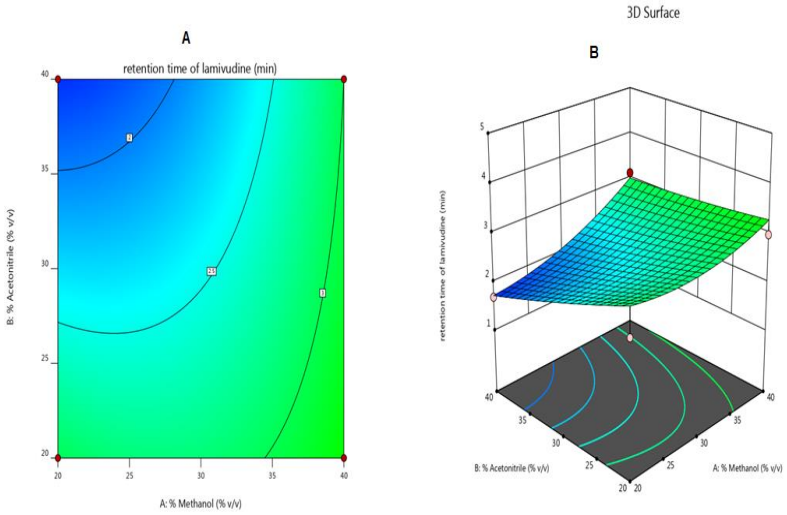

Figure 2. 2D (A) and 3D (B) contour plots showing the effect of % methanol (X_1) and % acetonitrile (X_2) on retention time of LAM (Y_1)

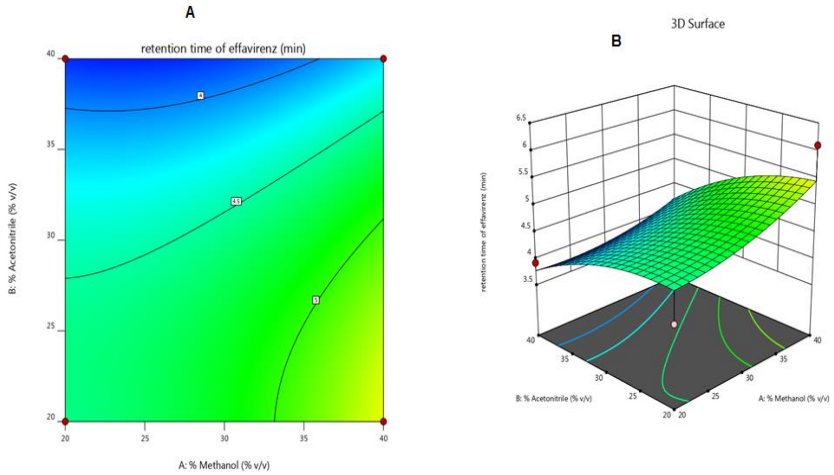

Figure 3. 2D (A) and 3D (B) contour plots showing the effect of % methanol (X_1) and % acetonitrile (X_2) on retention time of EVZ (Y_2)

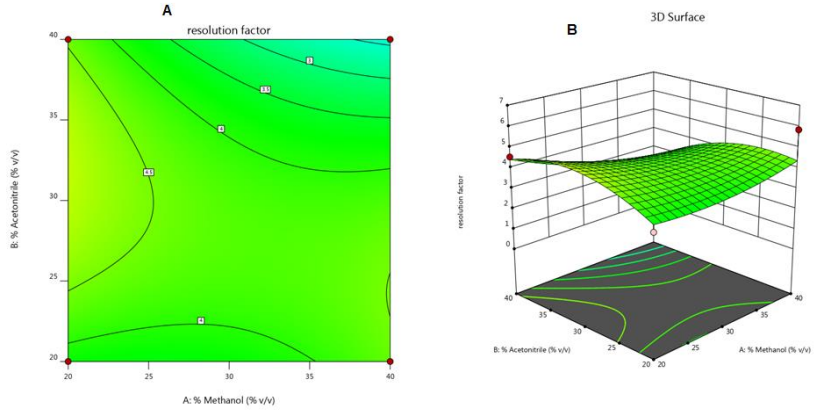

Figure 4. 2D (A) and 3D (B) contour plots showing the effect of % methanol (X_1) and % acetonitrile (X_2) on resolution (Y_3)

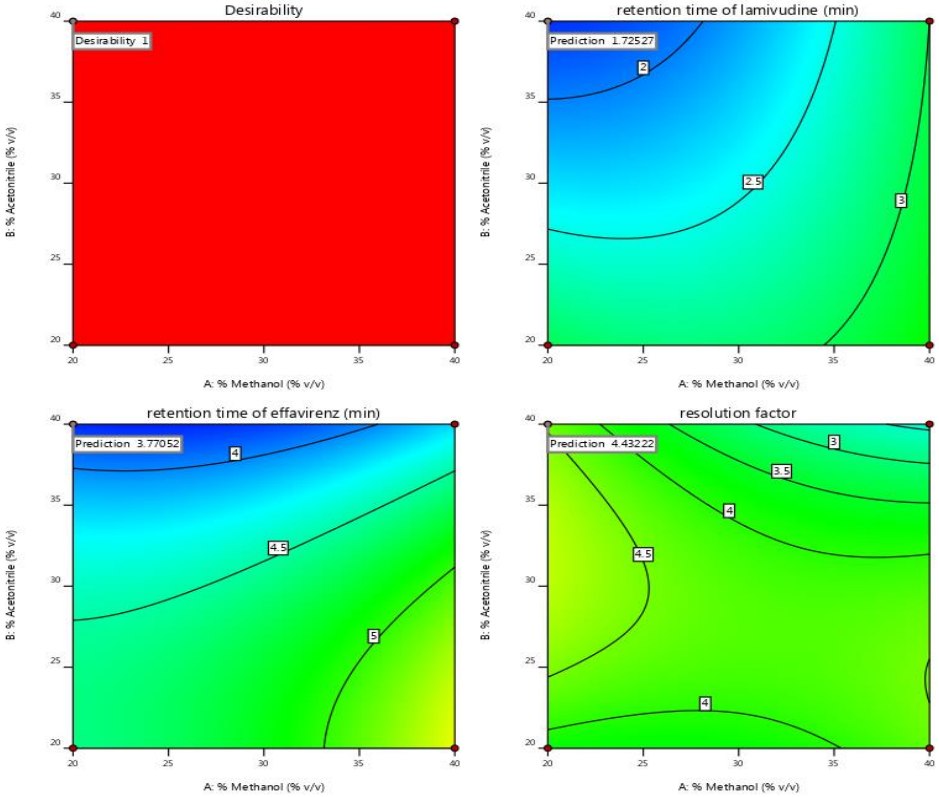

Figure 5. 2D desirability plot

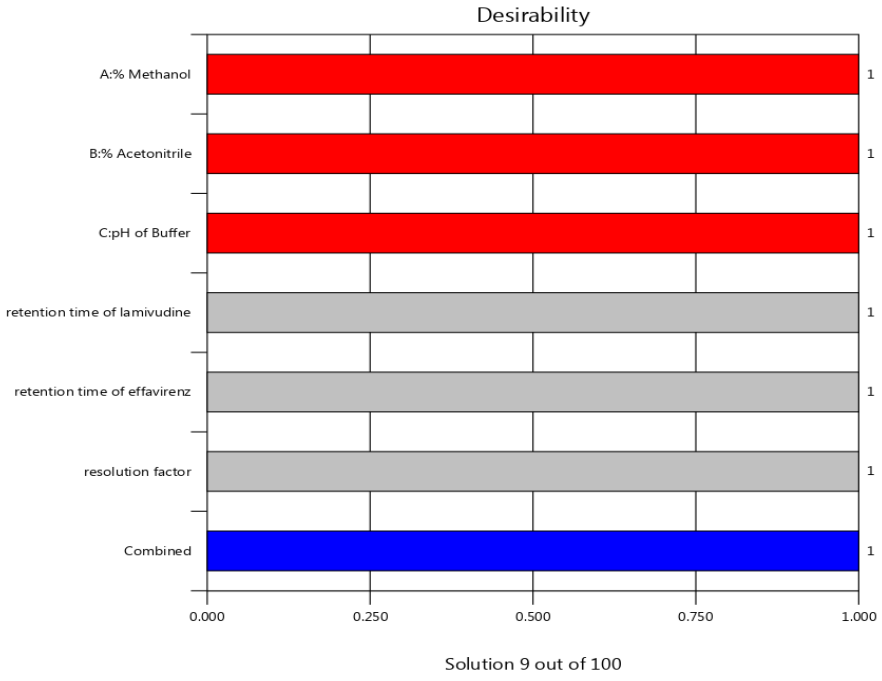

Figure 6. Desirability plot

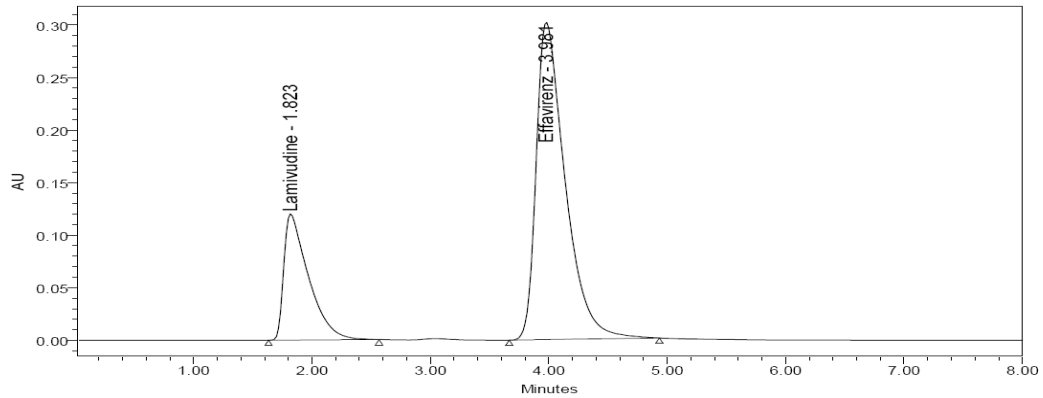

Figure 7. Optimized RP-HPLC chromatogram for LAM and EVZ at 215 nm

Method Validation

System suitability tests, according to the ICH, are an essential aspect of liquid chromatographic procedures. The performance of columns, as evaluated by theoretical plates count for both medicines, was greater than 2000, the resolution was 4.52, and tailing was less than 2. For six replicate injections, the percent relative standard deviation was 0.87 at the indicated concentration of 15 µg/ml for LAM and 1.09 at the indicated concentration of 30 µg/ml for EVZ, respectively. Because the % RSD < 2%, it demonstrated high injection repeatability. The proposed method's linearity was verified by plotting the linearity curve for LAM and EVZ over a range of concentrations, from 5 to 25µg/ml and 10 to 50µg/ml, respectively, with correlation coefficients of (r^2=0.999) for both medicines, as shown in Table 4.

Table 4. Validation results for LAM and EVZ

PARAMETERS		LAM	EVZ
System Suitability Parameters			
No. of theoretical Plates	Mean±SD*	2716.6 ± 88.98	2546.8 ± 73.88
	% RSD	1.65	1.81
Resolution	Mean±SD*	-	4.52 ± 0.021
	% RSD		0.24
Tailing factor	Mean±SD*	1.05 ± 0.003	1.01 ± 0.0019
	% RSD	0.21	0.18
Linearity			
Range (µg/ml)		5-25	10-50
Slope		48973	15368
Intercept		-48000	43802
Correlationcoefficient		0.9991	0.9992
Accuracy			
% Recovery		99.3-101.43	99.02-99.89
% RSD**		1.87	1.73
Precision			
Repeatability		0.87	1.09
Intermediate precision		0.91	1.23
LOD(µg/ml)		0.12	0.18
LOQ((µg/ml)		0.36	0.54

*Mean of six determinations, **Set of three determinations

The peak area and concentration have a strong correlation, as shown by the obtained correlation coefficient (r2=0.999). Samples at 50%, 100%, and 150% of the nominal concentrations for both drugs

were created for the recovery investigation and recovery rates for LAM and EVZ were 99.3-101.43% and 99.02-99.89%, respectively. Table 4 displays data demonstrating a low level of error (% RSD 1.87 and 1.73 for LAM and EVZ, respectively) with the established approach. The outcomes of the intermediate precision and repeatability tests are presented in Table 4. For both medicines, the procedure was repeatable and accurate, since the precision values were below 2%. Results showed that the LOD and LOQ for LAM were 0.12 and 0.36 µg/ml, while those for EVZ were 0.18 and 0.54 µg/ml. The robustness of the RP-HPLC technique to slight variations in the optimum experimental modifications revealed its insensitivity to such modest alterations. The mobile phase composition and buffer pH had a substantial influence on LAM and EVZ retention time and resolution.

For the first time, the current study involves the methodical QbD-based study and development of a rapid, accurate, and cost effective RP-HPLC technique for concurrent quantification of LAM and EVZ. The experimental design covers the exploration of important components, such as methanol volume, acetonitrile volume, and buffer pH. The modeling software aided in a superior comprehension of the elements impacting optimization of the procedure and separation of LAM and EFZ. CCD was utilized in order to improve the resolution in a reasonable period of time(6 min) in response to LAM and EFZ. In the optimized model, the 20:40:40 v/v ratio of methanol, acetonitrile, and pH 7.0 phosphate buffer confirms the appropriateness for estimating LAM and EFZ. The validation study indicated that the method was selective, specific, accurate, linear, precise, and robust, which contributed in the determination of the optimal conditions. As a result, using the response surface methodology gives a better understanding for method development and robustness testing. This approach proposed meets the design space principle and is in line with regulatoryflexibility and suitable for regulatory submission.

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AUTHOR CONTRIBUTIONS

Concept: B.R.B.K., R.P.Y., K.M.P.A., S.R.Y., V.D., H.K.T.; Design: B.R.B.K., R.P.Y., K.M.P.A., S.R.Y., V.D., H.K.T.; Control: R.P.Y., K.M.P.A.,S.R.Y.; Sources: R.P.Y., H.K.T.; Materials: S.R.Y., V.D.; Data Collection and/or Processing: S.R.Y., V.D.; Analysis and/or Interpretation: B.R.B.K., H.K.T.; Literature Review: B.R.B.K., V.D., H.K.T.; Manuscript Writing: B.R.B.K.; Critical Review: B.R.B.K., R.P.Y., K.M.P.A., S.R.Y., V.D., H.K.T.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

BAZI DÜZCE HALK İLAÇLARININ KİMOTRİPSİN, ÜREAZ İNHİBE EDİCİ VE ANTİOKSİDAN AKTİVİTELERİ

CHYMOTRYPSIN, UREASE INHIBITORY AND ANTIOXIDANT ACTIVITIES OF SOME DÜZCE FOLK MEDICINES

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ÖZ

Amaç: *Bu araştırmada Düzce'de halk ilacı olarak kullanılan yedi bitkinin [Dioscorea communis (L.) Caddick & Wilkin, Mentha longifolia (L.) L. subsp. typhoides (Briq.) Harley, Origanum vulgare L., Rubus ulmifolius Schott, Salvia tomentosa Mill., Thymus longicaulis C.Presl subsp. longicaulis, Trachystemon orientalis (L.) D. Don] üreaz, kimotripsin inhibe edici ve antioksidan aktivitelerinin tespiti amaçlanmıştır.*
Gereç ve Yöntem: *Bu amaçla bitkilerden metanol ve su ekstreleri hazırlanmıştır. Daha sonra in vitro üreaz ve kimotripsin inhibitör aktiviteleri belirlenmiştir. Ayrıca farklı yöntemlerle antioksidan aktiviteleri (ABTS, CUPRAC, DPPH), toplam fenol ve flavonoit içerikleri de tespit edilmiştir.*
Sonuç ve Tartışma: *D. communis, O. vulgare, S. tomentosa ve T. longicaulis'in metanol ekstreleri orta düzeyde üreaz inhibitör aktivite (%34.26 ile %44.94 aralığında inhibisyon) gösterirken, diğerlerinde aktivite daha düşük bulunmuştur. En güçlü kimotripsin inhibe edici aktivite R. ulmifolius metanol ekstresi ve T. orientalis su ekstresinde gözlenmiştir (IC_{50} değerleri sırasıyla 65.32 ve 78.65µg/ml). Genel olarak, çalışılan bitkiler yüksek fenol ve flavonoit içeriklerine uygun olarak yüksek antioksidan aktivite göstermiştir. Sonuç olarak R. ulmifolius ve T. orientalis kuvvetli kimotripsin inhibe edici aktiviteleri ile dikkat çekmiştir. Bu bitkilerin kimotripsin aktivitesi üzerinde daha kapsamlı çalışmaların yapılması gerekmektedir.*
Anahtar Kelimeler: *Antioksidan, halk ilacı, kimotripsin, Rubus ulmifolius, üreaz*

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ABSTRACT

Objective: *In this study, determining the urease, chymotrypsin inhibitory and antioxidant activities of seven plants [Dioscorea communis (L.) Caddick & Wilkin, Mentha longifolia (L.) L. subsp. typhoides (Briq.) Harley, Origanum vulgare L., Rubus ulmifolius Schott, Salvia tomentosa Mill., Thymus longicaulis C.Presl subsp. longicaulis, Trachystemon orientalis (L.) D. Don] used as folk medicine in Düzce was aimed.*

Material and Method: *For this purpose, methanol and water extracts were prepared, then in vitro urease and chymotrypsin inhibitory activities were determined. Antioxidant activities (ABTS, CUPRAC, DPPH), total phenol and flavonoid contents were also determined.*

Result and Discussion: *While D. communis, O. vulgare, S. tomentosa and T. longicaulis methanol extracts showed moderate urease inhibitory activity (inhibitions between 34.26% and 44.94%), the activity was lower in others. The strongest chymotrypsin inhibitory activities were observed in R. ulmifolius methanol extract and T. orientalis water extract (IC_{50}: 65.32 and 78.65 µg/ml, respectively). In general, the studied plants showed high antioxidant activity in accordance with their high phenol and flavonoid contents. In conclusion, R. ulmifolius and T. orientalis attracted attention with strong chymotrypsin inhibitory activities. More comprehensive studies on the chymotrypsin activity of these plants are required.*

Keywords: *Antioxidant, chymotrypsin, folk medicine, Rubus ulmifolius, urease*

GİRİŞ

Üreaz, bitkiler, mantarlar ve bakteriler dâhil olmak üzere çok çeşitli canlıda bulunan, amonyak ve karbondioksit oluşturmak üzere ürenin hidrolizini katalize eden nikel bağımlı bir enzimdir [1]. Birincil fizyolojik rolü, organizmanın dışarıdan aldığı veya kendisi tarafından üretilen ürenin bir azot kaynağı olarak kullanmasını sağlamaktır [2]. *Helicobacter pylori*'nin midenin düşük pH'sında yaşaması ve kolonizasyonu için üreaz enzimine gereksinim duyduğu ve böylece peptik ülser, mide kanseri gibi patolojileri indüklediği bilinmektedir. Bununla birlikte üreaz enzimi üretebilen ve insanda enfeksiyonlara neden olan farklı bakteriler de bulunmaktadır. Örneğin *Proteus mirabilis*'in ürettiği üreaz enzimi ürolitiazis ve akut piyelonefrite neden olurken, *Yersinia enterocolitica'*nın ürettiği üreaz enzimi ise reaktif artrit gelişimine katkıda bulmaktadır. Üreaz enziminin aktivitesinin kontrolü, tarım ve ekoloji açısından da oldukça önemlidir. Tarımda üre içeren gübre uygulamalarından sonra topraktaki bakterilerin ürettiği üreaz aktivitesiyle atmosfere önemli derecede amonyak salınır ki, bu da ekolojiyi olumsuz yönde etkilemektedir. Diğer taraftan üreaz, ihtiyaç duydukları azottan mahrum bırakmanın yanında toprağın pH'sında artışa da neden olarak bitkiler için de zararlıdır [1]. N-(*n*-butil) tiyofosforik triamit (NBPT) gibi üreaz inhibitörleri kullanılarak bu durumun önüne geçilmeye çalışılır [3]. Fosforodiamidatlar, hidroksamik asit türevleri ve imidazol yapısındaki bazı bileşiklerin üreaz enzimini inhibe ettiği tespit edilmiş olsa da klinik kullanıma geçen sadece bir bileşik (asetohidroksamik asit) vardır. Dolayısıyla yan etkisi hiç olmayan veya kabul edilebilir düzeyde az olan yeni üreaz inhibitörlerinin keşfi oldukça önemli olup bu alanda yapılan çalışmalar son zamanlarda hız kazanmıştır [4].

Serin proteazlar, hidrolazlar grubuna ait proteazların önemli bir alt sınıfıdır. Gastrointestinal sistemde yer alan serin proteazlar insan vücudunda önemli fizyolojik fonksiyonlara sahiptir ve protein sindiriminde de anahtar rol oynamaktadır [5]. Serin proteazların inflamasyon sırasında doku hasarına karıştığı bilinmektedir. TNF-α, IFN-γ, interlökinler, nitrik oksit ve prostaglandinler de dâhil olmak üzere proinflamatuvar mediyatörlerin üretimini uyararak inflamatuvar yanıta katılırlar. Proinflamatuvar sitokin zimojenlerinin olgunlaşmasında yer alırlar, proinflamatuvar aktivitelerini indüklerler ve monositlerden salınmalarını da etkilerler. Bu nedenle, inflamatuvar sitokinlerin üretiminde yer alan serin proteazların aktivitesinin inhibisyonu veya modülasyonu, inflamatuvar hastalıklarının tedavisinde potansiyel bir strateji olarak kabul edilmektedir [6]. Bununla birlikte, aktivitelerindeki artma veya azalma kronik obstrüktif akciğer hastalığı, pankreatit, pankreas/kolorektal kanserler, Alzheimer gibi önemli hastalıklarla da ilişkilendirilmiştir [5,7]. Öte yandan, amino asitlerin fazlası yağa dönüşüp depolanan trigliseritlerin sentezinin öncüsü olan glukoz veya asetil-CoA üretiminde substrat olarak kullanılmaktadır. Dolayısıyla protein sindiriminde rol alan bu enzimlerin inhibisyonu, obezite tedavisinde de faydalı olabilmektedir. Ayrıca serin proteaz inhibitörlerinin bağışıklık sistemini ve kan

üretimini destekleyici etkisi de bilinmektedir [8]. AIDS tedavisinde kullanılan HIV proteaz inhibitörleri (örn: ritonavir) ve hipertansiyon tedavisinde kullanılan ACE inhibitörleri gibi ilaçlar, piyasada bulunan serin proteaz inhibitörlerinin örnekleridir [7].

Önemli serin proteazlardan biri olan kimotripsin, pankreasda inaktif prekürsörü kimotripsinojen olarak üretilir. Bağırsakta tripsin ile hidroliz edilip aktif formu kimotripsine dönüşür. Aktif hale geçen kimotripsin enzimi tirozin, fenilalanin, triptofan ve lösinin C-terminal tarafındaki peptit bağlarının hidrolizini seçici olarak katalize eder. Proteinlerin, fibrin pıhtılarının sindirimi, kanser hücrelerinin etrafındaki proteinlerin uzaklaştırılmasıyla kanserden korunma gibi birçok fizyolojik olayda önemli role sahiptir [9]. Bu enzimin inhibisyonunun bakteri üremesini önlediği, kan basıncını düşürüp vazodilatasyon sağladığı, antiinflamatuvar ve antikarsinojenik etkiye sahip olduğu gösterilmiştir [10]. Diğer taraftan kimotripsin inhibitörlerinin irritabl bağırsak sendromu ve ülseratif kolit semptomlarını azaltmada etkili olduğu da bildirilmiştir [11]. Günümüzde enfeksiyon hastalıkları başta olmak üzere pek çok hastalık üzerinde yapılan çalışmada, kimotripsin enziminin inhibisyonu hedef alınmakla birlikte henüz akut pankreatit, kronik pankreatit, Stevens-Johnson sendromu, yanık, septik şok ve toksik epidermal nekrolizis durumlarında klinik kullanımına onay alan bir adet kimotripsin inhibitörü bulunmaktadır. Söz konusu ürün (Ulinastatin®), yan etkileri olabilen ve intravenöz yoldan uygulanabilen bir üründür. Bu nedenle yan etkisi olmayan veya daha az olan, ucuz, oral yoldan uygulanabilecek kimotripsin inhibitörlerine ihtiyaç vardır.

Bitkiler, zengin fitokimyasal çeşitlilikleri ile yeni ilaç geliştirme çalışmalarında son derece önemli bir başlangıç noktasıdır. Tarama çalışması olarak planlanan bu projede de Düzce'de mide ağrısı, karın ağrısı, inflamasyon, iç hastalıkları, yara-yanık gibi üreaz, kimotiripsin inhibe edici ve antioksidan aktivitenin etkili olabileceği durumları akla getiren hastalıklar başta olmak üzere halk ilacı olarak kullanılan [12] ve daha önce üzerinde üreaz/kimotripsintripsin enzimini inhibe edici aktivitesi konusunda çalışma bulunmayan yedi bitkinin [*Dioscorea communis* (L.) Caddick & Wilkin, *Mentha longifolia* (L.) L. subsp. *thyphoides* (Briq.) Harley, *Origanum vulgare* L., *Rubus ulmifolius* Schott, *Salvia tomentosa* Mill., *Thymus longicaulis* C.Presl subsp. *longicaulis*, *Trachystemon orientalis* (L.) D. Don] üreaz ve kimotripsin enzimini inhibe edici aktivitelerinin değerlendirilmesi amaçlanmıştır. Ayrıca ABTS, CUPRAC, DPPH yöntemleri ile antioksidan aktivite tayinlerinin yapılması, ilaveten toplam fenol ve toplam flavonoit içeriklerinin belirlenmesi de amaçlanmıştır.

GEREÇ VE YÖNTEM

Bitki Materyali

Dioscorea communis Düzce, Akçakoca, Kurukavak'dan (toplayıcı no: 09DZ066); *Mentha longifolia* subsp. *thyphoides* Düzce, Çilimli, Yukarıköy'den (toplayıcı no: 09DZ143); *Origanum vulgare* Düzce, Yığılca, Güney Köyü'nden (toplayıcı no: 09DZ155); *Rubus ulmifolius* Düzce, Merkez, Yukarıyahyalar'dan (toplayıcı no: 09DZ154); *Salvia tomentosa* Düzce, Yığılca, Mengen'den (toplayıcı no: 09DZ157); *Thymus longicaulis* subsp. *longicaulis* Düzce, Gümüşova, Yeşilyayla Köyü'nden (toplayıcı no: 09DZ109); *Trachystemon orientalis* Düzce, Akçakoca, Kurukavak Köyü'nden (toplayıcı no: 09DZ068) toplanmıştır. Bitkilerin bilimsel isimlerinin tayini Prof. Dr. Galip Akaydın tarafından yapılmıştır. Herbaryum örnekleri Gazi Üniversitesi Eczacılık Fakültesi Herbaryumu'nda (GUEF) muhafaza edilmektedir.

Ekstraksiyon

D. communis'in kökleri, *Rubus ulmifolius*'un sürgünleri, *M. longifolia* subsp. *thyphoides*, *O. vulgare*, *T. longicaulis* subsp. *longicaulis* ve *T. orientalis'in* toprak üstü kısımları, *Salvia tomentosa'nın* çiçek, yaprak ve toprak üstü kısımları gölgede kurutulmuş ve bitki değirmeninde kaba toz olacak şekilde öğütülmüştür. Daha sonra her bitki örneğinden 2'şer gram alınmış ve ayrı ayrı 50'şer ml metanol veya su ile ultrasonik banyoda 30 dakika çalkalanarak masere edilmiştir. Süre sonunda ekstreler süzülmüş ve maserasyon işlemi aynı şekilde ikişer kez daha tekrar edilmiştir. Her bitkiden elde edilen metanol ekstreleri kendi içinde birleştirilip alçak basınç altında 45°C'yi geçmeyen sıcaklıkta evapore edilerek kurutulmuş, sulu ekstreler ise liyofilize edilmiştir. Elde edilen tüm ekstreler kullanılıncaya kadar buzdolabında (4-6°C'de) muhafaza edilmiştir.

Üreaz İnhibe Edici Aktivite Tayini

Üreaz aktivitesi, indofenol yöntemi kullanılarak amonyak üretiminin ölçülmesi ile tespit edilmiştir. İlk olarak mikroplağın kuyucuklarına 25'er µl jack bean üreaz enzim solüsyonu (10 U/ml) ve 5 µl test örneği (%80 etanol içinde) konulup 30°C'de 15 dakika inkübe edilmiştir. Daha sonra her bir kuyucuğa 55 µl 100 mM üre içeren tampon (0.01 M K_2HPO_4, 1 mM EDTA ve 0.01M LiCl) ilave edilip tekrar 30°C'de 15 dakika inkübe edilmiştir. İnkübasyonun ardından her kuyucuğa 45 µl fenol reajanı (%1 a/h fenol ve %0.005 a/h sodyum nitroprusit) ve 70 µl alkali reajanı (%0.5 a/h NaOH ve %0.1 aktif klorit taşıyan NaOCl) ilave edilmiş ve 50 dakika oda sıcaklığında inkübe edildikten sonra 630 nm'de absorbansdaki artış ölçülmüştür. Kontrol ve referans deneylerinde test örneği yerine sırasıyla %80'lik etanol ve tiyoüre kullanılmıştır. Deneyler üç tekrar olacak şekilde yapılmıştır [13]. Yüzde inhibisyon aşağıdaki formül ile hesaplanmıştır:

% inhibisyon= ($A_{kontrol}$ -A_{test}) / $A_{kontrol}$ x 100
A_{test}: Test örneğinin absorbansı
$A_{kontrol}$: Kontrolün absorbansı

Kimotripsin İnihibe Edici Aktivite Tayini

α-Kimotripsin inhibe edici aktivite Cannell ve ark. (1988) tarafından kullanılan metotta bazı değişiklikler yapılarak tespit edilmiştir. 60 µl 50 mM Tris-HCl tamponu, 10 µl test çözeltisi (etanol içinde) ve 30 µl enzim çözeltisi (36 U/ml) içeren reaksiyon karışımı 15 dakika 37°C'de inkübe edilmiş ve süre sonunda 410 nm'deki absorbansları Versa Max mikroplaka okuyucu ile ölçülmüştür. Bu işlemin ardından reaksiyonu başlatmak için 1,3 mM 30 µl substrat (N-süksinil fenilalanin-p-nitroanilit) eklenmiştir. 60 dakika 37°C'de inkübasyondan sonra 410 nm'deki absorbanslar tekrar ölçülmüştür. Pozitif kontrol olarak fenilmetansülfonil florit kullanılmıştır. Tüm deney üç tekrar olacak şekilde yapılmıştır. IC_{50} değerleri GraphPad Prism 6 yazılımı kullanılarak hesaplanmıştır. Yüzde inhibisyon aşağıdaki formül ile hesaplanmıştır [14]:

% inhibisyon = ($A_{kontrol}$ -A_{test}) / $A_{kontrol}$ x 100
A_{test}: Test örneğinin absorbansı
$A_{kontrol}$: Kontrolün absorbansı

DPPH (1,1-difenil-2-pikrilhidrazil) Radikal Süpürücü Aktivite Tayini

Mikroplakanın kuyucuklarına test örneklerinden (metanol veya su içinde) 150'şer µl ve $1x10^{-3}$ M DPPH çözeltisinden (metanol içinde) 50'şer µl konulup 30 dakika karanlıkta, oda sıcaklığında inkübe edilmiştir. İnkübasyonun ardından absorbanslar 517 nm'de ölçülmüştür. Kontrol deneyinde test örneği yerine su veya metanol konulmuştur. Standart antioksidan ajan olarak gallik asit (metanol içinde) kullanılmıştır. Her deney üç kez tekrarlanmıştır. Yüzde inhibisyonlar aşağıdaki formülden hareketle hesaplanmıştır [15].

Yüzde inhibisyon= [($A_{kontrol}$ – A_{test})]/($A_{kontrol}$) x 100
$A_{kontrol}$: kontrolün absorbansı
A_{test}: test örneğinin absorbansı

Bakır İndirgenme Antioksidan Kapasitenin (CUPRAC) Tayini

İlk olarak standart antioksidan bileşik olarak kullanılan gallik asitten seri dilüsyon (metanol içinde) hazırlanmıştır. Mikroplakanın kuyucuklarına 27.5 µl test örneği (metanol veya su içinde) veya farklı konsantrasyonlardaki gallik asit çözeltilerinden konulmuştur. Daha sonra her bir kuyucuğa sırasıyla 27.5 µl su, 50 µl 10^{-2} M $CuCl_2$ çözeltisi, 50 µl amonyum asetat tamponu (1 M, pH 7.0), 50 µl neokuproin çözeltisi (7.5 x 10^{-3} M, %96'lık etanol içinde) konulmuş ve 30 dakika inkübasyona bırakılmıştır. İnkübasyonun ardından 450 nm'deki absorbanslar köre karşı ölçülmüştür. Deney sonucunda gallik asit çözeltileri ile elde edilen absorbanslar konsantrasyona karşı grafiğe geçirilerek kalibrasyon eğrisi hazırlanmıştır. Örneklerinin deney sonucunda verdiği absorbans ve gallik asit ile

hazırlanan kalibrasyon eğrisinin eğiminin denkleminden hareketle sonuçlar "mg gallik asit eşdeğeri (G.A.E.)/g ekstre" cinsinden hesaplanmıştır. Her deney üç kez tekrarlanmıştır [16].

ABTS [2,2'-azino-bis(3-etilbenzotiazolin-6-sülfonik asit)] Radikal Katyonu Renk Giderici Aktivitenin Tayini

İlk olarak 0.1 M fosfat tamponu (pH 7.4) içerisinde 2 mM ABTS çözeltisi hazırlanmıştır. Daha sonra bu çözeltiye 2.45 mM'lık potasyum persülfat ilave edilip manyetik karıştırıcı ile 12 saat karıştırılıp ABTS radikali üretilmiştir. Hazırlanan ABTS radikal çözeltisi kullanılmadan önce 3:1 oranında fosfat tamponu ile seyreltilmiştir. Standart antioksidan olarak kullanılan gallik asitten (metanol içinde) seri dilüsyonlar hazırlanmıştır. Mikroplakanın kuyucuklarına 100 µl test örneği (metanol veya su içinde) veya farklı konsantrasyonlardaki gallik asit çözeltisi ve 100 µl ABTS çözeltisi konulmuştur. Kontrol olarak test örneği/gallik asit çözeltisi yerine metanol veya su kullanılmıştır. 30 dakika oda sıcaklığında karanlıkta inkübe edildikten sonra absorbansları 734 nm'de okunmuş ve gallik asit çözeltileri ile elde edilen absorbanslar konsantrasyona karşı grafiğe geçirilerek kalibrasyon eğrisi hazırlanmıştır. Örneklerin deney sonucunda verdiği absorbans ve kalibrasyon eğrisinin eğiminin denkleminden hareketle sonuçlar "mg G.A.E./g ekstre" cinsinden hesaplanmıştır. Her deney üç kez tekrarlanmıştır [17].

Total Flavonoit Miktar Tayini

Total flavonoit miktarının tayini "aluminyum klorür kolorimetrik yöntemi" ile belirlenmiştir [18]. Bu yöntemde ilk olarak rutinden (%80 etanol içinde) bir seri dilüsyon hazırlanmıştır. Test örnekleri metanol veya suda çözülmüştür. Mikroplakanın kuyucuklarına 20 µl test örneği veya farklı konsantrasyondaki rutin çözeltilerinden konulmuştur. Üzerine 60 µl %75'lik etanol, 10 µl %10'luk aluminyum klorür, 10 µl 0.4 M sodyum asetat çözeltisi ve 100 µl distile su ilave edilip oda sıcaklığında 30 dakika inkübe edilmiştir. İnkübasyonun ardından 415 nm'deki absorbanslar ölçülmüş ve rutin dilüsyonları ile elde edilen absorbanslar konsantrasyona karşı grafiğe geçirilip kalibrasyon eğrisi hazırlanmıştır. Rutin ile hazırlanan kalibrasyon eğrisinden hareketle örneklerin total flavonoit miktarı "mg rutin eşdeğeri (R.E.)/g ekstre" cinsinden hesaplanmıştır. Her deney üç kez tekrar edilmiştir.

Total Fenol Miktar Tayini

Toplam fenol miktar tayini "Folin Ciocalteu yöntemi" ile belirlenmiştir [19, 20]. Öncelikle gallik asitten seri dilüsyonlar hazırlanmıştır. 100 µl test örnek çözeltisi (su içinde) 125 µl Lowry C çözeltisi* ile karıştırılmış ve oda sıcaklığında 10 dakika inkübe edilmiştir. Daha sonra reaksiyon karışımına 12.5 µl Folin Ciocalteu reajanı (%96 etanol ile 1/3 oranında seyreltilerek) ilave edilmiştir. Oda sıcaklığında 30 dakikalık inkübasyondan sonra oluşan mavi rengin absorbansı 750 nm'de Versa Max mikroplaka okuyucu ile ölçülmüştür. Gallik asit çözeltileri ile elde edilen absorbanslar konsantrasyona karşı grafiğe geçirilip kalibrasyon eğrisi oluşturulmuştur. Gallik asit ile elde edilen kalibrasyon eşitliğinden hareketle test örneklerinin total fenol miktarı "mg G.A.E./g ekstre" cinsinden hesaplanmıştır. Her deney üç kez tekrar edilmiştir. Bitkilerin metanol ekstrelerinin sudaki çözünürlüğü oldukça düşük olup ancak metanol-etanol gibi organik çözücülerde çözülebilmiştir. Organik çözücüde çözülüp bu prosedüre uygulandıklarında ise çökelek oluşması nedeniyle sonuçlar değerlendirilememiş ve sadece sulu ekstrelerin fenolik içerikleri tespit edilmiştir.

* Lowry C çözeltisinin hazırlanması:
Lowry C çözeltisi = 1 ml Lowry B çözeltisi + 50 ml Lowry A çözeltisi
Lowry B solüsyonu = 25 mg $CuSO_4$ + 5 ml %1 $NaKC_4H_4O_6$ çözeltisi
Lowry A çözeltisi = 50 ml 0.1M NaOH çözeltisi + 1 g Na_2CO_3

SONUÇ VE TARTIŞMA

Çalışılan ekstrelerin üreaz ve kimotripsin inhibe edici aktiviteleri Tablo 1'de verilmiştir (sadece %40 ve üzeri inhibisyon gösteren ekstreler için IC_{50} değeri hesaplanmış, daha düşük aktivite görülen durumlarda %50 ve üstü aktiviteye ulaşmak için gerekecek yüksek konsantrasyonlar deney prosedürü için uygun değildir). Bu tablodan anlaşılacağı üzere çalışılan bitkiler üreaz enzimi üzerinde genel olarak orta ve düşük inhibe edici aktiviteye sahip olmakla birlikte en yüksek üreaz inhibe edici aktivite *D.*

communis (kök) bitkisinin metanol ekstresinde görülmüştür (IC_{50}: 66.03 µg/ml). *R. ulmifolius*'un metanol ekstresi ve *T. orientalis*'in su ekstresi ise kimotripsin enzimini önemli derece inhibe etmektedir (IC_{50} değerleri 65.32 ve 78.65 µg/ml).

Tablo 1. Çalışılan bitkilerin üreaz ve kimotripsin inhibe edici aktiviteleri

Bitkiler/referans maddeler	**Ekstre**	**İnhibisyon (%) ± standart hata**	
		Üreaz[a]	**Kimotripsin[b]**
D. communis (kök)	Su	38.93 ± 2.45	9.36 ± 1.21
	Metanol	44.94 ± 0.79 (IC_{50}: 66.03 ± 1.19 µg/ml)	19.88 ± 1.20
D. communis (toprak üstü)	Su	27.22 ± 3.44	-
	Metanol	27.42 ± 1.08	25.39 ± 1.58
M. longifolia subsp. *thyphoides* (toprak üstü)	Su	24.42 ± 1.58	18.25 ± 1.37
	Metanol	20.65 ± 1.19	46.21 ± 0.90 (IC_{50}: 160.63 ± 1.10 µg/ml)
O. vulgare (toprak üstü)	Su	17.44 ± 0.72	19.71 ± 0.47
	Metanol	34.82 ± 0.83	22.11 ± 1.07
R. ulmifolius (sürgün)	Su	18.24 ± 1.14	33.33 ± 1.37
	Metanol	26.58 ± 0.24	76.69 ± 0.95 (IC_{50}: 65.32 ± 0.43 µg/ml)
S. tomentosa (çiçek)	Su	15.77 ± 3.52	38.22 ± 1.40
	Metanol	31.89 ± 0.94	15.03 ± 1.89
S. tomentosa (yaprak)	Su	16.56 ± 1.07	44.33 ± 1.13 (IC_{50}: 206.70 ± 3.16 µg/ml)
	Metanol	37.41 ± 1.30	27.70 ± 1.63
S. tomentosa (toprak üstü)	Su	7.55 ± 1.01	47.41 ± 1.05 (IC_{50}: 192.36 ± 5.35 µg/ml)
	Metanol	29.79 ± 0.87	34.89 ± 0.84
T. longicaulis subsp. *longicaulis* (toprak üstü)	Su	2.58 ± 1.16	52.91 ± 0.83 (IC_{50}: 153.96 ± 4.57 µg/ml)
	Metanol	34.26 ± 1.16	39.86 ± 0.59
T. orientalis (toprak üstü)	Su	15.63 ± 1.13	72.27 ± 0.80 (IC_{50}: 78.65 ± 0.47µg/ml)
	Metanol	23.37 ± 1.27	26.96 ± 1.36
Tiyoüre[c]	-	100.0 (IC_{50}: 6.70 ± 0.24 µg/ml)	-
FMSF[d]	-	-	100.0 (IC_{50}: 0.98 ± 0.19 µg/ml)

[a] 50 µg/ml konsantrasyondaki % inhibisyon
[b] 153.84 µg/ml konsantrasyondaki % inhibisyon
[c] Standart üreaz inhibitörü (25 µg/ml konsnatrasyonda)
[d] Fenilmetanesülfonil florit (standart kimotripsin inhibitörü, 4.8 µg/ml konsantrasyonda)
- İnhibitör aktivite yok

Çalışılan bitkilerin antioksidan aktivite deneylerinden elde edilen sonuçlar Tablo 2'de bir arada sunulmuştur. Tüm ekstreler genel olarak kuvvetli DPPH radikal süpürücü aktivite göstermekle birlikte en yüksek aktivite *S. tomentosa* (çiçek), *T. longicaulis* subsp. *longicaulis*, ve *M. longifolia* subsp. *thyphoides*'un metanol ekstrelerinde gözlenmiştir (sırasıyla IC_{50} değerleri; 9.11, 11.92 ve 12.70 µg/ml). ABTS testinde ise *M. longifolia* subsp. *thyphoides*, *O. vulgare* ve *S. tomentosa* (yaprak) sulu ekstreleri

ön plana çıkmıştır (sırasıyla 56.47, 56.43 ve 56.35 mg G.A.E./g ekstre). *T. longicaulis* subsp. *longicaulis*'in metanol, *S. tomentosa* (toprak üstü)'nın metanol ve *O. vulgare*'nin sulu ekstrelerinin sırasıyla 343.24, 270.77 ve 166.85 mg G.A.E./g ekstre değerleri ile en yüksek bakır indirgeyici antioksidan aktiviteye sahip oldukları tespit edilmiştir. Antioksidan aktivite sonuçları ile uyumlu olarak *S tomentosa*'nın farklı kısımlarından hazırlanan ekstrelerin en yüksek fenolik içeriğe sahip oldukları belirlenmiştir. Benzer şekilde kuvvetli antioksidan aktiviteye sahip olan *T. longicaulis* subsp. *longicaulis* (170.04 mg R.E./g ekstre), *S. tomentosa* (145.82 mg R.E./g ekstre) ve *M. longifolia* subsp. *thyphoides*'in (145.37 mg R.E./g ekstre) yüksek flavonoit içeriğine sahip olduğu anlaşılmıştır (Tablo 3).

Tablo 2. Çalışılan bitkilerin radikal süpürücü ve bakır indirgeyici antioksidan kapasiteleri

Bitkiler/referans maddeler	Ekstre	DPPH[a]	ABTS[b]	CUPRAC[b]
D. communis (kök)	Su	42.95 ± 1.57	19.12 ± 0.42	5.89 ± 2.86
	Metanol	49.08 ± 1.00	26.02 ± 0.08	80.80 ± 0.80
D. communis (toprak üstü)	Su	21.39 ± 1.04	27.23 ± 0.08	26.08 ± 3.75
	Metanol	66.54 ± 1.61	21.12 ± 0.09	26.79 ± 3.90
M. longifolia subsp. *thyphoides* (toprak üstü)	Su	31.29 ± 0.32	56.47 ± 0.11	76.63 ± 3.33
	Metanol	12.70 ± 0.87	27.11 ± 0.07	89.56 ± 3.72
O. vulgare (toprak üstü)	Su	70.56 ± 1.65	56.43 ± 0.01	166.85 ± 3.94
	Metanol	29.09 ± 0.85	27.89 ± 0.04	124.84 ± 1.47
R. ulmifolius (sürgün)	Su	22.28 ± 0.95	56.25 ± 0.19	76.82 ± 2.38
	Metanol	19.18 ± 0.19	27.59 ± 0.06	106.26 ± 2.35
S. tomentosa (çiçek)	Su	29.84 ± 1.51	56.26 ± 0.14	61.51 ± 3.34
	Metanol	9.11 ± 0.34	27.73 ± 0.04	107.59 ± 3.33
S. tomentosa (yaprak)	Su	14.58 ± 1.58	56.35 ± 0.20	127.62 ± 4.92
	Metanol	33.32 ± 1.11	27.64 ± 0.032	104.07 ± 2.78
S. tomentosa (toprak üstü)	Su	31.68 ± 1.47	52.93 ± 0.32	98.30 ± 3.72
	Metanol	14.37 ± 0.32	27.67 ± 0.02	270.77 ± 2.23
T. longicaulis subsp. *longicaulis* (toprak üstü)	Su	18.52 ± 0.62	56.34 ± 0.03	112.83 ± 3.61
	Metanol	11.92 ± 0.39	28.14 ± 0.03	343.24 ± 3.51
T. orientalis (toprak üstü)	Su	42.62 ± 1.10	54.98 ± 0.15	31.45 ± 2.58
	Metanol	26.50 ± 1.53	28.06 ± 0.01	68.88 ± 3.98
Gallik asit	-	2.51 ± 0.01	-	-

[a] IC_{50} (µg/ml) ± standart hata
[b] mg G.A.E./g ekstre ± standart hata

Yaptığımız literatür çalışmasına göre çalışılan bitkilerden *O. vulgare* dışındakilerin üreaz ve kimotripsin enzimini inhibe edici aktiviteleri daha önce araştırılmamıştır. Tablo 1'den de anlaşılacağı üzere *M. longifolia*'nın toprak üstü kısımlarından hazırlanan su ve metanol ekstreleri, üreaz enzimine karşı çok yüksek inhibitör aktivite göstermemiştir (sırasıyla %24.42 ve 20.65 inhibisyon). Daha önceki bir araştırmada *M. longifolia*'nın antibakteriyel etkisi 10 ayrı klinik *H. pylori* suşu izolatı üzerinde araştırılmıştır. Araştırmanın sonucunda bitkinin organik ekstresinin 18-40 mm arasında değişen inhibisyon zonları ve 62.5-250 µg/ml aralığında değişen MİK değerleri ile *H. pylori*'nin üremesini engellediği ortaya çıkmıştır [21]. Fakat Bakr ve ark. (2021) daha yakın tarihteki çalışmalarında *M. longifolia*'nın %70 etanol ekstresinin *H. pylori* üzerinde antimikrobiyal aktiviteye sahip olmadığını belirlemiştir [22]. Gul ve ark. (2015), bitkinin etanollü ekstresinin sıçanlarda 200 mg/kg dozda etanol ülserini %47, aspirin ülserini ise %63 oranında önlediğini tespit etmiştir [23]. Bitkinin antioksidan aktivitesinin ve total fenol-flavonoit içeriğinin incelendiği daha eski çalışmalara da rastlanmıştır. Bu çalışmalardan ilkinde üç farklı lokaliteden toplanan *M. longifolia*'nın su ve metanol ekstrelerinin fenol içeriklerinin 2.99 ile 28.27 mg pirokateşol/g arasında; flavonoit içeriklerinin ise 1.27-25.42 mg kersetin

eşdeğeri (K.E.)/g ekstre değerleri arasında değiştiği tespit edilmiştir. DPPH radikalini ise 100 µg/ml konsantrasyonda %38.08-95.77 oranında inhibe ettiği belirlenmiştir [24]. Bir başka çalışmada ise etanol ekstresi ve infüzyonunun total fenol içeriğinin sırasıyla 67.05 ve 92.38 mg G.A.E./g ekstre; flavonoit içeriğinin ise 23.68 ile 46.18 mg R.E./g ekstre olduğu kaydedilmiştir. DPPH testinde 162.08 ve 195.96 mg troloks eşdeğeri/g ekstre; ABTS testinde 242.06 ve 269.25 mg troloks eşdeğeri/g ekstre; CUPRAC testinde ise 371.30 ve 454.99 mg troloks eşdeğeri/g ekstre antioksidan aktivite sergilemiştir [25]. Yakın tarihli bir araştırmada ise bitkinin yapraklarının metanol ekstresinin 9.1 µg/ml IC_{50} değeriyle DPPH radikalini inhibe ettiği belirtilmiştir [26]. Üreaz enzimi üzerinde çok yüksek aktivite tespit edilmese de daha önce yapılan mikrobiyolojik ve *in vivo* deney sonuçları dikkate alındığında [21,23], biyoaktivite yönlendirmeli fraksiyonlama, yapı-aktivite gibi daha ileri çalışmaların yapılması halinde *M. longifolia*'nın *H. pylori* pozitif olan ve olmayan ülserlerin tedavisinde umut verici olabileceği ve gastroprotektif etkisine antioksidan aktivitesinin de katkı sağladığı görüşü akla gelmektedir.

Tablo 3. Çalışılan bitkilerin total fenol ve flavonoit içerikleri

Bitkiler	Ekstre	Total fenol içeriği[a]	Total Flavonoit içeriği[b]
D. communis (kök)	Su	49.50 ± 1.73	4.71 ± 1.92
	Metanol	-	70.26 ± 2.90
D. communis (toprak üstü)	Su	40.00 ± 2.70	17.82 ± 1.38
	Metanol	-	97.15 ± 1.01
M. longifolia subsp. *thyphoides* (toprak üstü)	Su	78.87 ± 4.16	57.6 ± 4.80
	Metanol	-	145.37 ± 3.00
O. vulgare (toprak üstü)	Su	129.63 ± 9.12	106.04 ± 4.33
	Metanol	-	88.26 ± 2.66
R. ulmifolius (sürgün)	Su	170.93 ± 6.55	78.04 ± 4.28
	Metanol	-	99.37 ± 0.76
S. tomentosa (çiçek)	Su	221.50 ± 3.62	97.15 ± 4.07
	Metanol	-	122.48 ± 2.69
S. tomentosa (yaprak)	Su	313.28 ± 2.79	143.82 ± 4.07
	Metanol	-	145.82 ± 4.23
S. tomentosa (toprak üstü)	Su	387.17 ± 3.53	135.15 ± 3.79
	Metanol	-	126.48 ± 1.38
T. longicaulis subsp. *longicaulis* (toprak üstü)	Su	204.03 ± 5.23	135.82 ± 3.90
	Metanol	-	170.04 ± 1.01
T. orientalis (toprak üstü)	Su	99.73 ± 6.53	42.48 ± 4.01
	Metanol	-	57.37 ± 2.52

[a]mg G.A.E./g ekstre ± standart hata
[b]mg R.E./g ekstre ± standart hata
- Çalışılmadı

Araştırmamızda *R. ulmifolius*'un su ve metanol ekstresinin de üreaz enzimini çok yüksek olmasa da (sırasıyla %18.24 ve 26.58 inhibisyon) inhibe ettiği görülmüştür. Daha önceki bir çalışmada yaprak ekstresinin iki *H. pylori* suşuna karşı 134 µg/ml ve 270 µg/ml minimum bakterisidal konsantrasyonu ile antibakteriyel aktiviteye sahip olduğu belirlenmiştir. Aynı çalışmada bitkinin antioksidan kapasitesi ABTS testi ile belirlenmiş ve ABTS radikalini %55 oranında inhibe ettiği görülmüştür [27]. Bu sonuçlar bitkinin *H. pylori* üzerindeki antibakteriyel aktivitesinin kısmen üreaz enzimi inhibisyonu ile gerçekleşebileceğini, bununla birlikte farklı mekanizmaların da rol oynuyor olabileceğini düşündürmüştür. Antioksidan aktivite testi sonuçları da dikkate alındığında, bitkinin *H. pylori* pozitif olan peptik ülser hastalığı üzerindeki etkisinin daha ayrıntılı bir şekilde araştırılmasının gerektiği düşünülmektedir. Elde ettiğimiz sonuçlar bitkinin metanol ekstresinin kimotripsin enzimini oldukça

önemli derecede inhibe ettiğini göstermiştir (IC_{50}: 65.32 µg/ml). Bu araştırmanın bir tarama çalışması olup biyoaktivite yönlendirmeli fraksiyonlama yapılmaması nedeniyle bu etkiden hangi madde/maddelerin sorumlu olduğu bilinmemektedir. Ancak bitkinin tanen ve polifenoller bakımından zengin olduğu bilinmektedir [27,28]. Kimotripsinin de arasında bulunduğu proteazların aktivitesini tanenlerin inhibe ettiğini gösteren çalışmalar mevcut olup [29,30] *R. ulmifolius*'da gözlenen yüksek kimotripsin inhibe edici aktivitede tanenlerin etkili olabileceği düşünülmüştür.

Araştırmamızdan elde edilen sonuçlar *D. communis*'in köklerinden hazırlanan metanol ekstresinin üreaz enzimini önemli derecede inhibe ettiğini göstermiştir (IC_{50}: 66.03 µg/ml). Bu araştırmanın bir tarama çalışması olması bakımından biyoaktivite yönlendirmeli fraksiyonlama yapılmamıştır ve dolayısıyla bitkideki hangi maddenin bu etkiden sorumlu olduğu bilinmemektedir. Ancak bitkinin kökleri üzerinde yapılan fitokimyasal çalışmalar fenantren ve 9,10-dihidrofenantren (nudol, konfusarin, krizotoksen, herorensol, orkinol gibi) bakımından zengin olduğunu göstermiştir. Ayrıca *β*-sitosterol, stigmasterol ve kampesterol gibi steroller taşıdığı da bilinmektedir [31]. Bildiğimiz kadarıyla fenatrenlerin üreaz enzimi üzerindeki etkisi henüz değerlendirilmemiştir ancak daha önceki bir çalışmada sterol yapısındaki ursolik asidin üreaz enzimini %52 oranında inhibe ettiği gösterilmiştir [32]. Dolayısıyla bitkinin üreaz enzimini inhibe edici aktivitesinde taşıdığı sterol yapısındaki bileşiklerin rol oynayabileceği akla gelmektedir. *D. communis*'in metanol ekstresi DPPH, ABTS ve CUPRAC yöntemlerinde çok yüksek olmasa da belirli bir aktiviteye sahiptir. Daha önceki bir araştırmada *D. communis*'in köklerinden hazırlanan sulu-metanol esktresi ve ondan elde edilen kloroform, etilasetat ve kalan su fraksiyonlarının antioksidan aktivitesi DPPH yöntemi ile değerlendirilmiş, kloroform ve etilasetat fraksiyonlarının, kalan su fraksiyonundan önemli ölçüde daha yüksek radikal süpürücü aktivite gösterdiği (BHT'e yakın aktivite) anlaşılmıştır [33]. Daha yakın tarihli bir çalışmada bitkinin köklerinden hazırlanan metanol ekstresinin fenolik içeriği ve DPPH radikal süpürücü aktivitesi belirlenmiştir. Fenol içeriği bizim bulgularımızla benzer şekilde 55.2 mg G.A.E./g ekstre olarak tespit edilmiş, DPPH radikalini ise 0.128 mg/ml IC_{50} değeri ile inhibe ettiği ifade edilmiştir [34].

Çalışmamızda *S. tomentosa,* antioksidan aktivite, toplam fenol ve toplam flavonit içeriği bakımından ön plana çıkan bitkilerden biri olmuştur. Daha önceki bir araştırmada farklı bölgelerden toplanmış yabani olarak yetişen veya kültüre alınmış *S. tomentosa* örneklerinin antioksidan aktivitesi, toplam fenol ve toplam flavonoit içeriği kıyaslanmıştır. Çalışmanın sonucunda %80 metanol ekstresinin 49.27-66.15 mg G.A.E./g ekstre aralığında değişen oranlarda fenol içeriğine ve 36.27-40.83 mg kateşin eşdeğeri/g flavonoit içeriğine sahip olduğu belirlenmiştir [36]. Fenol içeriğinin bizim çalışmamızdakinden farklı olmasının nedeninin, bitkinin toplandığı lokasyon, ekstraksiyon çözücüsü, ekstraksiyon yöntemi ve fenol içeriğinin tespitinde kullanılan yöntem farklılıkları gibi nedenler olabileceği düşünülmelidir.

Çalışmaya dâhil edilen bitkiler arasında *T. orientalis* kuvvetli kimotripsin inhibe edici aktivite göstermesiyle ön plana çıkmıştır. Bu aktivitenin taşıdığı hangi sekonder metabolitten kaynaklandığı bilinmemekle birlikte bitki üzerinden yapılan fitokimyasal çalışmalar son derece kısıtlıdır. Ancak bir araştırmada bitkinin toprak üstü kısımlarının *β*-karoten, likopen, flavonoit, antosiyanin taşıdığı belirlenmiştir [37]. Bu sebeple taşıdığı terpen ve fenolik bileşiklerin bu aktiviteye katkı sağlayabileceği akla gelmektedir. Literatür taramamız sonucunda *T. orientalis*'in toplam fenol, toplam flavonoit içeriği ve antioksidan aktivitesinin değerlendirildiği daha eski tarihli bir çalışmaya rastlanılmıştır. Bu çalışmada bitkinin toprak üstü kısımlarından %80 etanol ekstresinin 82.1 mg pirokateşol eşdeğeri/g ekstre toplam fenol ve 3.63 mg K.E./g ekstre değerinde flavonoit içeriğine sahip olduğu, DPPH radikalini 100 µg/ml konsantrasyonda yaklaşık %47 oranında inhibe ettiği belirtilmiştir [38]. Kullanılan birimin farklı olması nedeniyle fenol ve flavonoit içerikleri bizim çalışmamızla kıyaslanamamıştır. Ancak antioksidan aktivitede gözlenen farklılığın kullanılan ekstraksiyon çözücüsü, bitkinin toplandığı lokasyon, çalışılan konsantrasyonlar gibi farklılıklardan kaynaklanabileceği akla gelmektedir.

T. longicaulis de antioksidan aktivite ve yüksek flavonoit içeriği ile dikkat çeken bitkilerden biridir. Daha önce yapılan çalışmalarda da kuvvetli antioksidan aktiviteye sahip olduğu belirlenmiştir [39,40]. Bu çalışmalardan ilkinde toprak üstü kısımlarından hazırlanan %70 etanol ekstresinin 25 µg/ml konsantrasyonda %87.82 DPPH radikal süpürücü aktiviteye sahip olduğu belirtilmiştir [39]. Aynı yıl yapılan bir başka araştırmada ise metanol ekstresi ve infüzyonunun fenol içerikleri 188.98 ve 119.43 mg G.A.E./g ekstre; flavonoit içerikleri ise 21.43 ve 11.43 mg R.E./g ekstre olarak hesaplanmıştır. DPPH testinde 3.6 µg/ml konsantrasyonda %65.28 ve %54.37 inhibisyon göstermiştir [40].

Araştırmamızda *O. vulgare* su ve metanol ekstreleri 50 µg/ml konsantrasyonda düşük üreaz inhibe edici aktivite göstermiştir (sırasıyla %19.71 ve %22.11 inhibisyon). Hanganu ve ark. tarafından 2020 yılında yapılan çalışmada ise bitkinin toprak üstü kısımlarının %70 etanol ekstresinin 200 µg/ml konsantrasyonda üreaz enzimini %90.47 oranında inhibe ettiği sonucuna varmıştır. Yine aynı çalışmada total fenol içeriği ise 89.21 mg G.A.E./g ekstre olarak hesaplanmıştır [41]. Söz konusu araştırmada üreaz inhibe edici aktivitenin tespiti için bizim yöntemimizden (indofenol metodu) [13] oldukça farklı olarak Nessler Reajanı kullanılmıştır. Total fenol içeriğinin belirlenmesi için ise kullandığımız Lowry metodu [19,20] yerine sadece sodyum karbonat ve Folin Ciocalteu Reajanın kullandığı bir yöntemi tercih etmişlerdir. Dolayısıyla bizim çalışmamızda tespit edilen üreaz inhibe edici aktivite ve fenol içeriklerinden farklı sonuçların elde edilmesinin sebebinin kullanılan yöntemlerdeki bu farklılıklardan kaynaklanabileceği düşünülmüştür. Bitkinin *H. pylori* üzerindeki aktivitesi de daha önce araştırılmıştır. Bu çalışmalardan birinde %70 metanol ekstresinin 15 klinik *H. pylori* izolatı üzerindeki aktivitesi değerlendirilmiş ve 0.625 ile ≥ 5 mg/ml arasında değişen MİK değerleri ile çalışılan *H. pylori* suşları üzerinde orta derecede bir antibakteriyel etkiye sahip olduğu tespit edilmiştir [42]. Bir diğer araştırmada ise klonlanan ve ticari *O. vulgare*'nin dekoksiyonu ve %60 etanol ekstresinin fenolik içeriğinin 35.43 ile 55.35 mg G.A.E./g ekstre değerleri arasında değiştiği; %1'lik çözeltisinin (1 g bitki/100 mL çözücü) DPPH testinde %80-82, ABTS testinde ise %65-95 arasında değişen radikal süpürücü aktivite sergilediği tespit edilmiştir. Yine aynı çalışmada hazırlanan ekstrelerin *H. pylori* üzerindeki etkisi de araştırılmış ve disklere emdirilen 50 ile 200 µg aralığında değişen total fenol içeriğine sahip ekstrelerin 10 ile 23 mm arasında değişen inhibisyon zonları ile anti- *H. pylori* aktivite sergilediği belirlenmiştir [43]. Daha önceki çalışmalardan elde edilen bulgular da dikkate alındığında *O. vulgare*'nin *H. pylori* üzerindeki etkisinin kısmen üreaz enzim inhibisyonundan kaynaklanmakla birlikte antioksidan aktivite gibi farklı mekanizmaların da rol oynayabileceği akla gelmiştir. Biyoaktivite yönlendirmeli fraksiyonlama, doz-cevap ilişkisi gibi daha ileri çalışmalarla bu bitkiden *H. pylori* pozitif ülserlerde umut vaat edebilecek sekonder metabolitlere ulaşılması mümkün olabilir.

Düzce'de halk ilacı olarak kullanılan yedi bitkinin [*Dioscorea communis, Mentha longifolia* subsp. *thyphoides, Origanum vulgare, Rubus ulmifolius, Salvia tomentosa, Thymus longicaulis* subsp. *longicaulis, Trachystemon orientalis*] kimotripsin ve üreaz enzimlerini inhibe edici aktivitelerinin ve total fenol-flavonoit içeriklerinin tespit edildiği bu araştırmada, *D. communis* köklerinden hazırlanan metanol ekstresinin çok yüksek olmasa da dikkate değer üreaz inhitörü aktivite gösterdiği belirlenmiştir. *R. ulmifolius* metanol ekstresi ve *T. orientalis* su ekstresi de kimotripsin enzimi üzerinde önemli derecede inhibisyon sergilemeleriyle ön plana çıkmıştır. Dolayısıyla bu bitkiler üzerinde biyoaktivite yönlendirmeli fraksiyonlama, yapı aktivite, doz cevap ilişkisi çalışmaları gibi kapsamlı araştırmaların yapılması halinde ülser başta olmak üzere obezite, pankreatit gibi rahatsızlıkların tedavisinde umut vaat edebilecek ilaç aday-adayı moleküllerinin tespiti mümkün olabilecektir.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARISON OF SOME BIOLOGICAL ACTIVITIES AND CATECHIN TANNIN CONTENTS OF TWO *JUNIPERUS* AND *PRUNUS* SPECIES

İKİ JUNİPERUS VE PRUNUS TÜRÜNÜN BAZI BİYOLOJİK AKTİVİTELERİNİN VE KATEŞİN TANEN İÇERİKLERİNİN KARŞILAŞTIRILMASI

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ABSTRACT

Objective: *Qualitative and quantitative determination of catechin in fruits of J. communis var. saxatilis, J. oxycedrus subsp. oxycedrus, P. spinosa, and P. cerasifera was analyzed by LC-MS/MS using standards. Antidiabetic, antimicrobial, and antioxidant activities of fruit and cone extracts of these plants were evaluated. Qualitative analysis of secondary metabolites was also done.*

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Material and Method: *Antimicrobial activity was done with MIC. $ABTS^{\cdot+}$ and DPPH• scavenging activies were used antioxidant activity. α-Amylase and α-glucosidase inhibition assays were done for antidiabetic activity.*
Result and Discussion: *JCS (IC_{50} = 578 ± 20 µg/ml), JOO (IC_{50} = 3706 ± 52 µg/ml), and PS (IC_{50} = 313 ± 7 µg/ml) extracts were observed to show a higher activity than acarbose (IC_{50} = 4061 ± 146 µg/ml). J. communis var. saxatilis fruit extract was observed with MIC values between (312.5 - 2500 µg/ml) against all microorganisms. JCS extract has the highest phenolic composition and JOO has the lowest phenolic composition (µg GAE/ mg extract). JCS extract indicated the highest antioxidant activity. It was determined that plant containing the highest catechin (1173.3±5.77 ng/ml) and epigallocatechin (208±7.21 ng/ml) was JCS. In this research, it can be said that JCS with the highest tannin content shows the highest effects.*
Keywords: *Antidiabetic, antimicrobial, catechic, Juniperus, Prunus*

ÖZ

Amaç: *J. communis var. saxatilis, J. oxycedrus subsp. oxycedrus, P. spinosa, ve P. cerasifera meyvelerinde standartlar kullanılarak LC-MS/MS ile analiz edilmiştir. Bitkilerin meyve ve kozalak ekstrelerinin antidiyabetik, antimikrobiyal ve antioksidan aktiviteleri değerlendirilmiştir. Sekonder metabolitlerin kalitatif analizi de yapılmıştır.*
Gereç ve Yöntem: *MIC ile antimikrobiyal aktivite yapıldı. $ABTS^{\cdot+}$ ve DPPH• süpürücü testler antioksidan aktiviteler için kullanılmıştır. α-Amilaz ve α-glukosidaz inhibisyon testleri antidiyabetik aktivite için kullanılmıştır.*
Sonuç ve Tartışma: *JCS (IC_{50} = 578 ± 20 µg/ml), JOO (IC_{50} = 3706 ± 52 µg/ml) ve PS (IC_{50} = 313 ± 7 µg/ml) ekstraktlarının akarbozdan (IC_{50} = 4061 ± 146 µg/ml) daha yüksek aktivite gösterdiği gözlemlenmiştir. Juniperus communis var. saxatilis meyve ekstresi tüm mikroorganizmalara karşı (312.5 -2500 µg/ml) arasında MİK değerleri göstermiştir. JCS en yüksek fenolik bileşime ve JOO en düşük fenolik bileşime sahiptir (µg GAE/ mg ekstrakt). JCS ekstresi en yüksek antioksidan aktiviteyi göstermiştir. En yüksek kateşin (1173.3±5.77 ng/ml) ve epigallokateşin (208±7.21 ng/ml) içeren bitki JCS'dir. Bu araştırmada en yüksek tanen içeriğine sahip JCS'nin en yüksek etkileri gösterdiği söylenebilir.*
Anahtar Kelimeler: *Antidiyabetik, antimikrobiyal, kateşik, Juniperus, Prunus*

INTRODUCTION

Type 2 diabetes mellitus (DM) epidemic is a substantial world health burden. It has been indicated that plant extracts can target the pathophysiology underlying sicks and get numerous mechanisms of action owing to the synergistic effects produced by the combinations of phytochemicals [1]. DM is in conjunction with diversified types of infections, primarily mucous membrane, soft tissue, skin, respiratory tract, urinary tract, and surgical and/or hospital-related infections. There are certain microbes related to each type of infection, and their existence demonstrates certain types of infection. For example, *E. coli* and *Klebsiella* are the most widespread initiative pathogens liable for the improvement of urinary tract infections. Diabetic foot infections (DFI) are usually seen in diabetic patients [2]. DFI are more serious and harder to cure than non-diabetic [3]. Diabetic wound healing is a great difficulty owing to its defenselessness to bacterial infection, as well as less vascularization and extended inflammatory period [4]. Increased oxidative stress in patients with DM and poor glycemic control or insulin resistance is most probably the effect of abnormal metabolisms like hyperglycemia, dyslipidemia, and high free fatty acids levels [5]. Various investigations have shown an important reduction in plasma antioxidants like retinol, lutein, α- and γ-tocopherol, ascorbic acid lycopene, β- and α-carotene, zeaxanthin, and β-cryptoxanthin during diabetes. Therefore, the justification for the therapeutical usage of antioxidants in the therapy and preservation of diabetic complications is powerful [6]. Tannins are potent antioxidants and have been recognized as being antidiabetic, anticarcinogenic, antiviral, antihypercholesterolemic, antiinflammatory, antibacterial, antimutagenic, and cardioprotective [7-8]. Catechin, gallic acid, epicatechin, and ellagic acid, proanthocyanidin are well-known tannins and have been observed to reduce the overexpression of TGF-β, IL-6, AMPK, PARP, and NF-kβ which are the main targets involved in the progression of DM [8]. The members of the *Juniperus* L. genus are a well-known resource of cedarwood oil is commonly distributed in the northern

hemisphere and is utilized in public medicine. *J. communis* L. (Cupressaceae) is a shrub or small evergreen tree [9]. *J. oxycedrus* L. is a species of plant in the Cupressaceae family, and it is one of ten species within the *Juniperus* genus. It is found throughout the Mediterranean region and can grow as both a shrub or tree. It has been traditionally used to treat a variety of ailments such as hyperglycemia, obesity, bronchitis, tuberculosis, and pneumonia [10]. The *Prunus* L. genus includes many economically important species whose fruits are commonly eaten fresh, frozen, or processed. Traditional medicine often uses these species to treat diabetes [11]. *P. spinosa* L., also known as "blackthorn or sloe," is a deciduous, dense shrub that can grow up to 4 meters tall. It is native to western Asia, northwestern Africa, and Europe and often grows wild in uncultivated regions near roads and canals. The stems of the plant are covered with spines [11-12]. *P. divaricata* Ledeb. (Rosaceae) is a wild plum and extensively grows from the Balkan Peninsula to Anatolia and the Caucasus to Central Asia, containing the Hyrcanian forests in the northern areas of Iran [13]. In an ethnobotanical research, it was seen that the fruits of *J. oxycedrus, P. spinosa,* and *P. divaricata* species are used as antidiabetics in folk medicine [14]. Tannins are polar compounds in polyphenolic structures found in many higher plants. Due to their pharmacological effects, they have been the subject of much research in the fields of food, medicine and medicine. This scope of work; qualitative and quantitative determination of catechin in the fruits of *J. communis* var. *saxatilis* (JCS), *J. oxycedrus* subsp. *oxycedrus* (JOO), *P. spinosa.* (PS), and *P. cerasifera* (PC) was analyzed by LC-MS/MS using standards (+)-catechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin, (-)-and epigallocatechin gallate. Additionally, the 70% methanolic extracts of the fruits and cones of these plants were analyzed for their ability to inhibit α-glucosidase and α-amylase enzymes, as well as for their antimicrobial activity by determining the minimum inhibitory concentration (MIC). The extracts were also tested for their antioxidant activity using the DPPH and ABTS methods. Furthermore, a qualitative analysis of secondary metabolites in these plant species was conducted.

MATERIAL AND METHOD

Plant Materials

JCS, JOO, PS, and PC were previously collected and data about the used parts, places and times of collection of are presented in Table 1. Collection and identification of plants were made by Mehmet Önal (Chief Engineer of the Eastern Anatolia Forestry Research Institute).

Figure 1. The photos of JCS, JOO, PS, and PC by Mehmet Önal.

Table 1. Used parts, places and collection times of JCS, JOO, PS, and PC.

Species	Used part	Places of collection	Times of collection
JCS	Cone	Erzurum/Aziziye/Rizekent Village	14.09.2015
JOO	Cone	Erzurum/Olur/Ormanağzı Village	04.05.2014
PS	Fruit	Erzurum/İspir/Özler Village	02.11.2014
PC	Fruit	Erzurum/Olur/Ilıkaynak Village	06.10.2014

Extraction

Collected fruits and cones were dried in a sun-free environment, away from moisture, by providing appropriate air circulation. Since tannins are polar substances and dissolve in water, 70% methanol was chosen as extraction solvent. After fruits were powdered, 50 grams of each were weighed and transferred to balloon and left to macerate overnight with 70% methanol. Then, it was extracted 3 times for 3 hours at 40°C utilizing a jacketed heater and a reflux cooler. Extracts obtained as a result of each extraction process were filtered and the filtrates were combined. It was concentrated to dryness in a rotavapor at 40°C, 120 rpm.

α-Amylase Inhibition Assay

Inhibitory activity on α-amylase was demonstrated by Nampoothiri et al. (2011) [15] in various modifications [16]. Entire extracts and 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9 including 6 mM sodium chloride) were incubated at 25°C for 10 minutes in a 24-well microplate. Afterwards, incubation 100 µL α-amylase solution (0.5 mg/ml) was annexed and reaction admixtures were incubated at 25°C for 10 minutes. The dinitrosalicylic acid was annexed as a colour reagent. Microplate was heated for 5 minutes and cooled to 25°C. Absorbance at 540 nm was registered.

α-Glucosidase Inhibition Assay

Inhibitory activity on α-glucosidase was established with respect to method of Bachhawat et al. (2011), [17] within several modifications [16]. Entire extracts (20 µL), an enzyme solution (10 µL, 1 U/ml), and potassium phosphate buffer (50 µL, 50 mM, pH 6.9) were admixed in a 96-well plate and incubated at 37°C for 5 minutes. Substrate, p-nitrophenyl-α-D-glucopyranoside, was annexed to each well, also 0.1 M sodium carbonate was annexed. Sum of p-nitrophenol was registered utilising a 96-well microplate reader at 405 nm.

Antimicrobial Activity

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 8739, *Klebsiella aerogenes* ATCC 13048, *Salmonella enterica* ATCC 14028, *Candida albicans* ATCC 10231, *Bacillus subtilis* ATCC 19659, *Pseudomonas aeruginosa* ATCC 9027, and *C. parapsilosis* ATCC 22019 were taken from Microbiologics (San Diego, CA), and used antimicrobial acitivity. Extracts of fruit were studied with a concentration range (5000 to 78.12 µg/ml) and diluted 2-fold initially. Ampicilline and terbinafine (64–0.125 µg/ml) were used as standard drugs and prepared within water and dimethyl sulfoxide. Activity was determined using a slight modification of microdilution methods for aerobic microorganisms (M-7-A7) and fungi (M-27-A3) reported by Clinical Laboratory Standards Institute (CLSI). Standard cultures were kept at -85°C. After incubation, it was got from single colonies that improved on growth medium and transferred to tubes with Mueller Hinton Broth (MHB) (RPMI medium for *Candida* species) and incubated again at 37°C for 24 hours. Afterwards 18-24 hours of incubation, cultures were prepared accordingly McFarland No: 0.5 tube (10^8 cfu/ml for bacteria, 10^6 cfu/ml for yeast culture).

Antioxidant Activity and Total Phenolic Content

Many methods have been developed for the determination of natural compounds in foods, medicinal plants, or biological systems antioxidant activity. However, the most popular ones are ABTS and DPPH radical scavenging capacity assays. Both methods are based on colorimetric measurements of the response of stable radicals to antioxidant activity. The reasons for the popularity of ABTS and DPPH radical scavenging capacity tests can be listed as the stability of the radicals used, ease of measurement, short analysis times, reproducibility of analyzes, and inexpensive spectrophotometric methods. The radicals used in the experiments are readily available commercially.

$ABTS^{\cdot+}$ Scavenging Activity

It was established with respect to method of [18] with slight modifications [19-20]. Prepared 2.45 mM potassium persulfate solution was added to ABTS solution and $ABTS^{\bullet+}$ was obtained. Trolox and *α*-tocopherol were utilised as reference antioxidants. All samples were prepared at diversified

concentrations of extracts. Activity of each specimen was detected via finding color of $ABTS^{\bullet+}$ radical and evaluating reduction in absorbance at 734 nm towards a blank of phosphate buffer. Whole evaluations were established in triplicate.

Total Phenolic Content

It was comparatively evaluated utilising method improved by Folin and Denis and modified by Singleton [21-22] with slight modifications [19-20]. Absorbances read at end of experiment were plotted against concentration. In standard graph, equation is $0.0016x + 0.0105$; r^2 was found to be 0.9977. Stock solutions were designed at a concentration of 1 mg/ml. Whole solutions were administered with FCR and aqueous Na_2CO_3. Absorbance was recorded at 760 nm. Findings are demonstrated in gallic acid equivalents (GAE) and micrograms. Whole evaluations were carried out in triplicate.

$DPPH^{\bullet}$ Scavenging Activity

It was established with respect to method of Blois et al. (2011) [23], with several modifications [19]. 1 mM $DPPH^{\bullet}$ solution was utilised. Trolox and *α*-tocopherol were utilised as reference antioxidants. Standard antioxidants were studied in concentration range of 1-100 µg/ml. 210 µL of stock solutions of extracts and standards at diversified concentrations and 70 µL of $DPPH^{\bullet}$ solution were added to 96 well plate. All measurements were performed in triplicate for all samples.

Analytical Procedure

After method was developed on pure substances, all standard substances were analyzed simultaneously on extracts prepared from fruits and cones of 4 plants belonging to *Prunus* and *Juniperus* genera. All analytical experiments were carried out using below systems:

LC: Spark Holland-SPH1240; MS/MS: AB SCIEX-4000 Q TRAP; Column: Intersil-ODS-3 (5µm, 4.0mm x 250.0mm); Mobile Phase A: MeOH 100%; System: Isocratic; Column Temperature: 30°C; Injection Volume: 10 µL; Flow Rate: 0.8 ml/min; Sample Concentration: 10 mg/ml

Validation of Method

Validation of method was established via testing following criteria: retention time (RT), standard deviation (SD), limits of detection (LOD), limits of quantification (LOQ), regression equation and coefficient (RE/C), and and regain (R).

Qualitative Analysis of Secondary Metabolites

Analysis of Alkaloid

0.5 g of extracts were taken and boiled with 10 ml of 70% ethanol containing 6% H_2SO_4 for 1 minute, cooled and allowed to settle. Mayer and Dragendorff reagents were added and it was checked whether precipitate was formed. After this control was done, ethanolic extract was taken into a small separating funnel, then alkalized by adding a sufficient amount of 25% Na_2CO_3 solution and rinsed with 15 ml of chloroform. Then, it was consumed with 15 ml of 10% acetic acid solution, and acetic acid phase was taken into 3 separate tubes. While one of tubes was kept for control, Mayer reagents were added to second and Dragendorff reagents to third, and it was checked whether precipitate formed [24].

Analysis of Coumarin

1 g of extracts was taken and 10 ml of 1 N H_2SO_4 was added. After boiling for 10 minutes under reflux and filtering while hot, filtrate was shaken with 15 ml of chloroform in a separatory funnel. Chloroform phase was separated and 5 ml of 10% NH_3 solution was annexed to 5 ml of it and shaken. Solution was put to stand for 5 min and then ammonia phase was checked for fluorescence at UV 366 nm [24].

Analysis of Cardioactive Heteroside

Extracts were taken and boiled in 10 ml of 70% ethanol in a water bath for 2 min and filtered. Filtrate was diluted 2 times with water, 1 ml of concentrated lead subacetate solution was added and

filtered. Filtrate was extracted with 10 ml of chloroform and chloroform phase was placed in 3 separate capsules and Keller-Kliani and Baljet reactions were done [24].

Analysis of Saponoside

0.5 g extracts were taken and put in a test tube with 10 ml of hot water and shaken vigorously for about 10 seconds after cooling. If there is saponosite, it was checked whether a foam layer of 1-10 cm height, which remains stable for at least 10 minutes, and which does not disappear when 1-2 drops of 2N HCl is dropped on it [24].

Analysis of Flavonoside

Qualitative analysis of flavonoside of extracts were done according to Cyanidin Reaction [24].

Analysis of Tannin

A 5% infusion was prepared from extracts and examinations were made on prepared infusion [24].

Analysis of Anthocyanoside

Extracts were extracted with 50% ethanol in a low flame, filtered, and filtrate was divided into five and reactions were applied [24].

Analysis of Anthracenoside

0.1 g of extracts were taken, boiled with 5 ml of diluted H_2SO_4 for 2 minutes and filtered while hydrolysis product was hot. Filtrate was cooled and extracted with a small amount of benzene. Upper benzene layer was removed, rinsed with 10% ammonia, and color of lower ammonia layer was observed [24].

Analysis of Cyanogenetic Heteroside

Extracts were placed in a 100 ml flask and only enough water to be heated was added. A filter paper impregnated with picric acid soaked with sodium carbonate solution was hung into flask close to water-soaked material and gently compressed with help of a cork stopper. Erlen was warmed in a light burner flame. Color formed on paper was observed [24].

Analysis of Resin

Extracts are shaken with 90% ethanol in a tube. Insoluble parts are separated by filtration. Ethanol part is taken into another tube. An equal volume of 1% copper acetate solution is added on it. Resulting green color indicates presence of resin [25].

Statistically Analysis

Whole tests were assessed in triplicate. Kruskal-Wallis test was utilised to detect statistical signification. Data were analysed utilising SPSS (IBM SPSS Statistics 20, IBM Corporation, Armonk, NY, USA) at signification level of $P = 0.05$. Percentile inhibition and IC_{50} value findings for extracts are represented as means ± standard deviation. Results of antioxidant activity experiments are given in percent inhibition ± standard deviation, and results of total phenolic component assay assays were represented in gallic acid equivalents ± standard deviation.

RESULT AND DISCUSSION

α-Amylase and α-Glucosidase Inhibition Assays

70% methanolic extracts prepared from fruits and cones of JCS, JOO, PS, and PC were assessed for determining their *α*-glucosidase inhibitory effect. JCS (IC_{50} = 578 ± 20 μg/ml), JOO (IC_{50} = 3706 ± 52 μg/ml), and PS (IC_{50} = 313 ± 7 μg/ml) extracts were observed to show a higher effect than reference compound (IC_{50} = 4061 ± 146 μg/ml). Interestingly, while PS displayed the highest activity, PC showed no inhibition against acarbose; even if their tannin contents were similar (Table 2). JCS and PS, which

have the highest total phenolic content, showed a good effect when compared to acarbose. *α*-Glucosidase inhibitory activity results showed similarity with total phenolic component assay results. Except for JOO extract (15%), other extracts did not show any activity against *α*-amylase at 5 mg/ml concentration. However, it was not found higher than acarbose (59%). In a study, bioactive potential of *Prunus* fruits were evaluated. As in our study, PS (blackthorn) displayed high activity against *α*-glucosidase with 0.78 mg/ml IC_{50} value when compared with acarbose (IC_{50}= 3.73 mg/ml). PC (white cherry plum) had no activity against both enzymes while PS (blackthorn) had'nt displayed any activity towards *α*-amylase when in comparison to acarbose (IC_{50}= 0.11 mg/ml) [26]. JCS hydroalcoholic extract indicated higher activity towards *α*-glucosidase (IC_{50}= 0.0044 mg/ml) than J. *oxycedrus* ssp. *oxycedrus* (IC_{50}= -) as in our study. However, they were not higher than acarbose (IC_{50}= 0.0009 mg/ml). Both of them had no inhibitory activity against *α*-amylase when with regard to acarbose (73.7%) at 3 mg/ml [27]. This was the first comparative study with different species from these two genus: *Prunus* and *Juniperus* towards *α*-glucosidase and *α*-amylase enzymes.

Table 2. *α*-Glucosidase inhibitory activity of extracts

α-Glucosidase Inhibitory Activity	**IC_{50} value (µg/ml)**							
	Mean	**Standard Deviation**	**Median**	**Minimum**	**Maximum**	**Chi-square**	**P**	**Post-hoc**
JCS	578	20	572	563	601	10.385	0.016	*Prunus spinosa* and Acarbose (P=0.013)
JOO	3706	52	3710	3640	3742			
PS	313	7	312	306	320			
PC	-	-	-	-	-			
Acarbose[a]	4061	146	4045	3964	4248			

[a] Positive control for *α*-glucosidase inhibitory effect

Antimicrobial Activity

Minimum inhibitory concentrations (MIC, µg/ml) were given as Table 3. JCS fruit extract was observed with MIC values between (312.5-2500 µg/ml) against whole microorganisms. The most sensitive strains were *Candida albicans* (312.5 µg/ml), and *C. parapsilosis* (312.5 µg/ml) followed by *B. subtilis* 1250 µg/ml), *Klebsiella aerogenes* (1250 µg/ml) and *E. coli* (1250 µg/ml). JOO fruit extract was found more active against *E. coli* (MIC= 625 µg/ml), followed by *B. Subtilis*, *C. albicans*, and *C. parapsilosis* with a MIC= 1250 µg/ml. MIC values were indicated between 156.25-1250 µg/ml for JCS berrries methanolic extract against *Staphylococcus aureus* ATCC 8538P, *S. epidermidis* G1, *Enterococcus hirae* V3, *B. subtilis* P3. Same extract was not found active against Gram-negative strains*; E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9021, *Proteus mirabilis G4*, yeasts *C. albicans* ATCC 10231 and *C. parapsilosis* P7 [28]. Antimicrobial potential of methanolic and aqueous extracts of branches of JCS and JOO were investigated against Gram-positive, Gram-negative, and yeasts by Taviano et al. (2011) [29]. Methanolic and aqueous extracts of *Juniperus* ssp. were found MIC=19.53 µg/ml towards *S. aureus* ATCC 6538P.

MIC values of extracts towards *S. epidermidis* G1 were between 78.12-156.25 µg/ml. MIC values of extracts towards *E. hirae* V3 were >250- 156.25 µg/ml. Any antimicrobial effect in Gram-negative strains and yeasts was not shown. These extracts also indicated a significant inhibiting effect on *S. aureus* biofilm formation (with a reduction of 66–84%) [29]. Antimicrobial effects of hexane, acetone and methanolic berry extracts of JOO were tested via disc diffusion method and hexane and methanol extrats of JOO indicated activity against some microorganisms. Acetone extract of JOO had no effect on any of microorganisms evaluated [30]. Based on result, methanol extract of JOO was observed effective towards *E. coli* with MIC 625 µg/ml. In our study, PS fruit extract was found more effective against *Candida* than bacteria strains. MICs generally were observed >2500 µg/ml for *Prunus divaricata* fruit extract. In literature, *P. divaricata* subsp. *divaricata* leaves exracts was prepared by petroleum ether, dichloromethane, methanol and distilled water and investigated and petroleum ether extract of *P.*

divaricata subsp. *divaricata* showed the highest antimicrobial activity against *E. faecalis* with 11 mm inhibition zone [31]. Antimicrobial effect of methanol extracts of *P. divaricata* subsp. *divaricata* fruits were carried out eight bacteria and two yeast by tube dilution and microdilution methods. Highest antimicrobial effect among clinical isolates Gram negative organisms were observed towards *E. coli* and *K. pneumonia*, while *C. albicans* and *C. parapsilosis* were the least [32]. Antimicrobia effect of PS fruit ethanol extract was investigated by Gram-negative, Gram-positive bacteria, and yeast. No specific inhibitory action was obtained against the evaluated Gram-negative or Gram-positive bacteria. Antimicrobial effect of *PS* ethanol fruit extract showed MICs range of 4.36 mg/ml to 8.72 mg/ml [33]. In addition, comprasion these data with available results, MIC for PS (2500 µg/ml, this paper) is lower than that of PS (8.72 mg/ml) towards *S. aureus* ATCC 6538. For fungal strains, it was found that MIC of *C. albicans* was not similar (625 µg/ml vs. 8.72 mg/ml) to that observed in study [33].

Table 3. Minimum inhibitory concentrations (MIC, µg/ml)

Extracts	*E. coli* ATCC 8739	*S. enterica* ATCC 14028	*S. aureus* ATCC 6538	*B. subtilis* ATCC 19659	*P. aeruginosa* ATCC 9027	*K. aerogenes* ATCC 13048	*C. albicans* ATCC 10231	*C. parapsilosis* ATCC 22019
JCS	1250	2500	2500	1250	2500	1250	312.5	312.5
JOO	625	>2500	2500	1250	2500	2500	1250	1250
PS	2500	2500	2500	2500	2500	2500	625	1250
PC	>2500	>2500	>2500	>2500	>2500	>2500	2500	2500
Ampicillin	0.125>	1.0	0.125>	0.125>	>64	32	-	-
Terbinafine	-	-	-	-	-	-	4.0	32

Total Phenolic Content and Antioxidant Activity

Many investigations have also indicated that there is a relationship between diabetes and free radicals. Again, studies have shown that antioxidant-effective compounds have antimicrobial effects on microorganisms [34]. Antioxidant effect of aqueous and ethanolic extracts prepared from *Juniperus communis* fruits was tested with various methods, it was noted that they showed strong antioxidant effects, especially at 20, 40, and 60 µg/ml concentrations. Especially, it was observed that ethanolic extract had better DPPH$^{\bullet}$ scavenging capacity and there was a statistically important difference with aqueous extract when compared ($p<0.05$) [35]. *J. communis* var. *communis* (JCc) and *J. communis* var. *saxatilis* (JCs) extracts indicated DPPH radical scavenging activity, which was much higher in *J. communis* var. *communis* ($IC_{50} = 0.63 \pm 0.09$ mg/ml) than in *J. communis* var. *saxatilis* ($IC_{50} = 1.84 \pm 0.10$ mg/ml). When compared in terms of total phenolic content, findings showed compatibility with antioxidant effect results (JCc 59.17 ± 1.65 mg GAE/g extract; JCs 17.64 ± 0.09 mg GAE/g extract) [28]. *P. domestica*, *P. cerasifera*, and *P. spinosa* extracts were contrasted in terms of antioxidant effect and phenol component *P. spinosa* extract was observed to be superior to other two species as compared with antioxidant effect ($p<0.01$) [36]. Difference of our study is that these four species are compared in terms of antioxidant effect, associated with tannin content, tested in terms of antidiabetic effect and antimicrobial effect. 70% methanolic extracts prepared from fruits and cones of PC, PS, JOO, and JCS were carried out for determining their antioxidant activity and total phenolic content. Findings of total phenolic content experiments were similar to those of antioxidant capacity tests. JCS extract has to highest phenolic composition and JOO has to lowest phenolic composition (µg GAE/ mg extract). [(JCS)19.38>(PS)17.21>(PC)2.94>(JOO)2.08 µg GAE/mg extract].

Results of this experiments were represented in Table 4. In ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging effect tests α-tocopherol (TK) and trolox (TR) were utilised as reference, *J. communis* var. *saxatilis* extract showed the best activity and *J. oxycedrus* subsp. *oxycedrus* showed lowest activity compared to other extracts. [(TR)99.14>(TK)47.92>(JCS)14.29>(PS)11.14>(PC)7.84>(JOO)3.65 %; at 100 µg/ml for ABTS$^{\bullet+}$)]. [(TR)92.78>(TK)90.78>(JCS)10.63>(PS)8.09>(PC)6.65>(JOO)3.48 %; at 100 µg/ml for

DPPH•)]. Findings of antioxidant effect experiments are presented in Table 5. When all results are evaluated, it is seen that phenolic compounds are effective in antioxidant effect of extracts. On the basis of findings of this investigation, it can be said that JCS with the best tannin content shows the highest effects.

Table 4. Total phenolic content results of *Juniperus* and *Prunus* plant extracts

Extracts	**Total Phenolic Content (µg GAE/mg extract ± SD*)**
JCS	19.38 ± 0.000854
JOO	2.08 ± 0.000416
PS	17.21 ± 0.000681
PC	2.94 ± 0.003161

*****SD: Standard deviation

Table 5. $ABTS^{\bullet+}$ and DPPH• scavenging activity results of *Juniperus* and *Prunus* plant extracts.

$ABTS^{\bullet+}$ Scavenging Activity (% Inhibition ± SD*)		
	% Inhibition (60 µg/ml)	**% Inhibition (100 µg/ml)**
JCS	9.32 ± 0.006149	14.29 ± 0.01195
JOO	1.10 ± 0.00315	3.65 ± 0.011817
PS	7.69 ± 0.002023	11.14 ± 0.002205
PC	4.36 ± 0.006338	7.84 ± 0.006894
Trolox	57.21 ± 0.005551	99.14 ± 0.000557
***α*-Tocopherol**	28.04 ± 0.004752	47.92 ± 0.0065
DPPH• Scavenging Activity (% Inhibition ± SD*)		
	% Inhibition (60 µg/ml)	**% Inhibition (100 µg/ml)**
JCS	7.86 ± 0.001429	10.63 ± 0.015451
JOO	1.31 ± 0.039647	3.48 ± 0.016417
PS	6.21 ± 0.033602	8.09 ± 0.003219
PC	2.64 ± 0.071712	6.65 ± 0.044022
Trolox	92.14 ± 0.001877	92.78 ± 0.0006
***α*-Tocopherol**	77.05 ± 0.004244	90.78 ± 0.000252

*SD: Standard deviation

Quantitative Analysis Secondary Metabolites

As a result of analyzes made; amounts of catechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate were determined in ng/ml. While catechin was not found in extracts of PC, PS, and JOO species; it was determined that these plants contained the most epigallocatechin (17.6±0.65, 15.1±0.56, and 20.3±2.01 ng/ml, respectively) compared to other substances. It was determined that plant containing the highest catechin (1173.3±5.77 ng/ml) and epigallocatechin (208±7.21 ng/ml) was JCS. Epigallocatechin gallate was found to be the least common substance in PC, PS, JOO, and JCS with 5.3±1.02, 4.2±0.19, 3.6±0.10, and 3±0.14 ng/ml, respectively. Tannin amounts determined as a result of analyzes are given in Table 6. Validation of method was performed by testing following criteria: retention time, LOD, LOQ, regression equation and coefficient, and regain. Results of validation method

was given in Table 7. LC-MS/MS chromatograms of standard compounds were represented in Figures 2-5. HPLC-MS analysis method parameters and Q1 and Q3 ion values of standard compunds are given in Table 8. MASS Spectrums of standard compounds are given in Figures 6-9. Catechin content was analysed in fresh fruit of PS and PC by using HPLC. It was found as 2.12 and 1.722 mg kg^{-1} fw, respectively [36]. However, catechin was not found in these two species in our study by LC-MS/MS. Qualitative analysis by using HPLC-DAD, all standards we used in our study except for epigallocatechin gallate were determined in PS [37]. Catechin was found in cones of *J. oxycedrus* and *J. communis*, while epigallocatechin was not found according to HPLC-MS analyse [38]. In contrast to, catechin and epigallocatechin was found in JCS in another research [39]. It was the first comprehensive and comparative study about qualitative and quantitative analyses of catechic tannins contents of these species.

Table 6. Tannin amounts determined as a result of analyzes.

Extracts (70% MeOH) (10 mg/ml)	(+)-catechin (ng/ml)	(-)-epigallocatechin (ng/ml)	(-)-epigallocatechin gallate (ng/ml)	(-)-epicatechin gallate (ng/ml)
JCS	1173.3±5.77	208±7.21	3±0.14	9.2±0.52
JOO	-	20.3±2.01	3.6±0.10	8.9±0.25
PS	-	15.1±0.56	4.2±0.19	9.6±1.38
PC	-	17.6±0.65	5.3±1.02	10.2±0.64

Table 7. LOD, LOQ values and calibration equation for standards (Concentration range 15.6-2000 ng/ml).

Standards	RT (dk)	SD	LOD (ng/ml)	LOQ (ng/ml)	RE/C	R (1000 ppb)
(+)-Catechin	2.41	1.56	4.67	15.57	y = 84.1x-185 r = 0.9999	905
(-)-Epigallocatechin	2.34	0.99	2.96	9.87	y = 169x+1.27e+003 r = 1.0000	927
(-)-Epigallocatechin Gallate	2.37	1.55	4.65	15.50	y = 71.8x-73.1 r = 0.9996	932
(-)-Epicatechin Gallate	2.30	1.10	3.30	11.02	y = 268x-684 r = 0.9999	1080

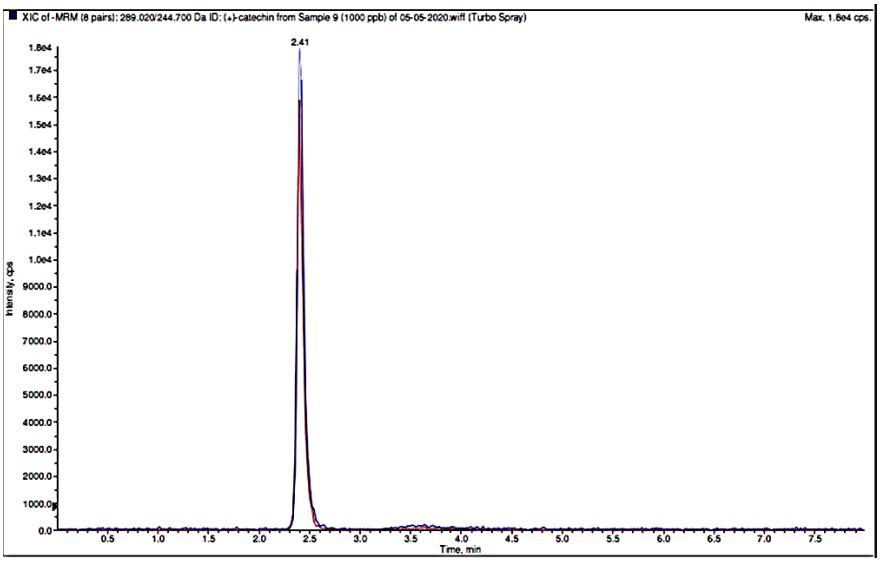

Figure 2. LC-MS/MS chromatogram of (+)-catechin.

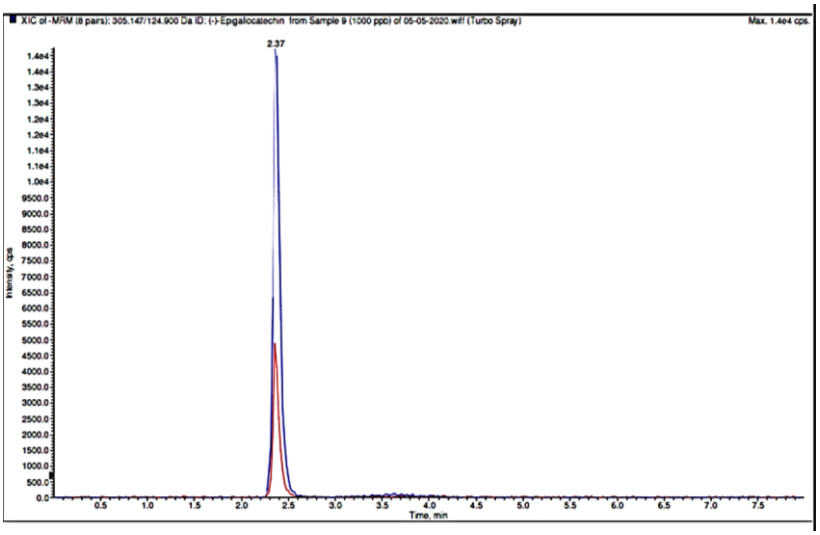

Figure 3. LC-MS/MS chromatogram of (-)-epigallocatechin.

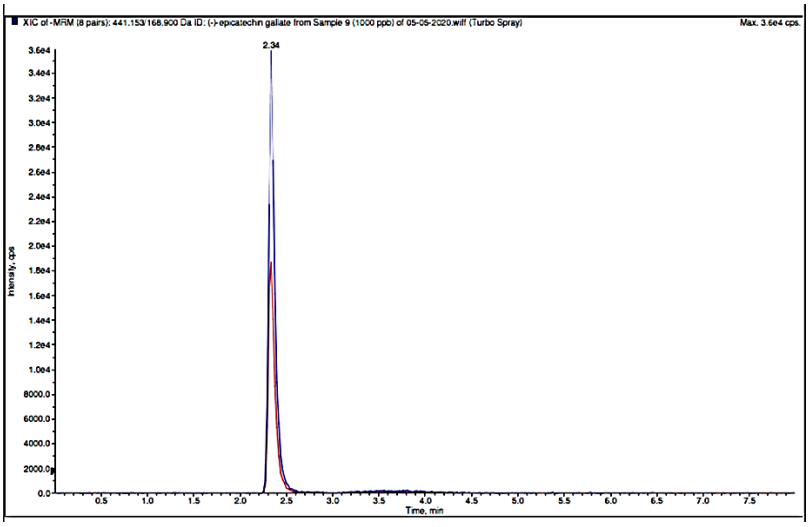

Figure 4. LC-MS/MS chromatogram of (-)-epicatechin gallate.

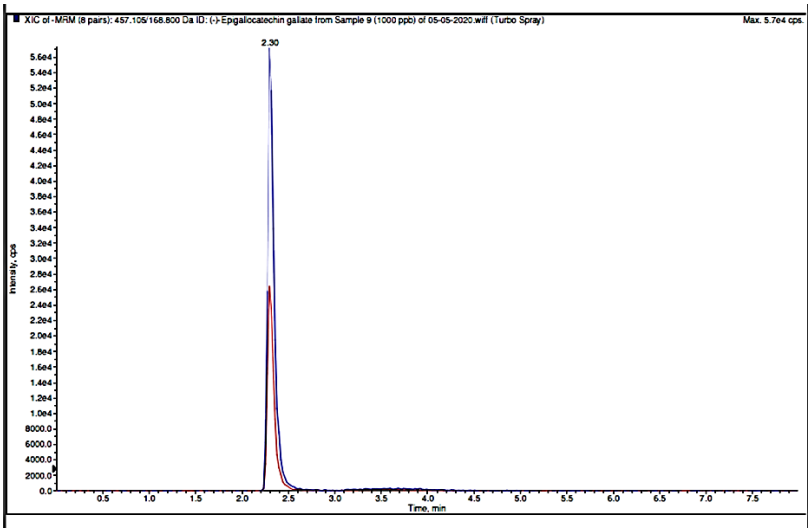

Figure 5. LC-MS/MS chromatogram of (-)-epigallocatechin gallate.

Table 8. HPLC-MS analysis method parameters and Q1 and Q3 ion values of standard compounds.

Q1 MASS (Da)	Q3 MASS (Da)	DEFINITION	DP (Volt)	EP (Volt)	CE (Volt)	CXP (Volt)
289.02	244.7	(+)-Catechin	-65	-10	-20	-13
289.02	108.8	(+)-Catechin	-65	-10	-32	-7
305.147	124.9	(-)-Epigallocatechin	-85	-10	-30	-7
305.147	178.8	(-)-Epigallocatechin	-85	-10	-22	-17
441.153	168.9	(-)-Epicatechin Gallate	-80	-10	-26	-1
441.153	288.7	(-)-Epicatechin Gallate	-80	-10	-22	-5
457.105	168.8	(-)-Epigallocatechin Gallate	-80	-10	-24	-13
457.105	124.7	(-)-Epigallocatechin Gallate	-80	-10	-58	-9
Ion spray voltage	-4500					
Temperature	250 °C					
Mode	Negative					

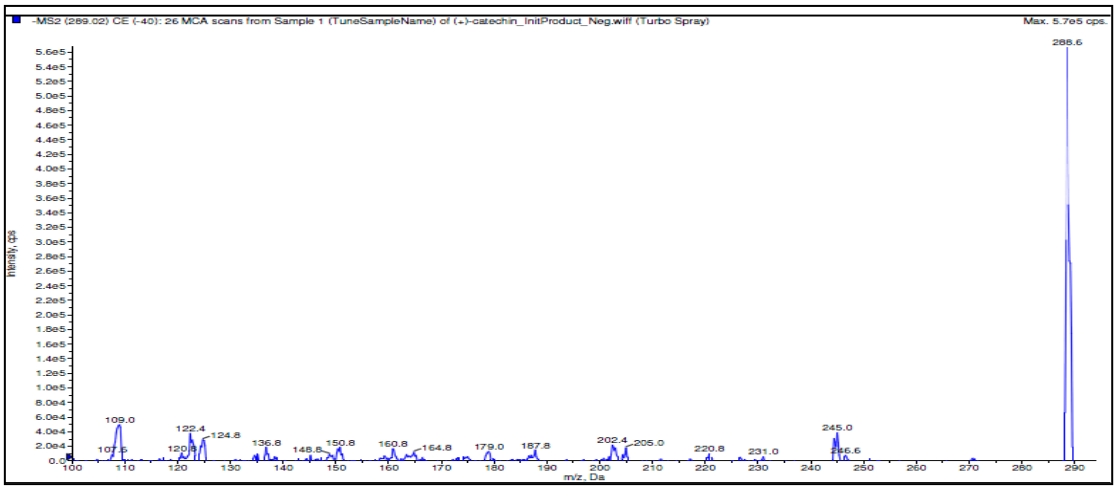

Figure 6. MASS spectrum of (+)-Catechine.

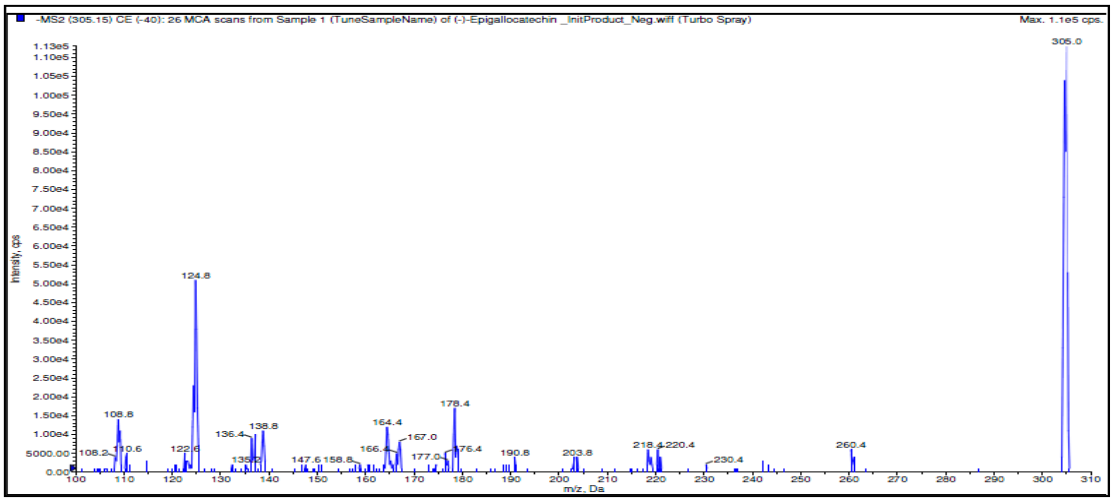

Figure 7. MASS spectrum of (-)-Epigallocatechin.

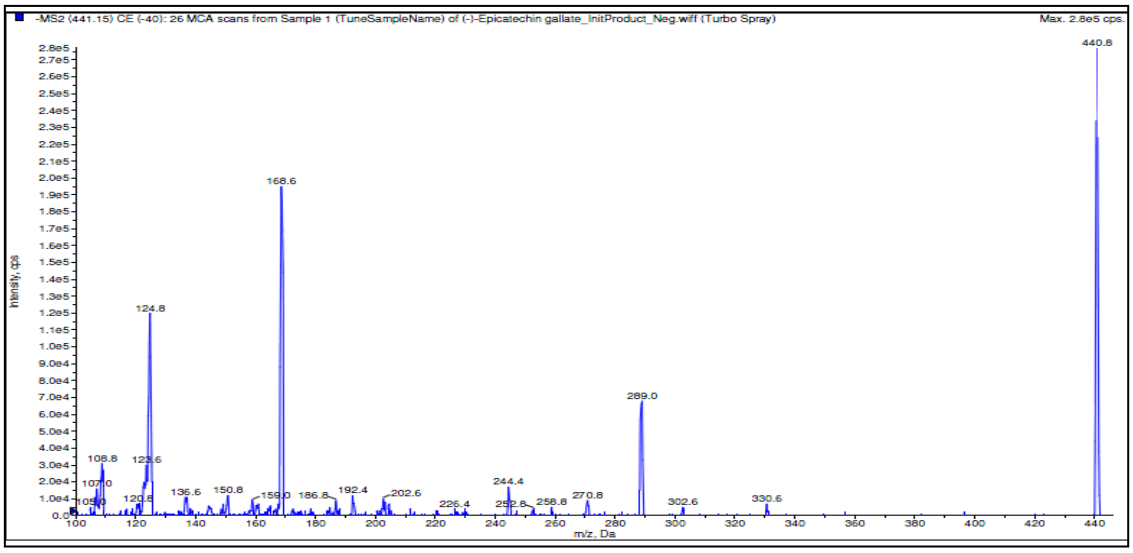

Figure 8. MASS spectrum of (-)-epicatechin gallate.

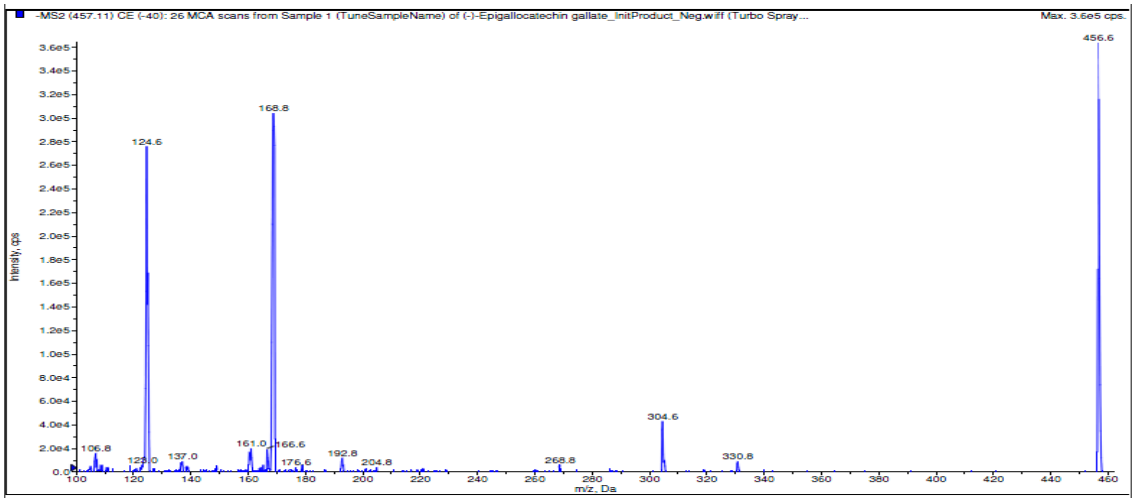

Figure 9. MASS spectrum of (-)-Epigallocatechin gallate.

Qualitative Analysis of Secondary Metabolites

Results of qualitative analysis of secondary metabolites of extracts is given in Table 9. Among extracts, alkaloid was observed in all extracts except PC with Dragendorff reagent. No coumarin and anthracenoside were observed among samples. Among extracts, cardioactive heteroside was observed in all extracts with Keller-Kliani reaction. Saponoside has been seen only in *Juniperus* species. Among extracts, flavonoside and tannin were observed in all extracts with Cyanidin reaction (for flavonoside), $FeCl_3$, gelatin reaction, brominated water, and Stiasny reactions (for tannin). Anthocyanoside has been seen in all extracts with lead acetate and amyl alcohol reactions. Cyanogenetic heteroside and resin were observed in all extracts except PC.

This is the first detailed research of the *in vitro* antimicrobial, antioxidant, antidiabetic effects, along with qualitative and quantitative analysis of JCS, JOO, PS, and PC. On the basis of the data of this research, it can be said that *J. communis* var. *saxatilis* with the highest tannin content shows the highest effects. We conclude that these species may be utilized as antimicrobial, antidiabetic, and antioxidant agents.

Table 9. Results of qualitative analysis of secondary metabolites of extracts.

Secondary metabolites	Chemical Reaction	JCS	JOO	PS	PC
Alkaloid	Mayer	-	+	-	-
	Dragendorff	+	+	+	-
Coumarin	UV 366	-	-	-	-
Cardioactive Heteroside	Keller-Kliani reaction	+	+	+	+
	Baljet reaction	-	-	-	-
Saponoside	Foaming test	+	+	-	-
Flavonoside	Cyanidin Reaction	+	+	+	+
Tannin	$FeCl_3$	+	+	+	+
	Gelatin reaction	+	+	+	+
	Brominated water reaction	+	+	+	+
	Stiasny reaction	+	+	+	+
Anthocyanoside	Diluted H_2SO_4	-	-	+	-
	NaOH-HCl	-	-	+	-
	Lead acetate reaction	+	+	+	+
	Amyl alcohol reaction	+	+	+	+
	Heat with diluted H_2SO_4 reaction	-	-	+	-
Anthracenoside	Borntrager reaction	-	-	-	-
Cyanogenetic Heteroside	Picric acid reaction	+	+	+	-
Resin	Abietat reaction	+	+	+	-

+ present, - absent

Phenolic compounds are a group of naturally occurring plant compounds that are known for their antioxidant properties. These compounds are found in a wide range of fruits, vegetables, and other plant-based foods. Recent studies have suggested that phenolic compounds may have a beneficial effect on individuals with diabetes. Diabetes is a metabolic disorder characterized by high blood sugar levels. Phenolic compounds have been found to improve insulin sensitivity and regulate blood sugar levels. Overall, while more research is needed to fully understand the relationship between phenolic compounds and diabetes, evidence suggests that incorporating phenolic-rich foods into the diet may have potential benefits for individuals with diabetes. In this study, the fruits of *J. communis* var. *saxatilis*, *J. oxycedrus* subsp. *oxycedrus*, *P. spinosa*, and *P. cerasifera* were analyzed for their catechin content using LC-MS/MS. The antidiabetic, antimicrobial, and antioxidant activities of the fruit and cone extracts of these plants were also evaluated. The JCS, JOO, and PS extracts showed higher activity than acarbose in terms of antidiabetic activity. *J. communis* var. *saxatilis* fruit extract showed good antimicrobial activity against all microorganisms tested. The JCS extract had the highest phenolic composition and antioxidant activity, and also contained the highest levels of catechin and epigallocatechin. It can be concluded that JCS with the highest tannin content exhibited the highest effects in this research.

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AUTHOR CONTRIBUTIONS

Concept: H.Y., H.C.D., B.A., M.Ö., E.T., A.C., M.N., G.G., S.K., Z.G.; Design: H.Y., E.T., A.C., M.N., G.G., S.K.; Control: H.Y., B.A., M.Ö., E.T., A.C., M.N., G.G., S.K., Z.G.; Sources: H.Y., M.Ö., S.K.; Materials: H.Y., M.Ö., E.T., A.C., M.N., G.G., S.K., Z.G.; Data Collection and/or Processing:

H.Y., H.C.D., B.A., M.Ö., E.T., A.C., M.N., G.G., S.K., Z.G.; Analysis and/or Interpretation: H.Y., B.A., M.Ö., A.C., G.G., S.K.; Literature Review: H.Y., B.A., G.G., S.K.; Manuscript Writing: H.Y., B.A., M.Ö., A.C., G.G., S.K.; Critical Review: H.Y., H.C.D., B.A., M.Ö., E.T., A.C., M.N., G.G., S.K., Z.G.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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DERLEME MAKALE / REVIEW ARTICLE

YEŞİL KOZMETİKLER

GREEN COSMETICS

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ÖZ

Amaç: *Son yıllarda, sentetik hammaddeler ile üretilen kozmetiklerin insan sağlığı ve çevre için oluşturduğu olumsuz etkiler, çevre bilinci doğrultusunda sürdürülebilir ürünleri tercih eden tüketicilerin sayısındaki artış ve üreticilerin kurumsal sorumluluğa verdiği önem nedeni ile yeşil kozmetiklere olan ilgi artmaktadır. Yeşil kozmetik ürünler, kontrollü veya organik tarımdan yola çıkılarak elde edilen doğal veya organik hammaddeler ile sürdürülebilir üretim metotları kullanılarak üretilen, geri dönüşümlü veya yeniden kullanılabilir ambalaj ile piyasaya sunulan ürünlerdir. Henüz hiçbir yönetmelikte bu ürün yelpazesi için yaptırım bulunmamaktadır. Ancak Amerika'da USDA/NOP ve Avrupa'da COSMOS, Ecocert, NaTrue, BDIH gibi standartlar ile ISO 16128 gibi harmonize standartlar aracılığıyla hammadde seçimi, üretim aşamaları ve ambalajlama, doğa ve insan sağlığı açısından ürünler kontrol altına alınmaya çalışılmaktadır. Türkiye İlaç Tıbbi Cihaz Kurumu-TİTCK tarafından yayınlanan doğal ve organik kozmetiklere yönelik yönetmelik eki uyarınca ülkemizde doğal ve organik kozmetiklerde ürün iddialarına yönelik sertifikasyon sağlanmalı ve sertifika logosu ambalajda yer almalıdır. Türkiye'de COSMOS sertifikaları ETKO ve IFC Global gibi sertifika sağlayıcıları aracılığı ile alınabilmektedir.*
Sonuç ve Tartışma: *Bu derlemede, doğal ve organik ürün standartlarının oluşum süreci ve şartları, yeşil formülasyonlarda tercih edilen hammaddeler, üretimde kullanılan yeşil kimya prensipleri ve sürdürülebilirlik açısından yeşil kozmetikler ele alınmıştır.*
Anahtar Kelimeler: *COSMOS, doğal kozmetikler, ecocert, organik kozmetikler, sürdürülebilirlik*

ABSTRACT

Objective: *Recently, the interest in green cosmetics increased due to the negative effects of cosmetics produced with synthetic raw materials on human health and the environment, the increase in the number of consumers who prefer sustainable products in line with environmental awareness, and the importance given to corporate responsibility by manufacturers. Green cosmetics are the products produced sustainable production methods using natural or organic raw materials that comes from the controlled or organic agriculture, and offered to the market with recyclable or reusable packaging. No regulation has yet imposed sanctions for this product range. However, by*

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means of standards such as USDA/NOP in the USA and COSMOS, Ecocert, NaTrue, BDIH in Europe and harmonized standards such as ISO 16128, products are tried to be controlled in terms of raw material selection, production stages and packaging, nature and human health. In accordance with the addendum to the regulation on natural and organic cosmetics published by the Turkish Pharmaceuticals and Medical Devices Agency-TİTCK, certification for product claims in natural and organic cosmetics should be provided and the certificate logo should be placed on the packaging. In TURKEY, COSMOS certificates can be obtained through certificate providers such as ETKO and IFC Global.

Result and Discussion: *In this review, the formation process and conditions of natural and organic product standards, raw material prefferred in green formulations, green chemistry principles used in production and green cosmetics in terms of sustainability are discussed.*

Keywords: *COSMOS, ecocert, natural cosmetics, organic cosmetics, sustainability*

GİRİŞ

Günümüzde kimyasal maddelerin kullanıldığı sentetik kozmetiklere alternatif olarak doğal ve organik kaynaklı hammaddeler ile hazırlanan kozmetik ürünlerin kullanımında artış gözlenmektedir. Doğal veya organik kozmetiklere duyulan ilgi artışının ana nedeni yeni nesillerin ekolojik çevre ve sorumluluk bilinci ile yetişmesi ve bunun sonucunda sağlık ve doğal arasında bağ kuran bir tüketici kesiminin ortaya çıkmasıdır. Bunun yanısıra, günümüz tüketicileri internet reklamları ve televizyon programları gibi sosyal medya aracılığı ile bilgi bombardımanına maruz kalmakta ve doğal malzemeler ile kendi kozmetiğini yapabilmeye teşvik edilmektedir. Doğal ve organik ürün kullanımı ile sentetik kimyasalların zararlı yan etkileri önlenirken, bu grup ürünler tüketiciye temizlik ve hijyen mesajı vermektedir. Yeşil kozmetiklerin üretim ve ambalajlaması yeşil kimya prensiplerine uygundur ve bu grup kozmetiklerde sürdürülebilirlik de önemli bir kriterdir.

Organik bir yaşam tarzını benimseyen tüketicilerin yukarda sayılan nedenlerden dolayı yeşil kozmetiklere doğru artan ilgisi sonucu kişisel bakım endüstrisi de tüketicinin artan talebine yanıt vererek her yıl organik ve doğal iddialarıyla birçok ürünü pazara sunmaktadır. Bu ürünler % 100 organik veya % 100 doğal olabildiği gibi, organik ya da doğal içerikli olarak da üretilebilmektedir. Küresel doğal ve organik kişisel bakım pazarı 2017 de 12 milyar dolar iken, 2021 yılında 29.9 milyar dolarlık bir pazar büyüklüğüne ulaşmıştır [1,2].

Bu grup kozmetiklerle ilgili en önemli tartışma konusu ürünlerin iddia ettiği doğal ya da organik veya çevre dostu, sürdürülebilir gibi özellikleri taşıyıp taşımadığı ve ürün güvenliğidir. Kozmetik ürün etiketindeki doğal, organik ya da yeşil gibi ifadeler tüketiciye üründe sentetik hammadde kullanımının kısıtlandığını göstermekle kalmayıp aynı zamanda sağlık ve çevre dostu olma mesajı da vermektedir. Araştırmalar organik ürün tüketicilerinin ambalaj ve etiketten çok etkilendiğini göstermektedir. Örneğin Fransa'da 2011 yılında yapılan bir anket çalışmasında organik şampuan ambalajı üzerinde üründe % 75'den fazla organik içerik olduğunun belirtilmesi fiyatında artışa rağmen tüketici tercihinde etkili olmuştur. Yine 2018 yılında İngiltere'de yapılan bir anket çalışması tüketicilerin güvenli, organik, geri dönüştürülebilir ambalaj gibi geri bildirimlerine rağmen yeşil kozmetik ile ilgili tanımları çok iyi bilmediğini ortaya koymuştur [3].

Kozmetik piyasasında gerçekte sürdürülebilir olmayan, sözde yeşil pazarlama (greenwashing) ürünleri de çokca bulunduğundan tüketicinin bu ayrımı yapabilecek şekilde bilinçlendirilmesi önem taşımaktadır. Etiket üzerinde ürünün ne kadarının doğal/organik içerik olduğunun belirtilmesi ve bu içeriğin uluslararası kuruluşlarca sertifikalandırılması bu tür ürünlerde güvenlik ve güvenilirliği arttırmakta ve sözde yeşil pazarlamanın engellenmesine katkı sağlamaktadır. Bu sertifikalandırmanın bir diğer avantajı da, sertifika sağlayıcı kuruluşların yönetmeliklerinde yer alan istekler doğrultusunda sürdürülebilirlik üzerine sağladığı katkıdır.

Bu derlemede günümüzde kozmetikte önemli bir eğilim ve ticari pazar haline gelen doğal ve organik kozmetikler konusu tartışılmıştır. Kozmetiklerde yeşil ve sürdürülebilirlik kavramlarının ortaya çıkışı ve sürdürülebilirlik tanımı verildikten sonra bu kozmetik ürünlerin yasal durumu ve hangi kuruluşlarca sertifikalandırıldığı, sertifikalar ve kılavuzlarda yer alan ilgili tanımları açıklanmıştır. Yeşil kozmetiklerin üretimlerinde yeşil kimya prensipleri gözönüne alınmaktadır. Buna göre kullanılan

hammaddeler, tercih edilen üretim metotları, üretimde karşılaşılan zorluklar ve ambalaj malzemeleri hakkında bilgi verilmiş, yeşil kozmetiklerde sürdürülebilirlik açısından su ve plastik kullanımının azaltılmasının atık yönetimi üzerindeki etkileri anlatılmıştır.

Kozmetiklere Yeşil Yaklaşım

Kozmetik ve sürdürülebilirlik kavramı ilk bakışta çok ayrı gözükse de yeşil kozmetikler sentetik hammadde kullanımından uzak durulması, yeşil kimya prensiplerine uyum, karbon ayakizi ve atık yönetimi gibi dikkat edilen konular nedeniyle sürdürülebilirlik yaklaşımı ile üretilen ürünlerdir [4].

Kozmetik ürünlerde yeşil yaklaşım ilk olarak küresel ısınma ile birlikte önem kazanmaya başlamıştır. Küresel ısınma ve artan çevre kirliliği, her yıl yeni ürünler sunan kozmetik firmaları için depolama alanlarının çokluğu, kozmetik ürünlerin belli bir süre sonunda raf ömürlerini doldurarak atık haline gelmesi, sosyal medyadan yayınlanan yazılarda kozmetik ürünlerde kullanılan kimyasal içeriklerin güvenliliğinin tartışılması gibi faktörler kozmetik endüstrisinde bu yaklaşımın doğuş nedenleridir. Ayrıca, 1920'lerde Amerika Birleşik Devletleri'nde (ABD) başlayan ve 1960 -1970'lerde kapsamı genişleyerek şirketlerin çalışma stratejilerine çevre sorunlarını da ekleyen "Kurumsal Sosyal Sorumluluk" (Corporate Social Responsibility; CSR) anlayışı 2000'lerin başında doğal ve organik kozmetik markalarının "kozmetiklerde yeşil eylem" yaklaşımının nedenleri arasındadır [5].

Yeşil kozmetik tanımının temelinde ekonomik, sosyal ve çevresel açıdan sürdürülebilirliği hedefleyen yeşil kimya kavramı bulunmaktadır. ABD Çevre Koruma Ajansı (Environmental Protection Agency, EPA) sürdürülebilirliği; insanların ve doğanın üretken uyum içinde olabileceği prensibine dayanan; doğal kaynakların korunması ve gelecek nesillerin suya, malzemeye, kaynaklara ve sosyal ve ekonomik gereksinimlere erişiminin sağlanması amacıyla çevre üzerindeki insan etkisinin yeterince azaltılmasını sağlayan koşullar olarak tanımlamaktadır [6].

Genel olarak kozmetikte yeşil yaklaşım kapsamında, biyolojik olarak parçalanabilir ambalaj kullanmak, sentetik hammadde ve plastik ambalaj kullanımını azaltmak, yeniden kullanılabilir ürün veya ambalajlar geliştirmek, çoklu fayda sağlayan ürünler geliştirmek, geri dönüşebilen malzemeleri kullanmak, çevre için daha az toksik maddeler üretmek yoluyla sürdürülebilirlik hedeflenmektedir. Doğal veya organik kozmetikler bu açıdan değerlendirildiğinde sertifika sağlayıcı kuruluşlar tarafından da sürdürülebilirliğin teşvik edildiği görülmektedir. Örneğin, COSMOS standardı hammaddelerin organik tarım ile elde edilmesini, biyolojik çeşitliliğe saygı duymayı, doğal kaynakları sorumlu bir şekilde kullanmayı, kullanılan işlem ve imalatın temiz olmasını, insan sağlığına ve çevreye saygılı olunmasını teşvik eder. Benzer şekilde Ecocert Greenlife standardı da çevreye saygılı üretim süreçlerini, mevcut enerji ve doğal kaynakların (su, hava, toprak verimliliği) daha iyi yönetilmesini, ürün kalitesi ve güvenliğini sağlamayı amaçlamaktadır [7,8].

Yeşil Kozmetikler ile İlgili Yasal Durum

Avrupa Birliği ve ABD Gıda ve İlaç İdaresi (Food and Drug Administration; FDA) direktiflerinin yeşil kozmetiklerin üretimi ve pazarlaması konularında doğrudan yasal yaptırımı yoktur. Bu tür ürünler için mevcut mevzuatlarda standartlaştırılmış tanımlar bulunmamaktadır. Bu nedenle bu tür ürünler genellikle kozmetik marketi tarafından fonksiyonel hammaddelerinin doğal ya da doğadan türevlendirilmiş oluşu gözönünde bulundurularak "doğal" ya da "doğal kaynaklı" olarak sınıflandırılmaktadır. Bunun yanısıra, doğal ya da organik yerine "yeşil" ifadesi de kullanılmaktadır [3].

Yeşil kozmetikler ile ilgili farklı ülkelerin kullandığı farklı standartlar bulunmaktadır. Ancak bu durum tüketiciler için kafa karıştırıcı olmasının yanısıra yeşil kozmetik ürünlerin formülasyon tasarımında sorun yaratmaktadır. Bu nedenle bu grup kozmetik hammadde ve ürünlerin tanımlanmasında ortak kullanılan bir standart olan COSMOS standardı oluşturulmuştur [9]. Yeşil kozmetiklerde karşılaşılan bir diğer sorun da ABD ve Avrupa'da doğal ve organik kozmetik ürünlerin standartlarındaki farklılıktır. ABD'de doğal ve organik iddiaları, farklı formülasyon felsefeleriyle, farklı iddialar olarak görülürken, Avrupa'da organik ve doğal standartlar iç içe geçmiş durumdadır [10].

Yaygın olarak kullanılan ve devlet dışı piyasa güdümlü (NonState Market-Driven) olarak tanımlanan bu doğal ve organik kozmetik standartları arasında en bilinenler olarak Natrue (Belçika), BDIH (Almanya), Ecocert Greenlife (Fransa), CosmeBio (Fransa), Soil Association (İngiltere) ve ICEA (İtalya) ile bu standartların uyumlulaştırılmasıyla ortaya çıkan COSMOS sayılabilir. Bu standartlar

küresel olmakla birlikte daha çok Avrupa'da kullanılmaktadır. Bunlara ek olarak Brezilya'da IBD, Avustralya'da NASAA, Japonya'da JAS gibi organik/doğal sertifikasyon sağlayan farklı kuruluşlar mevcuttur [9-11]. Bu kuruluşların sağladığı sertifikalar hammaddeler ve hammaddelerin üzerinde uygulanan işlemler, üretim, saklama, ambalajlama, etiketleme gibi final ürün kalitesini arttırmaya yönelik konular dışında enerji kullanımı ve atık yönetimi ile de ilgili standardizasyon sağlamayı amaçlar. Standardizasyon kuruluşlarının herbirinin bu konuda kendi yönetmeliği bulunmaktadır ve ürün sertifikalandırıldığında ambalajında kullanılan sertifika logosu verilmektedir [4].

ABD'de ise organik kozmetikler ile ilgili sertifikalar USDA (Amerika Birleşik Devletleri Tarım Bakanlığı) altında kurulan NOP (Ulusal Organik Programı), NSF/ANSI organik kişisel bakım ürünleri standardı, OASIS (Organik ve Sürdürülebilir Endüstri Standartları) ve NPA (Doğal Ürünler Derneği) kuruluşları tarafından sağlanmaktadır [9].

FDA organik kozmetikler için tanım yapmamıştır. Ancak NOP tarafından "Cope of Federal Regulations" içerisinde tanımlanan organik kozmetikler hem USDA organik iddia gerekliliklerini, hem de FDA kozmetik ürün etiketleme ve güvenlilik gerekliliklerini sağlamak zorundadır [12]. Ayrıca NOP yönetmelikleri tarımsal içeriklerin tanımı karşılayacak koşullar altında üretildiğine dair sertifikasyon sağlar. Uluslararası USDA/NOP sertifikasyon sağlayıcıları arasında Ecocert (Fransa), ETKO (Türkiye), ICEA (İtalya), IBD (Brezilya), NASAA (Avustralya), Pro-cert (Kanada), BIOH (Yunanistan) gibi kuruluşlar bulunmaktadır [13].

Doğal ve Organik Standartları

Yeşil kozmetikler ile ilgili yapılan araştırmalar tüketicilerin sertifikalı ürünleri daha güvenilir bulduğunu göstermektedir. Ayrıca bu sertifikaların logolarının etikette yer alması ile ürün ile ilgili hammadde, üretim ve ambalaja yönelik işlemler hakkında tüketici daha kolay bilgi sahibi olmaktadır.

Sertifikasyon ve sertifika logolarının sağladığı bir diğer avantaj da sözde yeşil pazarlama (yeşil dolandırıcılık; greenwashing) olarak ifade edilen pazarlama yöntemi ile tüketicinin kandırılmasının önüne geçilebilmesidir. Sözde yeşil pazarlama adı verilen bu hileli pazarlama metodu ile ambalaj üzerinde yeşil renk, bitki ya da çiçek resmi veya doğal, çevre dostu gibi kelimeler kullanılarak tüketici algısına hitap edilmektedir. Ancak, herhangi bir resmi belgesi olmayan bu tür ürünlerde çoğu kez ürün gerçekte iddia ettiği şekilde sürdürülebilir değildir. Örneğin, geri dönüştürülebilir ambalaj için aslında ekolojik olmayan üretim süreçlerinin kullanılması gibi durumlar ile karşılaşılabilmektedir [3].

Yukarda sayılan nedenlerden dolayı doğal ve organik kozmetiklerde standardizasyon kuruluşlarından sertifika almak ve bu sertifika logolarını etikette vermek önem taşımaktadır. Yeşil kozmetiklerde sık görülen doğal ve organik standardizasyon kuruluşları ve verdikleri sertifikalarda aranan şartlar aşağıda anlatılmıştır:

COSMOS

COSMOS (COSMetics Organic Standard) Standardı, 2002 yılında Nürnberg'de BDIH, Cosmebio, Ecocert Greenlife ICEA, Soil Association ve sonradan geri çekilen BioForum tarafından Avrupa'da organik doğal standartların uyumlulaştırılması amacıyla kurulmuştur. Bu standart farklı doğal kaynaklardan gelen fiziksel veya kimyasal işlem görmüş hammaddeleri, su, mineraller ve sentetik (petrokimya) ürünleri ayrı değerlendirir [3, 9, 10]. COSMOS, birden fazla standardizasyon kuruluşunun gereksinimlerini başarıyla uyumlu hale getiren bir kuruluştur [10].

Günümüzde COSMOS standardının yetkili sertifika sağlayıcısı olarak 12 farklı kuruluş bulunmaktadır. Bunlar; Türkiye'de faaliyet gösteren ETKO ve IFC Global yanısıra Avustralya'da ACO, Fransa'da Bureu Veritas, Ecocert Greenlife ve Cosmecert SASU, Almanya'da IONC, İtalya'da ICEA, Güney Kore'de Control Union Korea Co. ve KTR, İspanya'da CAAE ve İngiltere'de Soil Association kuruluşlarıdır [14].

Ecocert Greenlife

Fransa'da 1981 yılında kurulan Ecocert Greenlife, Avrupa'daki organik ürünler için lider bir sertifikasyon ve denetim kuruluşudur. Hem doğal, hem de organik ürünleri sertifikalandırmaktadır. Günümüzde 80'den fazla ülkede aktif olmakla beraber, aslen Fransa'da organik ürünlerin yaklaşık %

70'ine sertifika vermektedir. Ecocert organik tarımı, hammadde kaynaklarının sürdürülebilirliğini ve diğer çevresel girişimleri teşvik etmektedir [3,9].

CosmeBio

CosmeBio ekolojik ve organik sertifikasyon sağlayan bir kuruluştur. Ecocert ve Tarım Bakanlığı işbirliği ile Fransa'da kurulmuştur. Biolabel ve Ecolabel olmak üzere iki farklı sertifikasyonu bulunmaktadır. Bunlardan Biolabel Ecocert ile benzer şekilde organik sertifikasyon sağlarken, Ecolabel Ecocert ile benzer doğal sertifika vermektedir [3].

Soil Association

İngiltere'de 1967 yılında kurulan Soil Association organik sertifikası veren ilk kuruluştur. Ana konuları organik tarım ve gıda olmakla beraber 2002 yılından beridir tekstil ve kozmetik gibi alanlarda da sertifikasyon sağlamaktadır.

Soil Association tarafından verilen sertifikalar sadece organik kozmetikler ve ilgili tarım ve gıda ürünlerini içermektedir. Bu standard göre tarımsal içeriğin % 95'i organik olmalıdır. Sadece su ve işlem için gereken maddeler non-organik olabilir. Su doğal ürün olarak kabul edilir, dolayısıyla su içeren ürünler % 100 organik olarak kabul görmez. Organik içerikli ürünlerde % 70-95 organik içerik aranır ve bunun etikette belirtilmesi istenir [3].

ICEA

İtalya'da 2002 yılında Organik Tarım Bakanlığı desteği ile kurulan ICEA organik sertifikasyonu sağlayan bir kuruluştur. Ek olarak ambalajlara yönelik standardizasyon da sağlamaktadır. ICEA sadece kozmetik değil, gıda sektörünü de içerecek şekilde vegan sertifikasyonu da (ICEA VEGAN ve ICEA VEGETARIAN) sağlamaktadır. GDO kullanımı bu sertifikada yasaktır [3].

NaTrue

NaTrue (The International Natural and Organic Cosmetics Association), kozmetik ürünlerin tanınmasında yaşanan karmaşıklığa dayanarak doğal ve organik ürünler için kozmetikler için yasal tanımlamalar getirmek amacıyla 2008 yılında kurulan ve kar amacı gütmeyen bir kuruluştur. Kurucu üyeleri Weleda, Wala-Heilmittel, Logocos, Laverana, Santaverde ve Primavera aynı zamanda bir başka standart olan BDIH'nin de kurucularıdır [9]. NaTrue ayrıca IBD (Instituto Biodinâmico, Brezilya) ile de sertifikalandırma kriterleri konusunda anlaşma imzalamıştır.

Kozmetik ürün grubunun sertifikasyon etiketi taşıyabilmesi için belirli bir markaya (veya alt markaya) ait ürünlerin en az % 75'inde sertifikaya sahip olması gerekir. Başka bir sertifikalandırma standardı tarafından sertifikalandırılmış ürünler varsa bunlar, NaTrue sertifikası almak için ilk 2 yılık için % 75 hesaplamaya dahil edilebilir. Bu süreden sonra markanın NaTrue sertifikasına sahip ürünler doğrultusunda % 75 eşiğe sahip olması gerekmektedir.

IBD

Latin Amerika'da bulunan IBD (Instituto Biodinâmico, Brezilya) bir organik sertifika sağlayıcıdır. IFOAM (Uluslararası Organik Tarım Hareketleri Federasyonu), ISO/IEC 17065 (Avrupa pazar yönetmeliği CE 834/2007), Demeter (uluslararası pazar), USDA/NOP (Kuzey Amerika pazarı) ve SISORG (Brezilya Organik Uygunluk Değerlendirme Sistemi) ile akreditedir ve bu nedenle küresel olarak kabul edilen bir sertifikadır. IBD gereklilikler açısından NaTrue ile benzer olmakla beraber bu sertifikada su girdi olarak kabul edilmemektedir [15].

BDIH

Almanya'da (Mannheim) kurulmuş olan Alman/Avrupa standardı BDIH (Bundesverband Deutscher Industrie- und Handelsunternehmen) sadece doğal sertifikasyonu sağlayan bir kuruluştur. IONC GmbH nin ve COSMOS standardının kurulmasında rol oynamıştır. Kurucuları üyeleri Weleda, Wala, Logocos, Laverana, Santaverde ve Primavera kozmetik firmalarıdır. Alman menşeili doğal kozmetik ürünlerin ithalat hacmi nedeniyle BDIH Avrupa'da doğal kozmetik için lider sertifika

kuruluşlarındandır. Organik ürün sertifikası vermemekle birlikte sertifikalı ürünlerin içerisindeki hammaddelerin organik statüsünü doğrular [9].

Doğal ve organik kozmetiklerde sık karşılaşılan bazı standartların temel gereklilikleri Tablo 1'de karşılaştırılmıştır [9,16,17]. Türkiye'de ve Avrupa'da sık görülen bazı sertifika logoları Şekil 1'de verilmiştir.

Tablo 1. Çeşitli standartların temel gerekliliklerinin karşılaştırılması [9,16,17].

Standart	Sertifika tipi	Gereklilikler
COSMOS	• Doğal • Organik	• Son ürünün en az %20'si (su dahil) organik olmalıdır. • Durulanan ürünler, tonikler, vücut spreyleri ve tozlarda bu oran %10'dur. • Fiziksel olarak işlenmiş tarımsal bileşenlerin en az %95'i organik olmalıdır. • Kimyasal olarak işlenmiş tarımsal bileşenlerin en az %30'u organik olmalıdır. • Doğal kozmetik ürünler için organik bileşen içerme zorunluluğu yoktur. • Sentetik olarak sadece koruyucular, şelat yapıcılar ve amfoteriklere izin verilir.
ECOCERT	• Doğal (Eco) • Organik (Bio)	• Son ürünün en az %95'i (su dahil) doğal/doğaldan modifiye içerik olmalıdır. • Doğal içeriğin en az %5'i organik ve bitkisel kaynakların en az %50'si organik sertifikalı olmalıdır. • Organik kozmetiklerin en az %10'u organik tarımdan gelmelidir. • Sentetik ürün en fazla %5 (genellikle koruyucu) olmalıdır. • Amfoteriklere izin verilir.
NATRUE	• Doğal • Organik kısım içeren doğal • Organik	• Doğal sertifika için minimum bir organik miktarı belirtilmemiştir. Doğal ve modifiye edilmiş doğal içerik seviyeleri ürün tipine göre belirlenir. Bitkinin kendisinden gelen haricinde su hesaplamaya dahil edilmez. • Organik kısım içeren doğal ürünlerde en az %15 kimyasal olarak modifiye edilmemiş doğal içerik ve en çok %15 modifiye edilmiş doğal içerik olabilir. Bu içeriklerin %70'i organik veya kontrollu tarımla elde edilmelidir. • Organik sertifika için en az %20 kimyasal olarak modifiye edilmemiş doğal ve en çok %15 modifiye doğal içerik aranır. Doğal içeriğin en az %95'i organik veya kontrollu tarım ile elde edilmelidir. • Amfoteriklere izin verilmez. Sadece izinli koruyucular sentetik olabilir.
BDIH	• Doğal	• Bitkisel olmalıdır, hayvansal hammaddeler kullanılamaz. • Üretim organik tarımdan gelmeli ya da kontrollu ortamda yapılmalıdır. • Minerallere izin verilir, ancak ışın ve genetik müdahaleye izin verilmez. • Sadece belirli sentetik korucuyulara izin vardır ve etikette belirtilmelidir. • Şirket politikası CSR ile uyumlu olmalıdır.

Şekil 1. Türkiye ve Avrupa'da doğal ve organik kozmetiklerde sık karşılaşılan sertifika logoları

Yukarda sayılan standartların dışında doğal ve organik kozmetikler ile ilgili yönergeler sağlayan bir diğer kuruluş da Uluslararası Standardizasyon Organizasyonu (International Organization for Standardization; ISO)'dur. ISO üye kuruluşlar aracılığıyla dünya çapında olan bir uluslararası standart

kuruluşları federasyonudur. ISO teknik komiteleri tarafından hazırlanan "ISO 16128-Guidelines on Technical Definitions and Criteria for Natural and Organic Ingredients and Products" standardı, doğal ve organik kozmetik bileşenler ve ürünler için tanımlar ve kriterler hakkında yönergeler sağlar ve bu yönergeler kozmetik sektörüne özeldir.

ISO 16128 standardı iki kısımdan oluşur. İlk kısım, 16128-1:2016, doğal ve organik kozmetik içerikler ve ürünler için teknik tanımlar ve kriterler hakkında bir kılavuzdur. ISO 16128-2:2017 ise ISO 16128-1'de tanımlanan içerik kategorileri için geçerli olan doğal, doğal kaynaklı, organik ve organik kaynak hesaplanmasına yönelik yaklaşımları açıklamaktadır [3,18].

Diğer sertifikasyonlardan farklı olarak ISO 16128 standardında nihai tüketiciye yönelik etiketleme ve sürdürülebilirliğe yönelik iddialara yer verilmez. Ayrıca minimum eşik değerler belirtilmez. Burada amaç hammaddelerin doğal kriterini sağlaması ve bitmiş üründe nicelik olarak ürünün doğal ya da organik içeriğinin belirtilmesidir [3].

Türkiye'de Doğal ve Organik Kozmetiklere Yönelik Yasal Durum

Türkiye'de organik kozmetiklerin içeriğine dair sınırlar 23.05.2005 tarihli 25823 Sayılı Kozmetik Yönetmeliğinin altıncı ve onuncu maddelerine istinaden Türkiye Tıbbi İlaç ve Cihaz Kurumu (TİTCK) tarafından hazırlanan Doğal ve Organik Kozmetik Bileşen ve Ürün İddialarına İlişkin Kılavuz'da belirlenmiştir [19]. Kılavuza göre "Organik/ekolojik kozmetik ürün, organik tarım faaliyetleri esaslarına uygun olarak üretilmiş ham maddeler kullanılarak, ham maddeden bitmiş ürüne kadar organik olma gerekliliklerini sağladığını kanıtlayan, ağırlıkça en az %95'i organik üretimle elde edilmiş organik/ ekolojik kozmetik bileşenlerinden oluşan bitmiş kozmetik ürün"dür [19].

Kılavuzda, doğal ve organik kozmetiklerin üretiminde kullanılabilecek ekstraksiyon ajanları, yardımcı maddeler, ürün kategorisine göre doğal ve organik kozmetiklerin içerebileceği minimum doğal/organik madde yüzdesi, etiket ve tanıtım materyallerine ilişkin iddialar ve uyarılar ve bitmiş ürünün sahip olması gereken belgeler hakkında detaylı açıklamalar vardır.

Kılavuzda bitkisel, inorganik-mineral veya ölü omurgalılar hariç hayvansal kökenli bileşenler doğal kozmetik bileşen olarak ve biyoteknolojik prosesler dahil türevlendirilmiş doğal kozmetik bileşen olarak tanımlanır. Organik kozmetik bileşenler organik tarım faaliyetleri esaslarına uygun olarak üretilmiş olmalıdır. Doğal ve türevlendirilmiş doğal kozmetik bileşen içeren doğal kozmetik ürünler seviye 1, organik bileşen içeren doğal kozmetik ürünler ise seviye 2 ile açıklanmaktadır. Seviye 2'de doğal kozmetik ürün tanımına uyan ve içeriğindeki doğal bileşen veya türevlendirilmiş doğal bileşenlerin en az % 70 i organik tarım faaliyetleri esaslarına uygun olarak üretilmiş veya doğrudan doğadan elde edilmiş bileşenler kullanılarak oluşan bitmiş kozmetik ürünler yer almaktadır. Seviye 3 ise organik kozmetik ürünleri tanımlar. Bunlar doğal kozmetik ürün tanımına uyan ve içeriğindeki doğal bileşen veya türevlendirilmiş doğal bileşenlerin en az %95'i organik tarım faaliyetleri esaslarına uygun olarak üretilmiş veya doğrudan doğadan elde edilmiş bileşenler içeren bitmiş kozmetik ürünlerdir.

Kılavuzun 7. maddesine göre, üreticilerin doğal kozmetik ürünler için kılavuz gerekliliklerini sağladıklarını kanıtlayan, uluslararası kabul gören belgelendirme kuruluşları tarafından gerekli belgelendirmenin yapılmış olması gerekmektedir.

Doğal ve/veya organik kozmetik bileşenler ve ürünlere ilişkin belgelendirmelerde, uluslararası kabul gören harmonize standartlar (TS ISO 16128-1/ISO 16128-1, ISO 16128-2) ve uluslararası kabul gören belgelendirme kuruluşlarının kriterleri dikkate alınır. Ürünün ticari adında tüketiciyi yanıltıcı ibarelere yer verilmemelidir. Yine Türkiye'de faaliyet gösteren ETKO (Ekolojik Tarım ve Kontrol Organizasyon) ve IFC Global (International First Certification Global) sertifikalandırma kuruluşları üzerinden Ecocert, Cosmos, USDA/NOP sertifikaları almak mümkündür.

Yeşil Kozmetiklerde Sık Karşılaşılan Tanımlar

Doğal kozmetiklerde hammaddelerin doğal kaynaklardan elde edilmesi bu kozmetiklerin renklendirici, sentetik kimyasal, petro-kimya türevi hammaddeler gibi doğal olmayan bileşenler içermediği anlamına gelmektedir. Ancak organik ürünlerde bu yeterli değildir. Bu grup formülasyonlarda kontrollu organik tarım ile elde edilen hammaddelerin kullanılması gerekmektedir. İyi Tarım ve Toplama Uygulamaları (Good Agricultural and Collection Practices; GACP) gereği

organik tarımda toksik pestisit, sentetik gübre, bitki büyütme ürünleri, hayvan yemi katkı maddeleri ve genetiği değiştirilmiş organizma (GDO) kullanımına izin yoktur [4,20].

Organik kozmetiklerde formülasyon bileşenlerinin en az %95'i organik hammaddelerden oluşur veya hammaddeler sertifikasyonlarca kabul edilen ekstraksiyon yöntemleri ile organik kaynaklardan elde edilmiştir. Dolayısıyla organik bir bileşen aynı zamanda doğaldır. Ancak doğal bileşen organik değildir [4]. Ayrıca, doğal ve organik ürünler söz konusu olduğunda ürünün içeriğindeki doğal veya organik hammadde miktarına bağlı olarak aşağıda açıklanan farklı tanımlar ile karşılaşılabilmektedir. Tüketicinin pazarlama hilelerini aşabilmesi açısından bu tanımlamalardaki farklılıkları bilmesi gerekmektedir [21].

Sentetik İçerik

Sentetik hammaddeler kimyasal işlemlerle üretilen ve doğal olarak oluşmayan madde veya malzemelerdir

Doğal İçerik

Doğal hammaddeler, doğal kaynaklardan hasat edilmiş, kazılmış veya toplanmış ve daha sonra kimyasal reaksiyon olmadan işlenmiş madde ve malzemelerdir. Bu doğrultuda malzeme "kimyasal reaksiyon olmadan" yıkanma, renklendirilme, damıtılma, öğütülme, saflaştırma veya konsantre edilme gibi fiziksel işlemlere tabi tutulur. Biyoteknolojik yöntemler veya fermantasyon yoluyla kozmetik hammadde üretimi de bu tanımlara eklenebilir.

Doğal Olarak Türetilmiş Bileşen

Doğal olarak türetilmiş bileşenler doğal bir hammaddeden, doğada veya başlangıç malzemesinde bulunamayan yeni bir kimyasal üretmek için, kimyasal bir işlemin başlangıç noktası olarak kullanılması ile elde edilir.

Doğala Özdeş İçerik

Doğala özdeş bir malzeme üretmek için, genellikle doğal bir başlangıç malzemesinden değil, sentetik olarak üretilen bir maddeden yararlanılır. Örneğin L-mentol, nane yağlarından özütlenmek yerine genellikle sentetik olarak üretilir; sentetik olarak üretilen seramid benzeri yapılar hayvan kaynaklı seramidlerden daha iyi kabul edilmektedir.

Organik

Organik tarım yöntemlerinden kaynaklanan doğal içeriklerdir. Organik tarım, sentetik gübreler, böcek ilaçları, bitki büyüme düzenleyicileri ve hayvan yemi katkı maddelerinin kullanılmasını önleyen bir tarım şeklidir. Üretim yapan çiftliklerin ürünlerinin, kozmetik bileşen olarak kullanılabilmesi için sertifikalı olması gerekir.

Doğal, organik, bitkisel kozmetik gibi tanımların ortak noktası ise doğada bulunabilen malzemeler ile üretilmiş olmalarıdır. Bu kozmetiklerden beklenen temiz teknoloji kullanılarak doğal kaynakların sorumlu kullanımı ile üretilmeleri ve biyolojik olarak parçalanabilen ya da yeniden kullanılabilen ambalajlar ile sunulmaları, kısaca çevre dostu olmalarıdır [4,22].

Yeşil Kozmetiklerde Kullanılan Hammaddeler

Yeşil kozmetiklerin üretiminde tercih edilen hammaddeler doğal/sürdürülebilir seçenekler göz önüne alınarak seçilmektedir. Sentetik veya petro-kimyasal türevli hammaddeler, alerji veya iritasyon potansiyeli olan veya endokrin sistem sorunlarına neden olabilecek kimyasal maddeler bu grup kozmetik ürünlerde tercih edilmemektedir. Kozmetiklerde riskli görülen hammaddelerin başında parabenler, mineral yağlar, aluminyum, triklosan ve ambalajlamada kulanılan fitalatlar bulunmaktadır [23].

Parabenler deriden emilimi zayıf maddelerdir, alkil yan zincir uzunluğu ve dallanmaya bağlı olarak östrojenik etkisi artmaktadır. Paraben türevleri arasında en zayıf östrojenik etkiyi metil paraben gösterir. Tüm parabenlerin ortak metaboliti olan hidroksibenzoik asit ise in vitro şartlarda inaktiftir. Özellikle bütil paraben DNA hasarına neden olmaktadır, ancak metil ve etil parabenler ile kanserojen

etkiye dair hiçbir kanıt saptanmamıştır. Tüketici Güvenliği Bilim Kurulu (Scientific Committee on Consumer Safety; SCCS)'na göre metil ve etil parabenler kozmetiklerde tek başına %0.4 ve kombine kullanımda %0.8'e kadar insan sağlığı için güvenli olarak kullanılabilir [23].

Benzer şekilde, mineral yağların eser miktarda içerdiği doymuş ya da alkillenmiş hidrokarbonların toksisite potansiyelinin yüksek olmadığı bildirilmektedir. Avrupa Kimyasallar Ajansı (European Chemicals Agency, ECHA)'na göre, kozmetikte de farmasötik kalite mineral yağ kullanılması halinde hidrokarbon kalıntılarıyla ilişkilendirilebilecek kanserojen risk oluşmamaktadır [23]. Ancak saflaştırılmış olsa da hidrokarbonların tahriş ve alerji potansiyelinin olması, ve petrol-kimya ürünlerinin azaltılmasının sürdürülebilirlikteki önemi gereği yeşil sertifikalarda bu grup kimyasallar tercih edilmemektedir.

Tablo 2'de yeşil kozmetiklerde kullanılması tercih edilmeyen hammaddelere örnekler verilmiştir.

Tablo 2. Organik kozmetiklerde kullanımı tercih edilmeyen hammadde örnekleri [21].

Kimyasal Sınıfı	**Kozmetik İşlevi**	**Kullanılmama Nedeni**
Parabenler	Koruyucu	Endokrin sorunlar (kanıtlanmamış)
Formaldehit	Koruyucu	Kanserojen etki
Qaternium15	Koruyucu	Formaldehit donörü
DMDM Hidantoin	Koruyucu	Formaldehit donörü
Mineral yağlar	Yumuşatıcı, taşıyıcı	Petrokimyasal köken
Sentetik glikoller	Nemlendirici	Petrokimyasal köken
Sentetik polimerler	Kıvam verici, stabilizatör	Petrokimyasal köken, monomer safsızlık
Etoksilenmiş veya propoksillenmiş bileşenler	Sürfaktan	1,4 dioksan kontaminasyonu (kanserojen)
Sülfonlu malzemeler	Sürfaktan	Sert kimyasal işlem
Sentetik koku ve tat malzemeleri	Parfüm/Tat	Alerji
Sentetik boyalar	Renklendiriciler	Alerji, iritasyon
EDTA	Şelat yapıcı ajan	Biyobozunur sorunlar
Bütillenmiş Hidroksi Toluen	Antioksidan	Endokrin bozukluk
Fitalatlar	Parfüm seyreltici ve çözücü	İnsan bağışıklık sistemine toksik
Etanolaminler	pH ayarlayıcı, emülgatör	Nitrozamin oluşumu
Talk	Toz taşıyıcı	Asbest kirlenmesi
Kimyasal güneş koruyucular (oktil dimetil PABA, homosalat, oktilmetoksisinamat gbi)	UV absorbanları	Biyokimyasal veya hücresel seviye değişiklikleri, kanser endişesi
Sodyum Lauril Sülfat	Sürfaktan/Emülgatör	Deri irritasyonu
Sodyum Lauret Sülfat	Sürfaktan	1,4-dioksan kontaminasyonu (kanserojen)
Sakkarin gibi yapay tatlandırıcılar	Tat iyileştirme	Potansiyel kanser riskleri

Genel olarak yeşil standartlar incelendiğinde kontrollü olarak doğadan toplanan doğal ve organik hammaddelerin yanısıra fiziksel işlem görmüş doğal maddeler ve kimyasal reaksiyon ile doğaldan türetilmiş hammaddelerin de kabul edildiği görülmektedir. Ancak sertifikalarca izin verilen kimyasal işlemler sınırlıdır. Örneğin COSMOS ve NaTrue sertifikasyonunda açilasyon, amidasyon, kondensasyon, dehidrojenasyon, dimerizasyon, esterifikasyon, glikozidasyon, hidrojenasyon, hidroliz, saponifikasyon, oksidasyon, nötralizasyon gibi işlemler kabul görürken; etoksillenmiş ya da propoksillenmiş (alkoksilasyon) hammaddeler, beyazlatma, fosfatlama ve polimerizasyon gibi kimyasal işlemler yasaklar arasındadır. Hammadde üzerinde uygulanacak kimyasal işlemler sürdürülebilirlik politikası gereği ekolojik dengeyi bozmamalı, enerji kullanımı ve atık yönetimi açısından duyarlı olmalıdır [16,17].

COSMOS standardı bitki, hayvan ve mikrobiyolojik kaynaklardan fiziksel işlem ile doğal hammadde eldesini de kabul etmektedir. Genel olarak ekstraksiyon yöntemleri izin verilen fiziksel

işlemlerdir. Bu yöntemlerde de aranan kriter yine ekolojik denge, enerji kullanımı ve atık yönetimi açısından duyarlılıktır. Yeşil kozmetiklerde sık karşılaşılan fiziksel işlemler arasında santrifüj, uçurarak ya da distilasyon ile buharlaştırma, filtrasyon/ultrafiltrasyon ya da diyaliz ve iyon değişimi yoluyla saflaştırma, dondurma, öğütme, liyofilizasyon, maserasyon, infüzyon, perkolasyon, UV sterilizasyon, ultrason ve vakumlama sayılabilir [16].

Sentetikler yerine kullanılabilecek doğal ve organik hammaddeler

Doğal ve organik kozmetiklerin üretiminde kullanılan hammadde gruplarına bakıldığında, emoliyanlar, emülgatör ve yüzey aktif maddeler, antioksidanlar, antimikrobiyal koruyucular ve çözücülerin sıklıkla kullanıldığı görülmektedir.

-Emoliyanlar

Cildi yumuşatarak nemlendiren emoliyan özellikli yağlı hammaddeler arasında doğal bitkisel yağlar ve esterleri sıklıkla kullanılmaktadır. Bu yağlar doğal yağ asitleri/trigliseridler açısından zengin hammaddelerdir [21,24].

-Emülgatör ve Yüzey aktif maddeler

Soya fasulyesi, yumurta sarısı veya ayçiçeği tohumlarından elde edilen bir fosfolipid olan lesitin iyi bir doğal seçenektir. Hidrojene lesitin veya lisolesitin gibi lesitin türevleri kullanılabilmektedir [24].

Sentetik noniyonik emülgatörler yerine doğal olarak türetilmiş sükroz, glukoz, sorbitan, gliseril veya poligliseril esterleri, alkil glukozit karışımları, inulin lauril karbamat, zeytinyağı esterleri ve bunların karışımları kullanılabilmektedir. Yine anyonik doğal emülgatörler olarak hidrofilik asitler (sitrik, laktik, glutamik gibi) ve yağ alkolleri ile yağ asitleri ve gliserin gibi doğal olarak türetilmiş hammaddelerin esterleştirilmesi ile elde edilen gliseril stearat sitrat, asil glutamatlar, asil laktilatlar, asil fosfatlar gibi hammaddeler kullanılabilmektedir [21].

Deterjan etkisi nedeniyle şampuan ve duş jeli gibi ürünlerde sık kullanılan sodyum lauret sülfat (SLS) gibi anyonik yüzey aktif maddeler yeşil kozmetiklerde tercih edilmez. Ancak SLS etoksillenmemiş (SLES) ise kabul edilebilmektedir. Bu grup kozmetiklerde köpük yapıcı olarak kullanımı olan amfoterik maddelere Ecocert gibi bazı standartlarda izin verilmektedir. Amfoterik yüzey aktif madde olarak ciltte tahriş potansiyelini azaltan ve hindistan cevizi yağından hareketle elde edilen kokoamidopropil betain (CAPB) ile SLES beraber kullanılabilmektedir.

Biyobozunur özellikli, hindistancevizi veya hurma yağı kaynaklı yağ alkolu ve mısır ya da patates kaynaklı glikozdan elde edilen alkil poliglukozitler (koko, lauril, desil ve kapriil/kapril türevleri gibi) ile *Yucca glauca* veya *Quillaja saponaria* gibi bitkilerden elde edilen saponinler yüzey aktif özellikleri nedeniyle kullanılabilmektedir. Ancak, saponin bakımından zengin özütler, genellikle oldukça renklidir ve kabul edilebilir bir köpük formüle etmek zordur [21].

Silikonlar

Yeşil kozmetiklerde kullanımı sınırlı hammaddeler arasında yer alan silikonlar ürünlere kayganlık hissi veren, ciltte yayılabilirliği arttıran malzemelerdir. Yeşil kozmetiklerde birkaç doğal alternatif olarak denizel ve bitkisel silikonlar kullanılmaktadır. Örneğin, *Chondrus crispus* özü, mantardan türetilmiş kitozan türevi (kitozan süksinamid) ve süt devedikeni özü (*Silybum marianum* etil esteri) sayılabilir. Bunlar, emülsiyonlar gibi su açısından zengin ürünlerde, ciltte oklüzif olmayan bir koruyucu film gibi, doğal silikon benzeri bir his vermek üzere tasarlanmıştır [21].

Polimerler

Yeşil kozmetiklerde kalınlaştırıcı, emülsiyon veya süspansiyon stabilizanı gibi amaçlar ile kullanılan polimerlerin doğal türevleri tercih edilmektedir. Doğal kaynaklı polimerlere ksantan zamkı (fermantasyon türevi), İrlanda yosunu (*Crondus crispus*), mısır nişastası (*Zea mays*), mikrokristalin selüloz, Tara sakızı (*Caesalpinia spinosa* gum) ve akasya (*Acacia senegal*), algler ve killer örnek verilebilir. Bu malzemelerin birçoğunun en büyük dezavantajı, ciltte yapışkan bir his vermesi veya üründe lifli bir görünüm potansiyelinin olmasıdır. Yüzey aktif madde ile beraber kullanıldıklarında berrak görüntü etmek de diğer bir zorluktur [21].

Çözücü ve hümektanlar

Propilen veya bütilen glikol gibi glikoller, botanik materyalleri ekstrakte etmek için yaygın olarak kullanılan ve hümektan etkili maddelerdir. Propilen glikol, cilde nüfuz ettiğine ve muhtemelen protein ve hücresel yapıları zayıflattığına inanılan bir petrol türevidir. Yerine gliserin, su, alkol gibi diğer uygun çözücüler veya doğal yağlar yeşil kozmetiklerde tercih edilmektedir. Yeşil bir alternatif olarak çözücüler birkilerden fermentasyon ile de elde edilebilir. Örneğin propandiol mısır şekerinden fermantasyon yoluyla üretilmektedir. Biyofermentasyondan türetilen butilen ve pentilen glikoller de mevcuttur [21].

Koku bileşenleri

Yeşil kozmetiklerde sentetik koku bileşikleri yerine tercihen esansiyel yağlar, oleoresinler ve diğer doğal aromatik bileşiklerin karışımları kullanılmaktadır. FDA esansiyel yağları kozmetik hammadde olarak kabul eder ve koku maddeleri için ek bir izin talep etmez. Ancak, koku karışımları esansiyel yağların içerecebileceği koku alerjenleri ile bunların azami sınırlarını tanımlayan IFRA (Uluslararası Koku Birliği; International Fragrance Association,) düzenlemelerine uymalıdır [21,25].

ISO standartları ise her bir esansiyel yağ içeriğini ayrı sertifikasyonla kodlamaktadır. Örneğin Rosmarinus officinalis L. ISO 1324: 1988 ile sertifikalandırılır. ISO Teknik Komitesi (ISO/TC54) ayrıca tüm esansiyel yağların spesifikasyonları ve kontrolları için gerekli olan analitik yöntemleri de standardize etmektedir [25]. Koku bileşenlerinin ekstraksiyonunda genellikle etanol veya süperkritik karbondioksit tercih edilmektedir [21].

Antioksidanlar

Sentetik antioksidanlara alternatifler arasında doğal E Vitamini bileşeni alfa-tokoferol ve E vitamini yönünden zengin yağlar örnek verilebilir. Biberiye (Rosemary) ekstresi veya yağı da yaygın olarak kullanılmaktadır [21]. Aynı zamanda, özellikle makro ve mikro alglerde yüksek oranda olmakla birlikte tüm deniz canlıları vitamin içermektedir. Algler özellikle E ve C vitamini ve provitamin A (β-karoten) bakımından zengindir [26]. Bunun dışında yulafta bulunan avenantramidler; nar polifenolleri (ellajik asit ve punikalajin); polifenol türevleri bakımından zengin maltlı arpa özü oligomerik proanto-siyanidinler ve ferulik asit ve sesamin ve sesamolin bakımından zengin sabunlaşmayan susam (*Sesamum indicum*) yağı doğal antioksidanlara örnek verilebilir [21].

Boyar maddeler

Kozmetik formülasyonlarda, genellikle gıda endüstrisinden gelen, bir dizi yağda veya suda çözünür doğal renk maddesi kullanılmaktadır. Doğal renkler esas olarak bitkilerin tohumlarından (örneğin annatto); kökler veya rizomlardan (örneğin zerdeçal); şekerlerden (karamel); yapraklar ve saplardan (örneğin ıspanak); çiçeklerden (örneğin Tagetes kaynaklı lutein); sebzelerden (kırmızı lahana, kırmızı pancar suyu ve havuç yağı özü gibi); meyvelerden (üzüm suyu); alglerden (beta karoten) elde edilir. Kırmızı renkli karmin de teknik olarak doğaldır, ancak bir hayvan kaynağından (kokain böceği) elde edildiğinden ve allerji riski nedeniyle kabul edilmemektedir. Dekoratif kozmetikler için Ecocert Greenlife onaylı mineral pigmentler, sedef ve mika bazlı parıltılı parçacıklar kullanılmaktadır [21].

UV Absorbanları

Henüz, Avrupa Birliği ya da ABD mevzuatının izin verdiği doğal kaynaklı güneş koruyucu maddeler bulunmamaktadır. Doğal güneş koruyucu ürünleri üreticilerinin çoğu, titanyum dioksit (TiO_2) ve çinko oksit (ZnO) gibi inorganik UV koruyucu malzemeler kullanır. Fito-kimyasallar açısından zengin bitki özlerinden UV absorbe edici olarak yararlanılabilir, ancak bunlar tek başına güneş koruyucu bileşen olarak kullanılamaz. Örnekleri arasında siyah çay (*Camelia sinensis)*, aloe (*Aloe vera*), ginkgo (*Ginkgo biloba*) yaprakları, nar (*Punica granatum*) ve domates (*Solanum lycopersicum*) meyveleri, kurkumin (*Curcuma longa*) rizomu, Propolis reçinesi, avokado (*Persea americana*) ve üzüm (*Vitis vinifera*) çekirdekleri, Peruvian maca (*Lepidium meyenii*) sayılabilir [21,27].

pH Ayarlayıcılar

Formülasyonlarda pH ayarlamak için kullanılan sodyum veya potasyum hidroksit sertifikasyon açısından sentetik sayılır. Daha doğal, ancak daha pahalı bir alternatif, L-Arginin aminoasit, doğal kaynaklardan elde edilen laktik veya sitrik asit gibi alfa hidroksi asitler kullanılabilir [21].

Şelat Yapıcılar

Kozmetiklerde sık kullanılan etilen diamin tetra asetik asit (EDTA) çevre ve insan sağlığı açısından şüpheli hammaddeler arasında yer almaktadır. EDTA yerine fındık, tohum ve tahılların gövdelerinde bulunan fitik asit veya sodyum tuzu, sodyum fitat kullanılabilir. Ancak bu maddelerin maliyetleri oldukça yüksektir. Başka bir olasılık, doğal olmasa da, aspartik asitten üretilen ve EDTA'nın aksine biyobozunur olan etilen diamin disüksinik asit (EDDS) kullanmaktır [21].

Koruyucular

Kozmetik formülasyonlarda mikrobiyal bozulma ürünün fiziksel olarak bozulmasına yol açabilir veya üründe patojen varlığı tüketici güvenliği için bir tehdit oluşturabilir. Mikrobiyal kontaminasyona karşı korumak için kozmetiklere, ilaçlara ve gıdalara kimyasal koruyucular eklenmektedir. Fakat son yıllarda geleneksel/kimyasal koruyucuların güvenli olup olmadığı ile ilgili birçok soru ortaya çıkmıştır. Tüketicilerin kimyasal koruyucular, özellikle de paraben türevleri ile ilgili şüpheleri kozmetik endüstrisini kozmetiklerin korunması için alternatif yaklaşımlar aramaya itmiştir [28]. Bu yaklaşımlardan derlemenin ilerleyen bölümlerinde bahsedilecektir.

Tablo 3'de doğal ve organik kozmetiklerde sık karşılaşılan hammaddelere örnekler verilmiştir.

Tablo 3. Doğal ve organik hammadde örnekleri [21].

İşlevi	**Örnek**
Emoliyan	• Kaprilik, kaprik trigliseridleri (*palm/hindistan cevizi yağından fraksiyonla elde edilir)* • Badem yağı, jojoba yağı, hindistan cevizi yağı, shea yağı ve zeytinyağı gibi doğal yağlar
Emülgatör	• Oleozom ve gliserin • Inulin lauril karbamat • Sukroz laurat • Setearil olivat • Sodyum steraol glutamate • Poligliseril-3 polirisinoleat
Sürfaktan	• Sodyum koko sülfat • Disodyum kokoil glutamat • Sodyum koko-glukozit tartarat • Sodyum lauroil laktilat • Sodyum kokoil aminoasitleri • Disodyum lauril sülfosüksinat
Silikonlar	• Deziel kaynaklı bitkisel silikon (*Chondrus crispus* ekstresi) • Mantar türevi kitozan ve S*ilybum marianum* (devedikeni) ekstresi • Hindistan cevizi alkan ve koko kaprilat / kaprat karışımı
Saç kremleri	• Pirinç ve brassica türevi yağ asidi alkollerinin esterleştirilmesi ile oluşan L-izolösin • *Pentaclethra macroloba* tohumu yağı
Çözücüler	• Propandiol
Koku verici bileşenler	• Uçucu yağlar ve oleoresinler

Tablo 3 (*devamı*). Doğal ve organik hammadde örnekleri [21].

Polimerler	• *Crondrus crispus* (Carrageenan) • Nişasta bazlı malzemeler • Mikrokristalin selüloz • Tara zamkı (*Caesalpinia spinosa*), *Acacia senegal*, Ksantan zamkı, Sklerotium zamkı • Süksinoglikan zamkı (fermentasyon türevi) • Alg (deniz yosunu)
Antioksidan	• Doğal E vitamini bileşenleri, E vitamini içeren yağlar • Biberiye ekstesi/yağı, • Yulaftan elde edilen avenantramidler, • Nar polifenolleri (ellagik asit ve punikalajin), • Polifenol türevlerince zengin maltlı arpa özütü • Oligomerik proanto-siyanidinolinler ve ferulik asit; • Susam yağında bulunan sesamin ve sesamolin
Koruyucular	• Benzoik asit, sodyum benzoat, • Sorbik asit, potasyum sorbat, • Salisilik asit, • Benzil alkol • Greyfurt çekirdeği ekstresi, hanımeli ekstresi, bazı baharatlar, uçucu yağlar ve usnik asit vb.
Renklendiriciler	• Annatto tohumları • Zerdeçal kökü/rizomu, • Şekerden elde edilen karamel, • Ispanak yaprakları, • Tagetes çiçeklerinden elde edilen lutein, • Kırmızı lahana, şekerpancarı suyu, havuç yağı, greyfurt, algden elde edilen beta karoten
UV absorbanları ve güneş koruyucular	• ABD ve Avrupada onaylı doğal bir UV absorbanı yoktur. • Siyah çay, yeşil kahve, shea yağı gibi bitkisel ekstreler • TiO_2 ve ZnO doğal emoliyan yağlar içerisindeki dispersiyonları doğal kabul edilir.
pH düzenleyiciler	• EDTA alternatifi olarak fitik asit (fındık, tohum ve tahılların kabuğunda bulunur) • Fitik asit sodyum tuzu, sodyum fitat

Kozmesötik hammaddeler

Doğal ve organik kozmetiklerde botanik, denizel ve mikrobiyal kaynaklı hammaddeler yaşlanma karşıtı etkileri nedeniyle kullanılmaktadır. Sıklıkla karşılaşılan denizel hammaddelerin başında *Laminaria digitata, Kappaphycus alvarezii* ve *Chondrus crispus* gibi antioksidan etkileri olan deniz algleri gelmektedir [29]. Denizel kaynaklardan elde edilen kollajen ve kitozan ve Porphyra, Wakame, Spirulina, Chlorella gibi alglerden elde edilen protein ve peptitler de doğal kaynaklı kozmesötik hammadde örnekleri arasındadır [21].

Doğal kaynaklı kozmesötik hammaddeler arasında *Pentaclethra macroloba* (Pracaxi) meyvesinden elde edilen tohum yağı, hindistan cevizi, jojoba ve argan gibi emoliyan bitkisel yağlar, Ektoin gibi halofilik mikroorganizmalardan elde edilen kozmesötik aktifler, *Myrothamnus flabellifolia*'dan elde edilen glikoin, yulaftan elde edilen beta glukan örnek verilebilir [21,30-32].

Çay, kahve, üzüm, limon, hindistan cevizi gibi birçok besin maddesi de çeşitli doğal aktifler içermektedir. Çoğu antioksidan etkili bu kozmesötik aktifler, nutrikozmetik amaçlar ile kullanılmaktadır. Sık karşılaşılan nutrikozmetik bileşenler, bulundukları besinler ve kullanım alanlarına örnekler Tablo 4'de verilmiştir [33].

Nanomalzemeler

COSMOS standardı nanomalzeme olarak güneş koruyucu ürünlerde fiziksel UV filtre olarak titanyum dioksit ve çinko oksit ile silika kullanımına izin vermektedir. Doğal alternatifleri bulunmayan bu malzemelerde nano boyutu 100 nm ve üzeri olmalıdır. Avrupa Birliği kozmetik yönetmeliği ile uyumlu olacak şekilde 1 ila 100 nm ölçeğinde % 50 veya daha fazla partikül boyutu dağılımına sahip olan diğer tüm nanomalzemeler yeşil kozmetiklerde yasaklanmıştır [16]. COSMOS teknik yönetmeliği üzeri kaplı nanopartiküllere de minimum partikül boyutu kaplama olmaksızın 100 nm'nin üzerinde ise izin verir ve UV filtreleri olarak kullanılan TiO_2 ve ZnO Tüketici Güvenliği Bilim Kurulu (SCCS) tarafından yayınlanan koşullar karşılanırsa kabul eder. Bu koşullara göre, TiO_2 ve ZnO SCCS kriterlerine uygun olmalıdır ve UV filtreleri olarak TiO_2 ve ZnO hiçbir şekilde (püskürtme başlığı olmayan ambalajlar hariç) püskürtme uygulamalarında kullanılmamalıdır [34].

Tablo 4. Kozmetiklerde sık karşılaşılan besin hammadde örnekleri [33].

Besin	**İçerik**	**Kozmetik Kullanımı**
Yeşil çay	Kateşin türevleri; epikateşin, epigallokateşin gibi	Serbest radikal tutucu
Arabica kahve çekirdeği	Proantosiyanidinler	Antioksidan , cilt beyazlatıcı, kırışıklık karşıtı
Üzüm (*Vitis vinifera*)	Resveratrol, prosiyanidin, proantosiyanidin	Antioksidan (UV kaynaklı yaşlanma karşıtı)
Nar	Ellajik asit	Antioksidan, antiinflamatuvar, kırışıklık azaltıcı
Soya fasulyesi (Glycine max)	Izoflavonlar (genistein)	Antioksidan, UV kaynaklı oksidatif DNA hasarı ve fotohasarı azaltır
Citrus limon	Flavonlar	Antioksidan, depigmentasyon ajanı
Ficus carica	Fenolik bileşikler	Ekstreleri epidermal hasarı onarır, sebumu dengeler, kırışıklıkları azaltır
Cynara scolymus	Fenolik bileşikler	Serbest radikal tutucu, fotokoruyucu, cilt elastikiyetini arttırır
Glycyrrhiza glabra (licorice)	Flavonoidler (glabridin)	Antioksidan, pigmentasyon düzensizliklerinde etkilidir
Kakao (*Theobroma cacao*)	Polifenoller, metilksantinler	Antioksidan, antiinflamatuvar, fotoprotektif, kolajen ve glukozaminoglikan üretimini düzenler
Hindistan cevizi	Yağ asitleri (miristik, laurik, palmitik asitler)	Antioksidan, antiinflamatuvar, UV kaynaklı yaşlanma etkilerini azaltır, nemlendirici
Prunus dulcis	Triterpenoidler, kateşin, fitosterol, yağ asitleri	Antioksidan

Yeşil Kozmetiklerde Formülasyon Tasarımına Yönelik Zorluklar

Yeşil kozmetiklerde formülasyon geliştirmedeki en önemli zorluklardan biri kullanılan doğal hammaddelerin yetiştirildiği veya toplandığı bölgelerdeki ışık, sıcaklık, nem, rakım, yağış ve toprak özelliği değişikliklerinden ileri gelen farklardır. Ayrıca toplama, kurutma, depolama, taşıma ve işleme yöntemlerinin hammadde üzerinde etkisi bulunmaktadır. Yeşil kozmetiklerde bu konudaki standardizasyon İyi Tarım Uygulamaları (Good Agricultural Practices, GAP) ve doğal/organik ürün sertifikasyonu ile sağlanmaktadır. İyi tarım uygulamalarına uygun şartlarda gerçekleştirilen organik tarım ile tohum çeşitliliği, büyüme ortamı ve gübre kullanımı, pestisit kullanılmaması, kurutma ve depolama gibi şartlar standardize edilerek elde edilen organik hammaddeler ve bu hammaddelerin kullanıldığı bitmiş ürünler ise standardizasyon kuruluşları aracılığı ile sertifikalandırılmaktadır [35].

Hammadde eldesinde kullanılan ekstraksiyon yöntemi de ürün üzerinde etkilidir. Ekstraksiyon için herhangi bir su formu veya etil alkol, gliserin, bitkisel yağlar gibi doğal malzemeler kullanılmalıdır. Başka çözücülerin kullanılması gerekiyorsa, bu çözücüler daha sonra geri dönüştürülmeli ve bitmiş üründen tamamen uzaklaştırılmalıdır. Aromatik, alkoksillenmiş, halojenlenmiş, nitrojen veya kükürt bazlı solventlerin kullanımına organik olarak sertifikalandırılan ürünlerin üretiminde izin verilmez [16].

Sürdürülebilirlik açısından en çok tercih edilen yöntem ise yeşil kimya prensipleriyle uyumlu olan süperkritik sıvı ekstraksiyonu ve süperkritik çözücü kullanımıdır. Süperkritik sıvı ekstraksiyonunda madde sıvıda çözünür ve ardından basınç azaltılarak ektraksiyon ürünü sıvıdan ayrılır. Süperkritik çözücüler organik çözücü kullanımını azalttığından çevre dostu kabul edilmektedir. Süperkritik çözücüler ile yapılan ekstraksiyon işlemlerinde en sık karşılaşılan süperkritik çözücü ise karbondioksit (CO_2)'dir. COSMOS sertifikasyonunda süperkritik CO_2 ekstraksiyonu da dahil su veya üçüncü bir çözücü ile yapılan tüm ekstraksiyonların doğal malzemeler kullanılarak gerçekleştirilmesi istenmektedir [16,21,35].

Yeşil kozmetiklerde formulasyon geliştirirken karşılaşılan bir diğer zorluk ürünün mikrobiyolojik olarak korunması ile ilgilidir. Yeşil kozmetiklerde sentetik koruyucuların kullanımları kısıtlıdır ve farklı standartların koruyucular ile ilgili farklı gereklilikleri bulunmaktadır. Bunlar arasında Ecocert standardı sadece satın alınan malzemeler için olmak kaydıyla fenoksietanol ve parabenlere hala izin vermektedir. BDIH standardı, doğala özdeş terimini kullanarak organik asitler, tuzları ve etil esterleri (benzoik asit, salisilik asit, sorbik asit) ve benzil alkol gibi bazı sentetik koruyuculara izin vermektedir. Tıpkı Ecocert gibi, NaTrue standardı da benzoik asit, salisilik asit, sorbik asit gibi doğala özdeş koruyuculara ve doğala özdeş terimini kullanan sülfatlama, hidrojenasyon ve sentetik olarak korunmuş bileşenlere izin vermektedir [21,36].

Doğal ve organik kozmetiklerde koruyuculara yönelik yaklaşım koruyucu içermeyen (preservative-free) veya kendini koruyan (self-preserving) ürünler imal etmektir. Koruyucu içermeyen kozmetikler üründe kimyasal koruyucuları azaltmak veya ortadan kaldırmak üzere sterilizasyon ve/veya uygun ambalaj tasarımının kullanımını öngörmektedir. Ancak çok dozlu ambalaja sahip ve su içeriği yüksek doğal formülasyonlarda bu yaklaşım yetersiz kalmaktadır [37]. Bu tür formülasyonlarda sentetik kimyasal koruyucular yerine antimikrobiyal etkileri olan doğal bileşikler veya çok fonksiyonlu hammaddelerin kullanımını da kapsayan, kendini koruyan ürün yaklaşımı günümüzde tercih edilmektedir.

Kendini koruyan ürün yaklaşımı ile kozmetiklerde mikrobiyal büyümeyi engelleyen veya mikroorganizmaları öldüren bir ortamın yaratılması hedeflenmektedir. Kullanılacak suyun filtrasyonu, hammaddelerin mikrobiyal açıdan test edilmesi, ekipman dezenfeksiyonu gibi İyi İmalat Uygulamaları (Good Manufacturing Process; GMP) şartlarına uyum ile potansiyel kontaminasyon riskleri önemli ölçüde azaltılabilir. Ayrıca üretimde dış fazı yağ olan emülsiyon tipinin tercih edilmesi, ortamın pH kontrolu, formülasyondaki su içeriğinin azaltılması gibi yaklaşımlar da faydalıdır. Mikroorganizmaların büyümek için suya ihtiyaç duymaları nedeniyle, formülasyona katılacak polioller, protein hidrolizatları, amino asitler, tuzlar ve hidrokolloidler yardımıyla ortamdaki serbest suyun azaltılması su aktivitesi olarak tanımlanır ve kozmetiklerde düşük su aktivitesi değeri elde etmek de mikroorganizma büyümesini engellemeye yardımcıdır [28,37]. Hava içermeyen ambalajlar da kendini koruyan kozmetiklerde sıklıkla tercih edilmektedir [37].

Uçucu yağlar gibi çok amaçlı bileşenlerin formülasyona dahil edilmesi kendini koruyan ürünlerin hazırlanmasındaki temel yaklaşımlardan biri diğeridir. Koruyucu etkinliği nedeniyle sık karşılaşılan örnekler arasında *Rosmarinus officinalis, Lavandula officinalis, Pteronia incana, Artemisia afra, Thymus vulgaris, Okaliptus globulus, Laurus nobilis, Salvia officinalis* ve *Melaleuca alternifolia* gibi bitkilerden elde edilen uçucu yağlar ve özleri sayılabilir [28]. Yönetmeliklerde koruyucu olarak izinli olmayan ancak ikincil fonksiyonları koruyucu etki olan gliseril kaprilat, gliseril kaprat, gliseril undesilat, fenil etil alkol, sodyum anisat/sodyum levulinat gibi hammaddeler de doğal kozmetiklerde karşılaşılan çok fonksiyonlu hammaddeler arasında yer almaktadır [21].

Her ne kadar antimikrobiyal aktivitesi olsa da doğal maddeler, büyük ölçekli imalatta gerekli olan geniş spektrumlu koruma için yetersizdir. Bu maddelerin çoğu kozmetik üretimde su kaynaklı kontaminasyonun ana kaynakları olan *Pseudomonas* türleri ve diğer Gram-negatif bakterilere karşı aktif değildir ya da uçucu yağların antimikrobiyal etki için çok yüksek konsantrasyonda kullanılması gerekir. Doğal malzemelerin emülsiyonlarda iyi karışmaması, diğer kozmetik hammaddeler ile oluşturabilecekleri geçimsizlik, tahriş nedeniyle güvenlik riski de sorun oluşturmaktadır. Ayrıca bir kozmetik üründe, doğal koruyucu kullanmak oldukça pahalıdır [21].

Yeşil Kozmetiklerde Ambalajlama

Küresel ısınma sonucu son yıllarda çevre dostu (eco-friendly) veya yeşil ambalajlar önem kazanmış ve tüketiciler tarafından tercih edilebilirliği artmıştır. Bu nedenle sürdürülebilir kozmetik üreticileri de sertifikalarının öngördüğü doğa dostu etiketleri ürünlerinde kullanarak tüketiciler tarafından tercih edilebilirliklerini ve piyasadaki imajlarını güçlendirmektedirler [38].

Çevre dostu ambalajlar, biyoparçalanabilir ambalajlar veya geri dönüştürülebilir, yeniden doldurulabilir ambalajlar (recycle, refillable/reuse) olarak iki grupta incelenebilir. Maliyet ve etkinlik kapsamında biyoparçalanabilir materyaller çok sık tercih edilmektedir [38].

Çevre dostu kozmetik ambalaj ürünlerinde sıklıkla kullanılan malzemeler arasında selüloz asetat, selüloz, seramik, cam, aluminyum, demir, paslanmaz çelik gibi metaller, kağıt/karton, polietilen, polietilen terefitalat, polietilen terefitalat glikol, polilaktik asit, polipropilen, lastik, ahşap, GDO içermeyen % 100 kaynaklı diğer materyaller sayılabilir [16,34]. Son yıllarda piyasada yeniden doldurulabilir ambalaj örneği ve genellikle organik makyaj markalarının ambalaj tercihi olarak bambu kullanmaktadır.

Yeşil ambalajların, çevreye ve küresel ısınmaya etkilerinin daha az olması, uzun vadede geri dönüşüm ile daha az ambalaj üretimi sayesinde maliyetin azalması ve enerji tasarrufu sağlanması, kullanılan klasik materyallere (özellikle plastikler) göre formülasyon ile etkileşimlerinin daha az olması, marka imajı ve tercih edilebilirliğin artması gibi avantajları vardır. Ancak başlangıç materyalinin maliyetli olması, bazılarının ürünü çevre koşullarından, mikrobiyal kontaminasyondan ve mekanik açıdan korumada yetersiz olmaları gibi dezavantajları da mevcuttur [38].

Yeşil Kozmetiklerde Sürdürülebilirlik ve Atık Yönetimi

Sürdürülebilirlik bir ürünün yaşam döngüsünü ifade etmektedir. Her ne kadar kapsam olarak doğal, organik ya da yeşil kelimelerinin ifade ettiği tarımsal kaynakların sorumlu kullanımından ve sentetik hammadde kullanmamaktan daha geniş kapsamlı olsa da çevreye duyarlı, yeşil, organik gibi iddialar ile üretilen kozmetiklerde sürdürülebilirlik kavramı önem taşımaktadır [3]. Sürdürülebilir kozmetikler yeşil kimya prensipleri ile hazırlanmaktadır. Yeşil kimya tanım olarak, tehlikeli maddelerin kullanım ve üretimini azaltmak veya ortadan kaldırmak üzere kimyasal ürünler ve süreçlerin tasarımıdır. Yeşil kimya prensipleri ile kirliliği oluşturan süreç ve maddelere müdahale ederek, kirliliğin oluşumunu en aza indirmek veya kaldırmak, geri dönüşüm ve enerji tasarrufu sağlamak amaçlanır [39,40]. Bu kapsamda kullanılan suyun, ambalaj ve plastik atıkların en aza indirilmesi, atıkların doğada çözünebilir olması beklenmektedir.

Yeşil kozmetik endüstrisinde su kullanımı önem taşıyan bir konudur. Pek çok formülasyonun içeriğinde su bulunmaktadır. ISO 16128-1 yönetmeliklerine ve doğal/organik standartların birçoğuna göre su doğal bir içeriktir ve formulasyonlarda kullanılan saf suyun yanısıra ekstre ya da meyve suyu gibi bitkilerden elde edilen suyu da kapsar. Bunun yanısıra su, organik ürünlerde başlangıç basamağı olan organik tarım ve üretimde, soğutma ve ısıtma gibi işlemlerde kullanılan enerjinin üretilmesinde, ekipman temizliğinde de gereklidir. Bir kozmetik ürünün yaşam döngüsünde ve tedarik zinciri süresince sıfır su kullanması mümkün değildir. Ancak yeşil kozmetik firmalarının amaçları arasında, temiz su kaynaklarını korumak ve su ayakizini azaltmak bulunmaktadır [41]. Örneğin, süperkritik sıvı ekstraksiyonu da su kullanımını azalttığı için sıklıkla tercih edilen bir yöntemdir. Ambalajda plastik kullanımını azaltmak da su tüketimini azaltan bir unsurdur. Örneğin, yüksek dansiteli polietilen gibi plastik ambalaj materyalleri, üretimlerinde büyük miktarda su tüketimi gerektirdiğinden sürdürülebilirlik açısından uygun değildir [41].

AB'nin 94/62/EC numaralı Ambalaj ve Ambalaj Atıkları yönetmeliğine (Packaging and Packaging Waste Directive) göre ambalaj, tüm ürünün güvenliği, hijyeni ve tüketici tarafından kabulü için gereken minimum hacim ve ağırlıkta üretilmelidir; zararlı veya tehlikeli bileşenler minimumda tutulmalıdır ve kullanımdan sonra yeniden kullanılmalı, geri dönüştürülmeli ve/veya geri kazanılmalıdır. Günümüzde pek çok kozmetik firması tüketici kullanımından sonra geri dönüştürülebilen plastik ambalaj tercih etmektedir. En çok kullanılan % 100 geri dönüştürülebilen plastik polietilen terefitalat (PET)'dır. PET kullanımının avantajı, üretimi ve geri dönüşümü için hala su gerektirmesine rağmen, doğal olarak oluşan etilen glikol ve tereftalik asit kullanılarak üretilen ve

biyoparçalanabilir bir reçine olmasıdır. PET ambalaja bir diğer alternatif de, sertifikalı üretilen yenilenebilir polipropilen (PP) ambalaj materyali kullanmaktır. Yenilenebilir PP ambalajın avantajı ise, tüketiciler tarafından fosil yakıtı bazlı plastikten görüntü olarak ayırt edilememesidir [41]. Yeşil ürünlere verilen sertifikalarda da genel olarak ambalajın azaltılması, geri dönüştürülmüş veya yenilenebilir kaynaklardan elde edilen alternatiflerle değiştirilmesi, daha az kaynakla en uygun boyut ve ağırlıkta paketleme tasarlanması önerilmektedir [16,17,41].

Yeşil kozmetiklerin yeşil kimya prensipleriyle uyuştuğu diğer bir konu atıkların doğada bozunabilir olmasıdır. Atık haline gelen kimyasalların ekosistemde yarattıkları sorunların en önemlilerinden biri mikroplastiklerdir [3,23,41,42]. Mikroplastikler, plastik atıkların mikro boyutlu fraksiyonunu oluşturan, 5 mm'den daha küçük plastik malzemeden yapılmış parçacıklardır. Kozmetik endüstrisi, çok çeşitli ürünlerde plastik bileşenler kullanmaktadır (Tablo 5). Söz konusu plastik malzemeler, malzemelere istenen özellikleri ve işlevselliği vermek için katkı maddeleri ile karıştırılmış polimerlerden oluşan, sentetik, doğada parçalanmayan ve suda çözünmeyen katı malzemelerdir. Mikroplastik atıkların çoğu çıplak gözle görülmez, plastik içeren durulama ürünü kozmetikler ve evsel atık içeren sular yoluyla nehirlere ve denizlere taşınır. Bu şekilde çevre kirliliğine yol açan mikroplastikler doğada bozunmadan yüzyıllar boyunca kalabilir [42].

Tablo 5. Kozmetik ürünlerindeki plastik bileşen örnekleri [42].

Polimer Adı	**Kullanım Amacı**
Naylon-12 (Poliamid-12)	Hacim arttırıcı, viskozluk arttırıcı, opaklaştırıcı
Naylon-6	Hacim arttırıcı, viskozluk arttırıcı
Poli (bütilen tereftalat)	Film oluşturucu, viskozluk arttırıcı
Poli (etilen izotereftalat)	Hacim arttırıcı
Poli (etilen tereftalat)	Yapıştırıcı, film oluşumu, saç fiksatörü; viskozluk arttırıcı, estetik ajan
Poli (pentaeritritil tereftalat)	Film oluşumu
Polietilen	Aşındırıcı, film oluşturucu, viskozluk arttırıcı, tozlarda bağlayıcı
Polipropilen	Hacim arttırıcı, viskozluk arttırıcı
Polistiren	Film oluşumu
Politetrafloroetilen	Hacim arttırıcı, kayma düzenleyici, bağlayıcı
Poliüretan	Yüz maskeleri, güneş koruyucu, maskara gibi ürünlerde film oluşturucu
Poliakrilat	Viskozluk arttırıcı
Akrilat kopolimer	Bağlayıcı, saç bakım ürünlerinde sabitleyici, film oluşturucu, süspansiyon ajanı
Alil stearat/vinil asetat kopolimerleri	Film oluşturucu, saç bakım ürünlerinde sabitleyici
Etilen / propilen / stiren kopolimeri	Viskozluk arttırıcı
Etilen / metilakrilat kopolimer	Film oluşturucu
Etilen / akrilat kopolimer	Su geçirmez güneş kremleri, ruj, çubuk ürünler, el kremlerinde film oluşturucu
Butilen / etilen / stiren kopolimeri	Viskozluk arttırıcı
Trimetilsiloksisilikat	Renkli kozmetikler, cilt bakımı, güneş bakımı ürünlerinde film oluşturucu

Yeşil kimya prensipleri ile üretim, yeniden kullanılabilen ya da geri dönüşümlü ambalaj tercihi, ürünlerde plastik içeriğinin azaltılması ya da plastik içermeyen bileşenlerin kullanılması ile yeşil kozmetiklerde mikroplastik emisyonunda azalma sağlanabilmektedir. Günümüzde sıfır atık (zero waste) kavramı da sürdürülebilir kozmetik firmaları için önem taşımaktadır [41-43].

SONUÇ VE TARTIŞMA

Doğal kaynakların gelecek kuşaklara aktarılma arzusu, sentetik hammadde ve ürünlerden kaynaklı sağlık sorunları gibi nedenler ile günümüzde kozmetiklerde de sürdürülebilirlik önem

kazanmıştır. Çevreye duyarlı, sürdürülebilir, doğal veya organik ifadeleri ile yanyana sunulan bu kozmetik ürünlerde doğal ve/veya organik hammadde kullanımı, bu hammaddelerin ve bitmiş ürünlerin standardizasyonu önem taşıyan konuların başında gelmektedir.

Günümüzde hemen her ülkede kendi yönetmelik ve ilgili standardizasyon kuruluşları bulunmakla birlikte, COSMOS ve Ecocert başta olmak olmak üzere NaTrue, ICEA, Soil Association ve BDIH gibi Avrupa standartlarıyla ülkemiz dahil pek çok ülkede karşılaşmak mümkündür. Türkiye açısından bakıldığında ABD'nin USDA/NOP standardı da dahil olmak üzere, ETKO ve IFC Global gibi kuruluşlardan organik ve/veya doğal sertifikasyonu sağlanabilmektedir. Bu standartlar ve TİTCK tarafından yayınlanan Doğal ve Organik Kozmetik Bileşen ve Ürün İddialarına İlişkin Kılavuz ile yeşil kozmetiklerde kullanılmasına izin verilen hammadde ve ambalaj materyalleri, fiziksel ya da kimyasal işlemler, üründeki doğal/organik içerik yüzdesinin hesaplanması gibi bilgilere erişilebilmektedir. Doğal ve organik kozmetik içerikler ve ürünler için teknik tanımlar ve kriterler hakkındaki bir diğer ortak sertifikasyon da ISO 16128 standardıdır.

Kozmetik ürünlerde organik veya doğal tanımlarının karşılıklarının ürün ambalajı üzerinde ilgili kuruluştan alınan sertifikasyon logosu ile verilmesi istenmektedir. Bu, hem tüketicilerin doğru bilgilendirilmesini sağlamak hem de ürünün yeşil kimya prensiplerine uygun, çevreye en az atık bırakacak şekilde üretildiğini göstermek için gerekmektedir.

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Yazar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan eder.

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DERLEME MAKALE / REVIEW ARTICLE

SİNERJİSTİK İLAÇ KOMBİNASYONLARININ ANALİZİNDE GÜNCEL MATEMATİKSEL VE İSTATİSTİKSEL YÖNTEMLER

CURRENT MATHEMATICAL AND STATISTICAL METHODS IN THE ANALYSIS OF SYNERGISTIC DRUG COMBINATIONS

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ÖZ

Amaç: *İlaç kombinasyon tedavisi, kanser gibi çeşitli ölümcül hastalıkların tedavisinde önemli rol oynamaktadır. İlaçlar kombine edildiğinde sinerjistik, additif veya antagonistik etkileşimler meydana gelir. Bu etkileşimlerin tanımlanması ve ilaç kombinasyonlarının kantitatif analizi basit değildir. Terminoloji, deneysel protokoller ve modellerin yanı sıra veri analizinde standardizasyon eksikliği başlıca sorunlardır. Bu çalışmada, sinerjistik ilaç kombinasyonlarının incelenmesi ve analizi ile ilgili mevcut matematiksel ve istatistiksel yöntemler derlenmiştir. Takibinde, yaygın kullanılan yöntemleri anlamak için gerekli olan farmakolojik ve matematiksel kavramlar da derlenmiş, avantaj ve dezavantajları tartışılmıştır. Son olarak ilaç kombinasyonlarının analizinde dikkat edilmesi gereken temel konular açıklanmıştır.*
Sonuç ve Tartışma: *Muhtemel tüm deneysel koşullar için uygun optimum bir model olmadığı için, ilaç kombinasyonlarının kantitatif analizinin, burada tartışılan farklı yaklaşımların kollektif kullanımı ile kolaylaşacağını umuyoruz. Bu çalışmanın ilaç kombinasyonlarının analizi için bir referans teşkil edeceğine inanıyoruz.*
Anahtar Kelimeler: *Bliss bağımsızlığı, chou-talalay, ilaç kombinasyonu, kombinasyon indeksi, loewe sinerjizm*

ABSTRACT

Objective: *Combination drug therapy plays an important role in treating various deadly diseases such as cancer. When drugs are combined, synergistic, additive or antagonistic interactions occur. The determination of these interactions and the quantitative analysis of drug combinations are not simple. The lack of standardization in terminology, experimental protocols, and models as well as data analysis are major challenges. In this study, the current mathematical and statistical methods*

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concerning the study and analysis of synergistic drug combinations are reviewed. Following, the pharmacological and mathematical concepts necessary to understand the widely used methods are also reviewed, and their advantages and disadvantages are discussed. Finally, the main issues to be considered in the analysis of drug combinations are explained.

Result and Discussion: *Because there is no optimal suitable model for all possible experimental conditions, we hope that the quantitative analysis of drug combinations will be facilitated by the collective use of the different approaches discussed here. We believe that this study will serve as a reference for the analysis of drug combinations.*

Keywords: *Bliss independence, chou-talalay, drug combination, combination index, loewe synergism*

GİRİŞ

Hastalıkların tedavisinde ilaçların kombinasyon olarak kullanımı oldukça yaygındır. Tek ajan ile tedaviye (monoterapi) kıyasla yüksek etki, azaltılmış yan etkiler ve ilaç direnci gelişmesinin engellenmesi gibi avantajları sebebiyle kombinasyon tedavi modaliteleri kanser gibi ölümcül hastalıklarda standarttır [1]. Örnek olarak, farklı tür lösemiler için kombinasyon kemoterapisi [2], veya kolorektal kanser için floropirimidinler (örneğin; 5-florourasil) ve platinyum bileşikleri (örneğin; okzaliplatin) ve/veya hedefli ajanlar (Örneğin, Panitumumab veya Bevacizumab) ile kombinasyon tedavi modaliteleri verilebilir [3].

Geçtiğimiz 20 yılda, birçok hastalıkta sorumlu mutasyonlar ve sinyal yolaklarını karakterize eden omik teknolojilerinde önemli gelişmeler meydana gelmiştir [4]. Hastalıkların moleküler özelliklerine göre optimize edilen kişiselleştirilmiş tıp yaklaşımları ortaya çıkmıştır [5]. Omik ve hücre biyolojisindeki önemli ilerlemeler, modern tıpta ilaç kombinasyonlarının artan kullanımına katkıda bulunmuştur [6]. Kombinasyon ilaç tedavisinin üstün etkilerinin incelenmesi güncel araştırma alanıdır [7]. Hastalar-arası ve hastaya-özgü genetik heterojenite, klonal evrim ve direnç mekanizmalarının belirlenmesindeki sorunlar devam etmektedir. Bu problemler, kombinasyon tedavi modaliteleri ile büyük oranda çözülebilmektedir.

İlaç-ilaç etkileşimleri, bir ilaç diğer bir ilacın aktivitesini etkilediğinde meydana gelir. Bu etkileşimler farmakokinetik veya farmakodinamik olabilir [8]. Farmakodinamik ilaç etkileşimleri sinerjistik, additif veya antagonistik olarak sınıflandırılır. Sinerjizm, iki ilacın kombinasyon etkisinin aynı koşullardaki bireysel etkilerinin toplamından daha fazla olmasıdır [9]. İlaçların kombinasyon etkisi, aynı deneysel ortamdaki ilaçların bireysel etkilerinden daha küçük olduğunda gerçekleşen etkileşim türü antagonizmdir.

Terminoloji, farmakolojik sinerjizmin tanımlanması, ilaç kombinasyonlarının belirlenmesi için referans metodoloji ve analizde standardizasyon eksikliği bulunmaktadır [10]. Bu kavramların, additif etkinin tanımıyla başlayan ve basit olmayan çözümlerin önerildiği geçerli bir metodolojiye uygulanması karmaşıktır. Etkinliği belirlenmiş ilaç kombinasyonlarının klinik araştırmalara uygulanabilmesi için bilimsel ve teknik problemlerin çözülmesi gerekir [11,12]. Bu bilgiler doğrultusunda, bu çalışmada sinerjistik ilaç kombinasyonlarının analizi ve sinerjizmin belirlenmesi için matematiksel ve istatistiksel yöntemler derlenmiştir. İlk aşamada, kombinasyon etkisinin ölçüsü olarak bilinen ve bu etkinin beklenen additif etkiden daha büyük, daha küçük veya eşit olduğu bir etkiyi gösteren kombinasyon indeksi (CI) tanımlanmıştır. İkinci basamakta, ilaç kombinasyonlarının analizinde kullanılan terminoloji ayrıntılı olarak açıklanmıştır. Üçüncü aşamada ise, ilaç kombinasyonlarının analizinde etkileşimsizlik hipotezinin uygulaması için en yüksek tek ajan (HSA) yaklaşımı, Chou-Talalay metodu, Bliss istatistiksel bağımsızlık modeli ve Loewe additif etki yaklaşımı dahil olmak üzere yaygın olarak kullanılan geleneksel modeller sunulmuştur. Bu yöntemlerin avantaj ve dezavantajları tartışılmış, kullanılamayacağı durumları da belirtilmiştir. Takibinde, güncel kullanılan yöntemler açıklanmıştır. Son olarak, ilaç kombinasyonlarının analizinde dikkat edilmesi gereken hususlar sıralanmıştır.

Terminoloji ve İlaç Kombinasyonlarının Analizi

İlaç kombinasyonlarındaki etkileşimleri tanımlamak ve sinerjizmi tespit etmek amacıyla matematiksel ve/veya istatistiksel yöntemler geliştirilmiştir. Ancak, analiz metodlarında kullanılan

terminoloji maalesef her zaman uyumlu değildir. Bu tutarsızlık, farmakoloji başta olmak üzere çeşitli araştırma alanlarında hala gözlenmektedir ve ilaç kombinasyonlarının analizinde hatalara neden olmaktadır [13]. Bu bölümde, ilaç kombinasyonlarındaki sinerjistik etkileşimlerin tanımlanması ve nicelleştirilmesindeki muhtemel tutarsızlıkları ve yanlış anlaşılmaları önlemek için, terminoloji kapsamlı şekilde açıklanmıştır.

İlaç kombinasyonları, kombinasyon etkisinin sıfır hipotezinden (etkileşimsizlik etkisi) sapmasına bağlı olarak sinerjistik, additif veya antagonistik olarak tanımlanır [12]. Sinerjizmin kantitatif tek ölçüsü CI değeridir. CI<1, =1, >1, sırasıyla sinerjizm, additif etki ve antagonizmi ifade eder. Sinerjizm additif etkiden daha büyük, antagonizm ise additif etkiden daha küçük olan etkidir [11]. Additif etkiden önemli sapma, sapmanın yönüne bağlı olarak sinerjizm veya antagonizm olarak tanımlanır. Sinerjizm ile ilgili olarak tanımlanması elzem ilk kavram, additif etkidir. Additif etki, etkileşimsizlik ve intertizm olarak da adlandırılır ve sinerjizmin belirlenmesinde temel kabul edilir. Bir ilacın etkisi, diğer bir ilacın etkisini maskelemediği veya artırmadığı durumda kombinasyon additiftir. Bu durum etkileşimsizlik olarak da bilinir ve birden fazla ilacın kombinasyonu sinerjizme yol açmadığında beklenen veya ölçülen etkiyi belirtir. Sinerjizmin belirlenmesi için additif etki kavramının doğru şekilde anlaşılması ve hesaplanması gereklidir. Additif etkinin matematiksel olarak tanımlanması önemli bir problemdir. Additif etkinin tanımıyla eş anlamlı olan iki terim, Loewe additif etkisi ve Bliss bağımsızlığıdır. Loewe additif etkisi, doz additivitesi ve konsantrasyon ilavesi olarak da isimlendirilir. Bliss bağımsızlığı ise, Bliss additivitesi, yanıt-çoğaltımı, yanıt-ilavesi, etki-ilavesi ve bağımsız-hareket olarak adlandırılır [13].

Sinerjizm, iki ilacın kombinasyon etkisinin aynı deney koşullarındaki bireysel etkilerinin toplamından daha fazla olmasıdır. Sinerjistik kombinasyon tedavi rejimleri, ilaç dozlarının azaltılması ve terapötik etki korunurken doz ile ilişkili yan etkilerin azaltmasına olanak sağlayabilir [11]. İlaç kombinasyonlarında sinerjizm, tamamlayıcı ilaç etkileri (aynı protein veya yolak üzerindeki farklı bölgelerin hedeflenmesi), anti-karşıt etkiler (bir ilaç diğer bir ilacın farmakolojik yanıtını etkilediği durum) veya fasilitasyon etkilerin (bir ilaç diğerinin etkisini artırdığı durum) sonucu olarak ortaya çıkar [12]. Sinerjizm, süper-additif etki, potansiyalizasyon, güçlendirme, supra-additif etki olarak da isimlendirilir. Koalizm terimi, kombinasyondaki ilaçların hiçbiri tek başına etkili olmadığında sinerjizme atıfta bulunmak için kullanılır.

İlaçların doz-etki eğrilerinin belirlenmesi ve ilaç kombinasyonlarındaki sinerjistik etkileşimlerin düzeyini ölçmek için yaygın kullanılan geleneksel yöntemler, HSA modeli, Loewe additif etki yaklaşımı, Bliss istatistiksel bağımsızlık modeli ve Chou-Talalay (kütle-eylem yasası temelli medyan-etki denklemi) yöntemidir [14] (Tablo 1). Bu bölümde, ilaç kombinasyonlarının analizi için yaygın kullanılan geleneksel yöntemlerin prensipleri, avantaj ve dezavantajları açıklanmıştır. Takibinde de güncel orjinal çalışmalara değinilmiştir.

Tablo 1. İlaç kombinasyonlarının analizi için yaygın kullanılan geleneksel yöntemler

Yöntem	Temeli	Sınırlama
Kombinasyon Alt Eşikleme Yaklaşımı	Etkisiz ilaç dozlarının kombinasyonunun önemli bir etki oluşturduğu prensibine dayanır.	Etkililik genellikle, istatistiksel analiz sonucu elde edilen *p*-değeri ile verilir. Bir eşiğe bağlı olarak hesaplanan etkililiğin, ilaç kombinasyon etkisi ile bireysel bileşenlerin etkileri arasında önemli bir fark olduğu anlamına gelmez.
HSA Yaklaşımı	Kombinasyon etkisi, bireysel olarak HSA'nın etkisi ile karşılaştırılır.	Yöntem, bireysel bileşenlerden beklenen additif etkisiye kıyasla üstün bir kombinasyon etkisini göstermekte başarısızdır. En az bir ilacın bireysel olarak etkisiz olduğu durumlarda kısıtlı veri sunar.
Yanıt-katkı Yaklaşımı	Kombinasyon etkisini, ilaçların bireysel etkileri yerine beklenen additif etki ile karşılaştırır. Yöntem, ilaçların sıfır kesişimli doğrusal doz-etki eğrilerine sahip olduğunu varsayar.	Yöntem, doğrusal olmayan doz-etki eğrilerine sahip kombinasyonların analizinde kullanılamaz.

Tablo 1 (*devamı*). İlaç kombinasyonlarının analizi için yaygın kullanılan geleneksel yöntemler

Yöntem	Temeli	Sınırlama
Bliss İstatiksel Bağımsızlık Modeli	İlaç etkilerinin olasılıksal süreçlerin sonuçları olduğu ve ilaçların birbirini etkilemediği (farklı etki mekanizmaları), ancak her birinin ortak bir sonuca katkıda bulunduğu varsayımına dayanır.	Model, yalnızca 0 ve 1 arasında değişen olasılık olarak ifade edilen etkiler için geçerlidir.
Klasik İzobologram	İzobologram çizgileri, aynı etkiye (izo etkisi) sahip dozları veya dozların kombinasyonlarını birleştirir.	Yöntem, çok sayıda deneysel veri gerektirir. Ayrıca, bilgisayar yazılımı ve istatistiksel yaklaşım yetersizdir ve sadece ikili-ilaç kombinasyonu değerlendirilebilir. Birbirini-dışlamayan iki ilaç durumunda yöntem uygulanamaz.
Modifiye İzobologram	Yöntemin temeli additif etkidir. Sitotoksik ajanların önemli ölçüde etkileşime girmediği güven aralığı ile sınırlandırılmış bölgede analiz yapılır.	
Medyan-Etki Modeli (Chou-Talalay metodu)	Enzim kinetiği sistemleri: Kütle eylem yasası, Michaelis-Menten ve Hill denklemleri.	Doğrusal regresyon analizinin uygulama zorluğu nedeniyle, doz-cevap eğrileri sigmoidal olmadığında yöntem uygulanmamalıdır.
3-boyutlu Analiz	Michaelis-Menten denklemleri ve Medyan-Etki prensibi.	Model, her farklı yanıt-yüzey tipi için birkaç matematiksel fonksiyon ve yazılım gerektirir. Karmaşık uygulama, preklinik çalışmalarda yaygın kullanımı önler.
Oransal-verim metodu	İki ilacın etkisinin toplamı, inhibisyon etkilerinin sonucu ile ifade edilir.	Yöntem, doz-cevap eğrilerinin (m> 1 veya m <1) muhtemel sigmoidalitesini dikkate almaz ve karşılıklı olarak birbirini dışlayan iki ayrı ilaç veya ikinci-derece karşılıklı olarak birbirini tamamen-dışlamayan iki ilaç için geçerli değildir.

Kombinasyon alt-eşik yaklaşımı, ilaçların etkili olmayan dozlarının kombinasyonunun önemli etkisinin olduğu düşüncesine dayanır. Etkinlik, ilaç kombinasyon grubunun etkisinin kontrol grubuna karşı istatistiksel analiz ile kıyaslanarak *p*-değerine bağlı gösterilir (istatistiksel anlamlılık, $p<0.05$). Ancak, belirli eşik değer referans alınarak gösterilen etkililik, kombinasyon etkisi ile ilaçların bireysel etkileri arasında önemli bir fark olduğu anlamına gelmez [15]. Kombinasyon etkisinin çok az anlamlı olduğu (örneğin, $p=0.049$) ve ilaçların bireysel etkileri anlamlı olmadığında (Örneğin, $p=0.051$), "anlamlı" ve "anlamlı olmayan" arasındaki fark her zaman anlamlı değildir ve tek ajanın etkisine kıyasla pozitif bir ilaç kombinasyon etkisini göstermez [10]. Dolayısıyla bu yaklaşım ilaç kombinasyonlarının analizinde tercih edilmemelidir.

HSA modeli [16], bir ilaç kombinasyon etkisinin (E_{AB}), bireysel bileşenlerin etkilerinden (E_A ve E_B) daha büyük olduğu fikrine dayanır [17]. HSA modeli, ilaç kombinasyon etkisi bileşenlerin etkisinden büyük olduğunda bir miktar etkileşimin gerçekleşmiş olması gerektiği sezgisidir. Modelde, ilaçların herhangi bir konsantrasyonunda (X,Y), inhibisyon etkisi IHSA=maks (IX,IY), hesaplanır. IX ve IY, aynı deney koşullarında ilaçların tek ajan olarak etkileridir (sırasıyla (X, 0) ve (0, Y)). Model değerleri, IX ve IY'nin tek ajanın etki verilerine sigmoid uyumu kullanılarak belirlendiği her bir doz matriks noktasında hesaplanır. HSA modeli ile CI değeri aşağıdaki formülle belirlenir. Denklemde E_A, A ilacının, E_B ise B ilacının tek ajan olarak etkisini, E_{AB} ise, A ve B kombinasyonunun etkisini gösterir [10].

$$\mathrm{CI} = \frac{\mathrm{maksimum}(E_A; E_B)}{E_{AB}}$$

HSA, hedefleri ortak olmayan ajanların kombinasyonlarından pozitif bir etki beklentisidir. Model basit olduğundan dolayı, ilaç kombinasyonlarının analizinde yaygın olarak kullanılır. HSA metodu ile elde edilen sonuç, hedefleri ortak olmayan ilaçların kombinasyonlarının ürünüdür [16]. Ancak, referansın üzerindeki kombinasyon yanıtları, etki artışlarından ve/veya potensideki sapmalardan kaynaklanabilir. Pozitif kombinasyon etkisinin önemi, kombinasyonun etkisini HSA ile karşılaştıran

istatistiksel analizin *p* değeri ile verilir. HSA modeli, kombinasyon alt eşik yaklaşımından daha gelişmiş bir yöntemdir ve anlamın farklılığından ziyade farklılıkların anlamını gösterir [10]. HSA modeli, işlenmemiş veriler, inhibisyon ve kat artışları dahil tüm etki ölçümleri için kullanılabilir. HSA hesaplaması, ikili-ilaç kombinasyonu için her iki ajanın da tüm konsantrasyonlarda pozitif etkileri olduğunda (gürültü hariç) anlamlıdır. Bu basit model, aynı deney koşullarında kombinasyondaki bileşenlerin bireysel etkilerine kıyasla, kombinasyon etkisinin üstünlüğüne dair kanıt sağlar. Fakat model, kombinasyonda yer alan bir ilacın herhangi bir konsantrasyonda etkisiz olduğu durumlarda kullanılabilir [14]. Hatta, bu modele göre preklinik çalışmalarda bir ilaç kendisi ile kombine edildiğinde bile HSA'dan fazla etki gösterebilir ve dolayısıyla bu durum anlamsızdır.

Yanıt katkı yaklaşımı (lineer etkileşim etkisi [18], kombinasyon etkisi, ajanların bireysel etkilerinin toplamı ile verilen additif etkiden daha büyük olduğunda pozitif kombinasyon etkisinin meydana geldiğini ifade eder [10]. Bu yöntemde CI değeri aşağıdaki formül ile hesaplanır.

$$CI = \frac{E_A + E_B}{E_{AB}}$$

p değeri, ajanların bireysel ve kombinasyon etkilerinin varyans analizindeki etkileşim etkisinin önemidir. Yanıt-katkı yaklaşımı, kombinasyon etkisini ajanların bireysel etkisi yerine beklenen additif etki ile karşılaştırır. Sinerjizmi değerlendirmek için HSA modelinin gelişmiş formudur. İlaçların sıfır-kesişimli lineer doz-etki eğrilerine sahip olduğunu varsayar. Ancak doz-etki eğrileri genellikle lojistik veya eğriseldir [19]. Yanıt katkısı, yukarı-eğimli kısımda sinerjizmi ve aşağı-eğimli kısımda antagonizmi gösterir ve kombinasyon etkisinin geçersiz çıkarımıyla sonuçlanır [10]. Bir başka ifade ile, bu çıkarım sinerjistik kombinasyon etkisinin, ajanların bireysel etkilerinden daha az olduğu anlamına gelir ki bunun da bilimsel bir temeli bulunmamaktadır.

Bliss istatistiksel bağımsızlık modeli, ilaç etkilerinin olasılıksal süreçlerin sonucu olduğu ilkesine dayanır ve ilaçların, birbirini etkilemediği (farklı etki mekanizmaları) ancak her birinin ortak bir sonuca katkıda bulunacak şekilde bağımsız etki gösterdiğini varsayar. Bliss modeli, bir yüzeyin Bliss bağımsızlığından sapmasını ölçer [13]. Yöntem, ilaç etkilerinin olasılıksal olarak bağımsız olaylar olduğunu varsayar. Olasılık olarak ifade edilen gözlemlenen kombinasyon etkisi ($0 \leq E_{AB} \leq 1$), olasılık bağımsızlık $E_A + E_B (1 - E_A) = E_A + E_B - E_A E_B$ için beklenen additif etki ile karşılaştırılır ($0 \leq E_A \leq 1$ ve $0 \leq E_B \leq 1$). Bliss modeli ile CI değeri aşağıdaki formül ile hesaplanmaktadır [10].

$$CI = \frac{E_A + E_B - E_{AB}}{E_{AB}}$$

Loewe additif etki yaklaşımına alternatif olarak kullanılan yöntem Bliss modelidir. Ancak, Bliss modeli Hill eğimine bağlı Loewe additif etkisini gösteremez [13]. Model, bir kombinasyondaki her ilacın birbirinden bağımsız etki mekanizmalarının olduğunu varsayar ve etkileşimsizlik fikrine dayanır [10]. Bliss modeli ile hesaplanan kombinasyon etkisi, beklenen additif etkiden daha büyük olduğunda etkileşim türü sinerjizmdir. Bliss modeli çoklu-ilaç kombinasyonlarına uygulanabilir [20]. Yakın zamanda yapılan bir çalışmada, ikili, üçlü, dörtlü ve beşli-ilaç kombinasyonlarının analizinde Bliss istatistiksel bağımsızlık modeli kullanılmıştır [21]. Şunu belirtmekte fayda vardır ki, en az üç ilaç kombinasyon olarak uygulandığında, formül karmaşık hale gelebilir. İlaç kombinasyonlarının analizi için yaygın kullanılmasına rağmen, Bliss modelinin bazı sınırlamaları mevcuttur. Bliss modelinin istatistiksel kesinlikten yoksun olduğu ve bazı durumlarda yanlış-pozitif sonuçlara yol açtığı bilinmektedir [22]. Yöntem, ikili ilaç kombinasyonundaki moleküllerin birbirinden bağımsız etki mekanizmalarına sahip olduğunu varsayar [10]. İlaç kombinasyonlarındaki farmakolojik etkileşimlerin büyük bir kısmı için bu durumun geçerli olmayabileceği gösterilmiştir [23,24]. Bliss modelinin geçerli olması için, doz-etki eğrisinin tüm doz ve/veya etki seviyelerine uygulanabilir olması gerekir. Bu durum birçok koşulda doğru olmayabilir [24]. Bliss metodu, Loewe additif etki modelinin temeli olan ‘’sham karışım’’ yaklaşımına uygulandığında hatalı analiz sonuçlarının elde edilmesi muhtemeldir. Sham karışım yaklaşımının sezgisel olduğu belirtilmiştir [13]. Farmakolojik sinerjizm, karmaşık ve

bilinmeyen birden çok etki mekanizmalarına sahip ilaçları kapsar. Dolayısıyla, metodolojiler ilaçların etki mekanizmalarına bağlı olmamalıdır. Bliss modeli, ilaçların üstel doz-etki eğrilerine sahip olduğunu varsayar. Yöntem, 0 ve 1 aralığında değişen olasılık olarak ifade edilen etkiler için geçerlidir [10]. Bliss modeli, sigmoidal doz-etki eğrileri için uygun değildir ve sabit oranlı bir kombinasyonun tek başına her iki ilaçtan daha az etkili olduğu ve dolayısıyla sinerjizm olarak kabul edilebileceği gibi mantık dışı sonuçlar üretebilir.

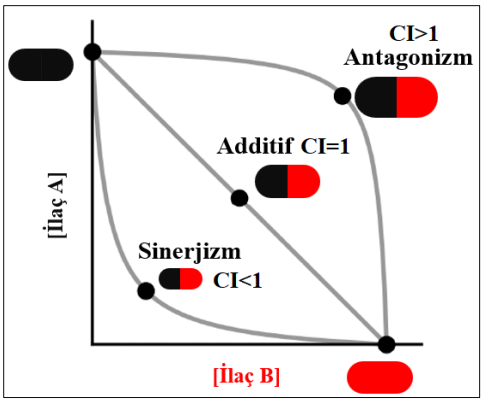
Doz-etki temelli analiz yöntemlerine göre non-lineer doz-etki eğrilerine sahip farklı ajanların etkilerini karşılaştırmanın yolu, her bir ilacın hangi konsantrasyonunun aynı etkiyi ürettiğini belirlemektir. Doz-etki temelli yaklaşımlar, ilk olarak Frei tarafından bildirilmiştir [25]. Loewe tarafından resmi olarak tanımlanmıştır ve Loewe additif etkisi olarak bilinen matematiksel modele dayanır. Loewe additif etkisi, doz denklik ilkesi ve sham kombinasyon ilkesini temel alır [14]. Örneğin; A ve B ilaçlarının additif etkisi, bireysel doz-etki eğrilerine bağlıdır. Loewe, ilaçların sabit bir etki (potensi) oranına sahip olduğunu varsayar (R=A/B). Sabit potensi oranına sahip doz-etki eğrileri, her etki düzeyinde sabit doz oranına sahiptir ve log-doz ölçeğinde paraleldir ve eşit bireysel ilaç maksimum etkilerine sahiptir [26]. E_{AB} kombinasyon etkisine sahip tüm doz çiftleri (a, b) ve bu etkiye ulaşmak için gerekli olan A ve B dozları arasındaki ilişki aşağıdaki eşitlikler ile tanımlanır.

$$a + a_b = A \quad ; \quad a + b \times R = A \quad ; \; a + b \times \frac{A}{B} = A$$

Yukarıdaki eşitlikler, Loewe additif etki modelinin matematiksel olarak ifade edilmesini ve CI parametresinin değerlendirilmesine olanak sağlar [27,28].

$$\frac{a}{A} + \frac{b}{B} = 1 \quad ; \quad CI = \frac{a}{A} + \frac{b}{B}$$

CI<1 değeri, a ve b dozlarının kombinasyon etkisinin additif etkiden daha düşük olduğunu ve sinerjizmi gösterir. Loewe additif etki modelinin bir diğer avantajı, cebirsel analizi izobologram olarak bilinen sezgisel ve yaygın kabul gören grafiksel yöntem ile tanımlanmasına olanak sağlamasıdır [29]. A ve B ilaçlarının sırasıyla a ve b dozlarının kombinasyon etkisi (E), a/A + b/B = 1 denklemi, E_{AB} kombinasyon etkisine yol açan tüm A ve B ilaç doz çiftlerini tanımlar. x- ve y- eksenlerinin A ve B ilaçlarının dozunu gösterdiği bir grafikte negatif eğimin additif etki eğrisi olarak çizilebilir. Bu gösterim, A ilacı A dozunda uygulandığında, belirtilen seviyeye ulaşmak için gereken B ilacı miktarının sıfır olduğunu ve B ilacının varlığında ise, model tarafından tahmin edilen miktarda gereken A dozuna ihtiyacın azaldığını ifade eder. Additif etki çizgisine göre bir kombinasyon etkisi (E_{AB}) için ihtiyaç duyulan dozlara karşılık gelen deneysel veri (a, b) sinerjizm, additif etki ve antagonizm açısından değerlendirilebilir [30]. Çizginin altındaki deneysel veri, CI<1 ve sinerjizmi belirtir. Çizgi üzerindeki bir nokta CI=1'e ve çizginin üstündeki nokta ise CI>1'e karşılık gelir ve sırasıyla additif etki ve antagonizmi gösterir (Şekil 1).



Şekil 1. İzobologram. CI: Kombinasyon indeksi.

Loewe additif etki temelli ilaç kombinasyon analizinin bazı sınırlamaları bulunmaktadır. Kombinasyon etkisini (E_{AB}) üreten dozların (A ve B) hesaplanması için belirlenen doz-etki eğrilerine dayanır. Doz-etki ilişkisi, aşağıdaki formül tarafından tanımlanan Hill denklemini (sigmoid veya lojistik fonksiyon) temel alır [10].

$$E = E_{maksimum} \times \frac{C^n}{EC_{50}^n + C^n}$$

Formülde E, c konsantrasyonunun etkisini, E_{max} maksimum etkiyi, EC_{50} maksimum etkinin yarısına denk gelen ilaç dozunu gösterir ve eğrinin bükülme noktasıdır. n, eğrinin şekil parametresidir. Kombinasyondaki ilaçlar için doz-etki eğrilerinin tahmini, belirli miktarda veri gerektirir. Bu durum, deneysel ve hesaplama açısından zor olmanın yanı sıra pahalıdır ve ilaç kombinasyonlarının analizini engelleyici hale getirebilir [16]. Loewe additif etki modeli, ilaç doz-etki eğrisi mevcut olmadığı veya modellemesinin zor olduğu durumlarda kullanışsızdır [22]. Sınırlı durumlarda additif izoboller düz çizgidir. Potensi oranı (R), genellikle sabit değildir. Dolayısıyla, bireysel log-doz-etki eğrileri paralel olmadığında ve/veya ilaç maksimum etkileri farklılık gösterdiğinde ve additif izobollere yol açtığında geçerlidir [30].

İlaç kombinasyonlarının kantitatif analizi için kullanılan en yaygın yöntem, Dr. Chou ve Dr. Talalay tarafından geliştirilmiş olan medyan-etki denklemidir [11]. Chou-Talalay, ilaç kombinasyon etkilerinin kütle-eylem ilkesi temelinde analizini sağlayan medyan-etki yaklaşımını önermiştir. Medyan-etki denklemi, kütle-eylem yasası ilkesinden yararlanılarak çeşitli reaksiyon mekanizmaları ve farklı inhibisyon türleri için Michaelis-Menten, Hill, Henderson-Hasselbalch ve Scatchard denklemlerinden matematiksel tüme-varım ve tümden-gelim ile türetilmiştir. Chou-Talalay yöntemi, kütle-eylem yasasının medyan-etki prensibinden türetilen ilaç kombinasyon etkisi denklemine dayanır. İlaç etkileşimlerinin sayısal analizi ve kombinasyonların simülasyonu için CompuSyn bilgisayar yazılım algoritması sağlar. Yöntem, her bir ilacın ve kombinasyonlarının doz-etki eğrisinin potensini ve şeklini temel alır [31]. Denklem, CI değerinin hesaplanması ve ilaç kombinasyonlarının analizinde sıklıkla tercih edilmektedir ve çok sayıda atıf almıştır. Medyan-etki denklemi, aşağıda verildiği gibidir.

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m$$

Medyan-etki eşitliği, ilacın dozunu ve etkisini ilişkilendirir. Denklemde D; ilacın dozu, f_a; ilaç dozunun etkisi ve f_u; ise ilaç dozundan etkilenmeyen kısımdır ($f_u = 1-f_a$). D_m, % 50 inhibisyona yol açan medyan-etki (IC_{50}, ED_{50} veya LD_{50}) dozu ve m, doz-etki eğrisini gösteren kinetik katsayıdır. m=1, >1 ve <1 sırasıyla hiperbolik, sigmoidal ve düz-sigmoidal doz-etki eğrisini ifade eder [32]. Medyan-etki denklemi, mekanizma-temelli (örneğin; Michaelis-Menten) ve etki-temelli denklemlerden türetilir ve ilaç etkilerinin boyutsuz olarak ölçülmesine olanak sağlar. Denklem, etkilerin oranı (E_d, ($1-E_d$) veya f_a ve f_{ua}) ve dozların oranından (gerçek doz d, medyan doz M) oluşur. Doz ve etkinin tanımlanmış parametreler ile değiştirilebileceği rapor edilmiştir [11]. Medyan-etki denklemi, analiz yoluyla türetilmiş olmasına rağmen mekanizmaya-özgü sabitler yerine doz ve etkiyi boyutsuz oranlarda ilişkilendirir. Medyan-etki denkleminde m eğim değeri, D_m ise x eksenini kesen noktanın anti-logaritmasıdır. m ve D_m değerleri biliniyorsa, herhangi bir etki (f_a) seviyesi için doz (D) veya herhangi bir doza karşılık gelen etki ve doz-etki eğrisi belirlenebilir [32]. Medyan-etki denklemi, eşitliğin her iki tarafının logaritması alınarak kolaylıkla doğrusallaştırılabilir. log (f_a/f_{ua}) ile log(d) arasındaki düz çizgi, Hill grafiğini verir [31].

Medyan-etki denklemi yeniden düzenlenerek ve/veya logaritması alınarak biyomedikal bilimlerdeki dört temel yaygın denklem Michaelis-Menten (birinci dereceden enzim kinetiği, m=1), Hill eşitliği (oksijen-hemoglobin etkileşimi (m=n)) gibi yüksek miktarda biyolojik reseptörlerde birincil ligand etkileşimi), Scatchard denklemi (ligand reseptör bağlanması ve ayrışması) ve Henderson-Hasselbalch (pH-iyonizasyonu) denklemi elde edilebilir [32]. Medyan-etki dozu (D_m), Michaelis-Menten denklemindeki K_m değerine (yarı-doygunluk), Hill denklemindeki K değerine (yarı-etkileşim),

Henderson-Hasselbach eşitliğindeki pK_a değerine (yarı-iyonizasyon) ve Scatchard denklemindeki K_d değerine (yarı-bağlanma ve -ayrışma) karşılık gelir [11].

Tek ilaç için geliştirilmiş olan medyan-etki denkleminin, çoklu ilaç kombinasyonlarına genişletilmesi mümkündür. n sayıda bileşenden oluşan ilaç kombinasyonları, medyan-etki prensibi ile analiz edilebilir. İkili-ilaç kombinasyonlarının analizi ve sinerjizmin sayısal tayini için CI terimi ve eşitliği tanımlanmıştır.

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_1}{(D_m)_1 \times \left[\frac{f_a}{1-f_a}\right]^{\frac{1}{m_1}}} + \frac{(D)_2}{(D_m)_2 \times \left[\frac{f_a}{1-f_a}\right]^{\frac{1}{m_2}}}$$

CI denkleminde $(D_x)_1$ ve $(D_x)_2$, % x inhibisyon için gereken D_1 ve D_2 dozlarıdır. $(D_x)_1$ ve $(D_x)_2$ değerleri, doz-etki denkleminden hesaplanabilir. Paydadaki dozların toplamı, $(D)_1+(D)_2$, sistemi % x oranında inhibe eder. Yukarıdaki eşitlikte CI değerinin <1, =1 ve >1, olması sırasıyla sinerjizm, additif etki ve antagonizmi gösterir. Medyan-etki prensibi sayesinde CI teoremi ve izobologram geliştirilmiştir [11,31]. F_a-CI grafiğindeki, x-ekseni etki seviyelerini (f_a) y-ekseni ise CI değerlerini belirtir. F_a-log(CI) grafiği, F_a-CI eğrisindeki büyük ölçekli noktaları küçülterek additif eksen (CI=1) ile simetrik hale getirir. Medyan-etki eşitliği, ilaç kombinasyonlarının kantitatif olarak tespit edilmesini sağlayan CI-izobologram denkleminin temelidir. İzobologram eğrisi, ilaçların dozlarını temel alır. CI grafiği ise etki-temelli bir grafiktir. Her iki yaklaşımın prensibi, CI teoremine dayanır. İzobologram iki boyutludur ve ikili-ilaç kombinasyonları için uygundur. İzobologram grafiği, ilaçların kombinasyon etkisi olarak çizilir. Her ilacın bireysel etkileri, eksenlerden belirlenebilir. İzobologram, IC_{50} (IC_{75} veya IC_{90}) ve bu etkiyi elde etmek için ilacın hangi konsantrasyonunun gerekli olduğunu gösterir.

Sinerjistik etkileşimin derecesi, ilaç kombinasyonunun farklı doz ve/veya etki seviyelerinde farklı olabilir. Chou tarafından, sinerjizmin dereceleri tanımlanmıştır (Tablo 2). Sinerjizm için CI değeri, 0 ile 1 aralığındadır. Log(CI), CI grafiğinin simetrik olarak gösterilmesine imkan sağlar (F_a-log(CI) grafiğinde, sinerjizm için CI = 0.1 ve 0.01, log(CI) = -1 ve -2'yi verir).

Dr. Chou, medyan-etki prensibi ile ilaç kombinasyon çalışmalarının tasarımı için moleküllerin ve/veya kemoterapötiklerin IC_{50} doz katlarının temel alınmasını ve ilaçların IC_{50}/4-4IC_{50} konsantrasyon aralığında sabit-oranlı dozların kombinasyon etkilerinin incelenmesini önermektedir [11]. Yakın zamanda yapılan bir çalışmada, polifenol-kemoterapötik ajan ikili ilaç kombinasyonlarının etkilerinin incelenmesinde IC_{50}/16-4IC_{50} doz aralığı kullanılmış ve kombinasyonlar analiz edilmiştir [21]. Elde edilen *in vitro* deneysel bulgulardan, Chou-Talalay metodunun ikili-ilaç kombinasyonlarındaki sinerjistik ve kuvvetli sinerjistik etkileşimleri başarılı bir şekilde gösterdiği anlaşılmaktadır.

Tablo 2. CI metodu ile sinerjizm ve antagonizmanın dereceleri [11]

CI aralığı	Log10 (CI) aralığı	Sınıflandırma
< 0.1	< -1	Çok güçlü sinerjizm
0.1 - 0.3	(- 1) - (- 0.52)	Güçlü sinerjizm
0.3 - 0.7	(- 0.52) - (- 0.15)	Sinerjizm
0.7 - 0.85	(- 0.15) - (- 0.07)	Orta sinerjizm
0.85 - 0.9	(- 0.07) - (- 0.05)	Az sinerjizm
0.9 - 1.1	(- 0.05) - (0.04)	Additif etki
1.1 - 1.2	0.04 - 0.08	Az antagonizm
1.2 - 1.45	0.08 - 0.16	Orta antagonizm
1.45 - 3.3	0.16 - 0.52	Antagonizm
3.3 - 10	0.52 - 1	Güçlü antagonizm
>10	>1	Çok güçlü antagonizm

Chou ve ekibi, ilaç etkileşim parametrelerinin belirlenmesini sağlayan CompuSyn yazılımını geliştirmiştir. Yazılım, preklinik çalışmalar için doz-etki eğrisini, medyan-etki grafiğini, CI grafiğini, izobolegramı ve poligonogramı gösterir [31]. CompuSyn güncel kullanılan yazılımlardan birisidir, basit ara-yüze sahiptir ve kullanımı kolaydır. Chou-Talalay metodu çok sayıda atıf almıştır ve yaygın olarak kullanılmaktadır [33,34]. Ancak, bazı sınırlamaları mevcuttur [9,12]. Yöntem, ilaçların sabit potensi oranına sahip olduğunu varsaymaktadır. İlaçların doz-etki eğrileri non-lineer olduğu için, medyan-etki dozunun ve doz-etki eğrisinin sigmoiditesinin doğru bir şekilde hesaplanması zordur. Lineer regresyon analizinin zorluğundan dolayı, doz-etki eğrileri sigmoidal olmadığında yöntem uygulanmamalıdır. Medyan etki yöntemi, Scatchard analizi gibi idealleştirilmiş kütle-eylem ilkesini temel alır varsayar ve log-linearizasyona dayanır.

Farmakolojide Scatchard analizi yerine, non-lineer regresyon kullanılmaktadır. Log-linearizasyondaki deneysel hatalardan dolayı zayıf model uyumunun olduğu ve medyan etki analizinde de benzer bir sorun yaşanabileceği öne sürülmüştür. Hatta, kombinasyon analizine uygun olmayan modelin, deneyde kullanılan ilaçlara verilen yanıtların analizinde hatalara neden olabileceği ve dolayısıyla kombinasyonunun yanlış yorumlanmasına yol açacağı bildirilmiştir [35].

Bahsedilen geleneksel metotlara ek olarak, bazı çalışmalarda ilaç etkileşim eşitliği (CDI) olarak adlandırılan basit denklem kullanılarak ilaç kombinasyonlarının monoterapiye kıyasla üstün etkisi tahmin edilmiştir [36]. Bu yöntem, ilaç kombinasyon etkisinin bireysel ilaç etkisine göre basit bir kıyaslamasıdır. Ancak bu metot oldukça ilkel bir kıyaslama yaklaşımıdır ve literatürde yaygın olarak tercih edilmemektedir. Bu denklemle hesaplama yapılırken ilaç doz-cevap eğrilerinin temel alınması ve analizi ile ilgili bir çalışma bulunmamaktadır. Hatta 0.7'den küçük olan her CDI değeri için sinerjizm değerlendirmesi yapmaktadır ve ''kuvvetli sinerjizm'' veya ''hafif sinerjizm'' gibi çıkarımlar vermemektedir.

İlaç kombinasyonlarının analizi için, son zamanlarda yeni yöntemler geliştirilmiştir. İlaçların bireysel ve kombinasyon etkileri arasındaki doz-etki eğrilerinin potensisindeki değişiklikler karşılaştırılarak ilaç etkileşimlerini belirleyen sıfır etkileşim gücü (ZIP) adlı model önerilmiştir. Yöntem, ilaç kombinasyonlarını tek bir parametre temelinde değerlendirmek yerine, sinerjistik doz bölgelerini belirlemek ve ölçmek için tam doz-yanıt matriksi üzerinde bir etkileşim bölgesinin kullanılmasına dayanır. Çalışma, literatürde 300'den fazla atıf almıştır [37]. Modelde sıfır etkileşim beklentisinden sapmayı ölçmek için bir delta değeri tanımlanmıştır. Sıfır delta puanı, olasılıksal bağımsızlığın ve doz additivitesinin göstergesidir. Boyutsuz CI parametresinden farklı olarak, delta skoru hücre proliferasyon inhibisyonunun yüzdesini gösterir ve ilaç etkileşim etkisinin doğrudan tahminini sağlayabilir. Örneğin, 0.1 delta değeri, kombinasyondan beklenen etkiye kıyasla ortalama % 10 daha fazla inhibisyon üreteceğini belirtirken, -0,1 delta skoru ise aynı büyüklükteki antagonizmi gösterir. Delta skoru 0 ile 1 aralığında ise kombinasyon sinerjistiktir. Bu değerin -1 ile 0 arasında olması, ilaç kombinasyonunun antagonistik etkileşime sahip olduğunu ifade eder. Antikanser ilaç kombinasyon çalışmasından elde edilen veriler kullanılarak, ZIP skorlama yaklaşımının yanlış-pozitif oranı düşük bir seviyede tutarken ilaç sinerjizmini başarılı bir şekilde göstermiştir. Etkileşim bölgesi, çeşitli ilaç kombinasyonları arasında ayrım yapılmasını sağlar. Bu sebeple kombinasyonların kliniğe uygulanmasına yönelik ilaç etki mekanizmalarını belirlemek için iyi bir metot olabilir.

İlaç kombinasyonlarının sıklıkla CI-temelli metotlar kullanılarak analizi oldukça yaygındır. Ancak yapılan çalışmalar, yanıt-yüzey temelli modellerin ilaç sinerjizminin belirlenmesinde daha güvenilir olduğunu göstermiştir [38]. Yanıt-yüzey temelli modeller, 3'lü ilaç kombinasyonlarına ve atipik ilaç kombinasyon yanıtlarına da uygulanabilir. Yakın zamanda, additif etkileşimli dozlara iki-değişkenli yanıt (BRAID) olarak adlandırılan BRAID yanıt-yüzey modeli rapor edilmiştir [39]. Yöntem, yanıt-yüzey temelli bir modeldir ve ilaç etkileşimlerini belirlemek için gerekli kolaylık, sezgisellik, farmakolojik ve toksikolojik kısıtlamalar temelinde analiz için çok yönlü modeldir. Model, tek bir ilacın doz-etki analizi için yaygın olarak kullanılan Hill veya log-lojistik denklemlerine dayanır. İlaç etkileşimlerinin sınıflandırılması ve kombinasyonun doz-etki eğrilerinin modellemesini de barındıran birleşik bir araç olarak düşünülebilir. Model, Ewing sarkomu üzerine yapılan deneylerde elde kombinasyon veri setinin analizinde kullanılmıştır. Yöntem temelinde yapılan analiz sonucunda, ilaç kombinasyonlarındaki etkileşimlerin sınıflandırmalarının, geleneksel yöntemlere göre daha tutarlı olduğu ve ilaç etki mekanizmaları ile etkileşim arasındaki ilişkiyi gösterdiği rapor edilmiştir.Çalışmalar

sonucunda, farklı modellerin keşfedilmesine ve spesifik deneysel veriler için en uygun olanın seçilmesine olanak tanıyan açık erişimli ve kullanımı kolay tek ve kombine ilaç etkisi analizi (SiCoDEA) olarak adlandırılan kombinasyon analiz uygulaması geliştirilmiştir [40]. Yöntem, yukarıda anlatılan etki- ve doz-etki temelli geleneksel yöntemleri referans alır. SiCoDEA sınır verilerinin çıkarılması veya analiz için farklı modeller arasında seçim yapma imkanı sunmak gibi seçenekleri barındırdığı için esnek bir uygulamadır.

İlaç kombinasyon etkilerinin kantitatif analizi için geliştirilen bir diğer model yanıt zarf analizidir [41]. Yöntem, mevcut yöntemlerin sınırlamalarını önemli ölçüde bertaraf eder. Bu yöntem, ilaç-reseptör etkileşimlerinin tanımlanmasında yaygın olarak kullanılan Hill denklemine dayanarak Loewe additif modeli ve Bliss bağımsızlık modelinden türetilmiştir. Bliss modeli ve genelleştirilmiş Loewe additif etki yaklaşımı, ilaç etkileşimlerinin iki sınırını gösterdiği için inhibisyon mekanizmalarına gerek kalmadan sinerjizm, additif etki ve antagonizmin belirlenmesi amacıyla bir yanıt zarfı tanımlar. Metot, aslında Loewe ve Bliss modellerini temel alan üç-boyutlu yanıt-yüzey yaklaşımıdır. Yöntem kullanışlıdır ve farklı ölçeklerde ve çeşitli ilaç kombinasyonlarının etkilerini incelemek için kullanılabilir. Yapılan çalışmada, antikanser ve antibiyotik ilaç kombinasyonları analiz edilmiş ve yanıt-zarf modelinin mevcut yöntemlere göre avantajlı olduğu gösterilmiştir. Bu model ile analiz edilen ilaç kombinasyon etkilerinin, moleküler veya klinik düzeyde karmaşık çalışmaların sonuçlarıyla daha uyumlu olduğu gösterilmiştir. Diğer yöntemlere kıyasla, doğruluğu yüksek bir yöntemdir.

İlaç Kombinasyonlarının Analizinde Dikkat Edilmesi Gereken Hususlar

İlaç kombinasyonlarının analizi için geliştirilen metotlar, matematiksel ve istatistiksel modellere rağmen, bütün deney koşulları için geçerli tek bir ideal analiz yöntemi bulunmamaktadır. Önceki bölümde açıklanan geleneksel metotlar ve güncel modellerin her biri sınırlı koşullarda geçerlidir. Bu sebeple, en uygun referans analiz modelini belirlemek zordur. Dolayısıyla, ilaç metabolizması veya olumsuz etkiler gibi ajanların bireysel etkileşimlerini etkileyen birçok faktörün dikkate alınması gereklidir [10,12]. Varsayımlarındaki temel farklılıklar sebebiyle, hangi referans modelin istatistiksel olarak doğru bir şekilde kullanılması gerektiği konusunda konsensüs yoktur ve hatta uygun referans modelin nasıl seçileceğine dair bir kılavuz bulunmamaktadır. Şunu belirtmekte fayda vardır ki; mevcut hiçbir analiz yöntemi, muhtemel tüm deney koşullarında sinerjizmi gösteremez. Hatta, aynı deneysel veri setine uygulanan farklı yöntemler birbirinden farklı sonuçlara yol açabilir [12,42]. Bu bölümde, ilaç kombinasyonlarının analizi için uygun yöntemin seçilmesi ve bu alanda dikkat edilmesi gereken hususlar açıklanmıştır.

İlaç kombinasyonlarının sayısal analizi ve CI değerlerinin hesaplanması için dikkat edilmesi gereken hususların birincisi, terminoloji ve yöntemlerin uygun bir şekilde kullanımıdır. İlaç kombinasyonlarının kantitatif analizi için uygun yöntem ve/veya yöntemler seçilmelidir. Sinerjizm kavramı yaygın olarak kullanılır. Ancak, literatürde tam anlaşılmamış ve açıkça tanımlanmayan teknik terimler mevcuttur [27] ve sinerjizm kavramının analiz yöntemlerine ve anlamına uygun olarak kullanılmadığı bilinmektedir [43,44]. İlaç kombinasyonlarının sayısal analizi için, ilaçların tek ajan olarak doz-etki eğrilerine ilişkin deneysel verilerin elde edilmesi zorunludur. Deneysel tasarım, ilaç kombinasyonlarının doğru analizi için önemlidir. Sinerjistik ilaç etkileşimlerini doğru bir şekilde belirleyebilecek bir yöntem seçilmelidir. Deneysel verilerin yanlış analizi sonucunda etkileşim türü sinerjizm olarak veya sinerjistik ilaç kombinasyonu additif olarak yorumlanabilir. Hatta antagonistik ilaç kombinasyonları, yanlış analizden dolayı additif olarak değerlendirilebilir. İlaç kombinasyon çalışmalarındaki sinerjistik etkileşimleri araştırmak için ilaçların en az üç farklı dozunun uygulanması gereklidir. Bir ilacın üç farklı dozundan daha az sayıda doz uygulaması yapılan çalışmalar, sinerjistik ilaç etkileşimlerini araştırmak için yeterli değildir [27]. Önceki bölümde anlatıldığı gibi, iki ilacın kombinasyonunun (Örneğin; A ve B ilaç kombinasyonu) bireysel ilaç uygulamasına kıyasla üstün bir etkileşim göstermesi sinerjizmden başka formlarda da tanımlanabilmektedir. Kombinasyondaki ilaçlardan biri tek ajan olarak etkili ise, bireysel ilaç uygulamasına kıyasla üstün bir kombinasyon etkisi "potansiyalizasyon" olarak adlandırılır [11,31]. Kombinasyon etkisi incelenen ilaçlar tek ajan olarak etkili olmadığında, bireysel ilaç uygulamasına kıyasla üstün bir kombinasyon etkisi koalizm olarak tanımlanır. Herhangi bir ilacın etkisine tamamen bağlı olmayan etkili kombinasyonlardaki etkileşim türü ise, "iş birliği etkisi" olarak isimlendirilir [45].

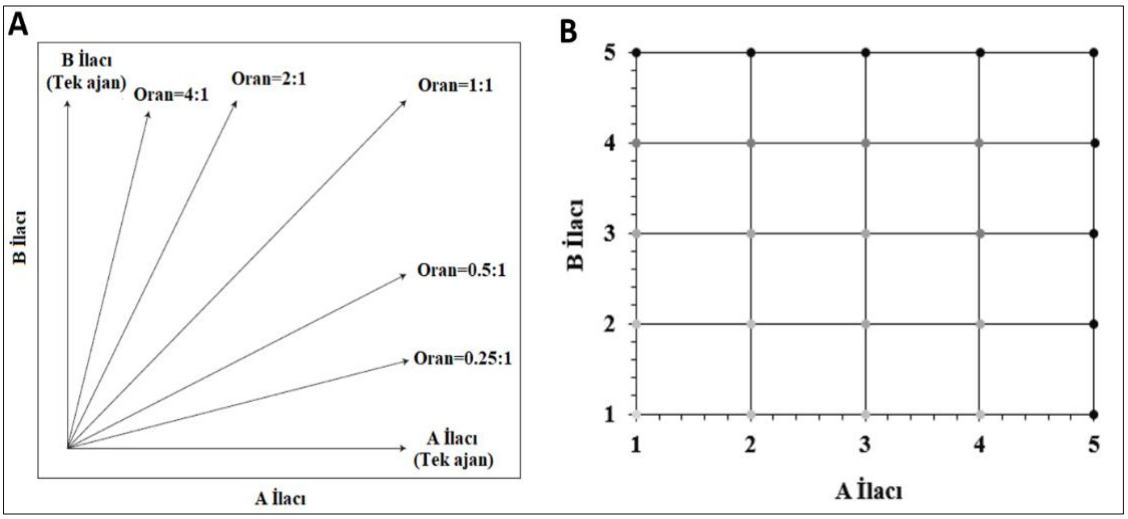
Dikkat edilmesi gereken ikinci husus, ilaç kombinasyonlarının analizinin standart bir referans model gerektirmesidir. İdeal analiz modeli, kesin olarak bilinmeyen ilaç etki mekanizmalarından bağımsız olarak additif, sinerjizm ve/veya antagonistik etkileşimi tanımlamalıdır. Aynı deneysel verinin farklı modeller ile analizi sonucunda farklı sonuçlar ve çıkarımlar ile karşılaşılabilir. İdeal analiz modeli, ilaçların etki mekanizmalarından bağımsız olmalıdır [46]. Ayrıca, ilaç kombinasyonlarının analizi için ideal metot, nadir ve özel durumları kapsayacak kadar genel olmalı, karşıt sezgisel sonuçlar ile sonuçlanmamalı, veri elde etmek için pratik ve etik konulara uygun ve bilimsel olarak kabul edilebilir olmalıdır. Ancak, ilaç kombinasyonlarının analizi için bütün bu nitelikleri karşılayan ideal bir model yoktur [47]. Loewe additif etki modeline dayanan doz-etki temelli yaklaşımlar, ilaç kombinasyonlarının analizi için uygundur. Fakat, bu yöntemler önemli miktarda deneysel veri gerektirir. Bu durum, verilerin elde edilmesinin pahalı ve/veya zor olduğu durumlarda çalışmayı sınırlayabilir. Yanıt-katkı yaklaşımı, HSA modeli ve Bliss yöntemi, en az üç ve/veya dört deneysel veriye ihtiyaç duyduğu için pratik sınırlamalara daha fazla uyumludur. Ancak, bu analiz yöntemleri çeşitli sınırlamalara ve bazı dezavantajlara rağmen bireysel ilaç uygulamasına kıyasla üstün pozitif bir kombinasyon etkisine dair yeterli kanıtlar sağlayabilir [10].

Dikkat edilmesi gereken üçüncü husus, ilaç kombinasyonlarının analizi için araştırma ve geliştirme süreçlerinin her aşamasına uygulanabilir yöntemlerin seçilmesi gerektiğidir. İlaçların kombinasyon etkileri farklı dozlarda uygulama yapılarak genellikle *in vitro* modeller kullanılarak araştırılır. Ancak *in vitro* ortamlarda ilaç dozu sabittir. Hücre dizileri ile yapılan *in vitro* deneyler, hastalıkların moleküler mekanizmalarını araştırmak için önemli kaynaktır. Hücre kültürü sistemleri, avantajlıdır. Örnek olarak; çeşitli insan hücre dizilerinin kullanılabilmesi, kültür koşullarının esnekliği ve protein/nükleik asit miktar tayininin kolaylığı verilebilir [9]. Fakat, her bir ilaç için doz belirleme deneylerinin, ilacın IC_{50} değerinden büyük ve küçük dozları kapsayacak şekilde yapılması önerilmektedir [11,31]. HSA yaklaşımı, Bliss metodu ve Loewe additif etki modeli, çeşitli araştırmalar için faydalı olabilir. Ancak, ilaçların, doz-etki eğrileri ayrıntılı olarak incelenmelidir. İlaç kombinasyonlarının analizi için, CI değeri ve izobologram eğrisi ile Loewe additif etki modeline dayanan doz-etki yaklaşımları uygun yöntemlerdir. Potansiyalizasyon veya koalizm gibi etkileşimlerde tüm ilaçlar için doz-etki eğrilerinin mevcut olmadığı kombinasyonlarda, HSA yaklaşımı kullanılabilir.

Preklinik ilaç kombinasyon çalışmaları, her ilacın maksimum tolere edilebilen dozlarında birlikte uygulanarak en yüksek etkinliğin elde edilebileceği varsayımına dayanan kombinasyon kemoterapi protokollerinin rasyonel tasarımına imkan sağlar [9]. Dolayısıyla preklinik kombinasyon çalışmalarının tasarımı yapılırken, ilaç konsantrasyonu, uygulama süresi, yöntemi ve ilaç kombinasyonlarını analiz etmek için kullanılan matematiksel ve istatistiksel metot gibi çeşitli faktörler dikkate alınmalıdır. Klinik çalışmalarda, ilaç kombinasyonlarının analizi kolay değildir. Bu aşamada, pratik ve etik kısıtlamalar söz konusudur ve sinerjistik ilaç etkileşimini bilimsel olarak belirlemek için yeterli veri elde etmek neredeyse imkansızdır [11,31]. Bu sebeplerle, resmi kuruluşlar tarafından kombinasyon ilaç tedavi modalitelerinin geliştirilmesi için gereken kriterler açıkça belirlenmiştir [48,49]. *İn vitro* deneylerden elde edilen sonuçların hayvanlara, *in vivo* çalışmalardan elde edilen verilerin insan klinik denemelerine ekstrapolasyonu önemlidir [11,31]. Klinikte kombinasyon tedavi geliştirme çalışmaları, kombinasyon etkisinin aynı dozda tek başına verilen ilaçların herhangi birinden daha yüksek etkinliğe ve/veya iyi güvenlik profiline sahip olduğunu kanıtlamalıdır [9-11,31]. Her aşama için gerekli veri miktarı, türleri ve uygun çalışma tasarımları, kombinasyonun yapısı, hastalık ve önceki aşamalardan elde edilen bilgilere göre değişmektedir. Dört-kollu klinik çalışma (plasebo veya standart bakım, ilaç A, ilaç B ve kombinasyon) yapılmalıdır [49]. Her ilacın kombinasyon etkisine katkısı önceki aşamalarda gösterilmiş ise, plasebo ve/veya sub-optimal doz terapisinin etik olmadığı durumlarda deneysel tasarım basitleştirilebilir [10].

Dikkat edilmesi gereken dördüncü husus, optimum doz oranının belirlenmesidir. Doz oranı, ilaç kombinasyonlarının analizinde kritik öneme sahiptir [50]. Çoğu durumda, kombinasyon tedavisi monoterapiden daha üstün ve yararlı etkiler sağlayabilir [1]. Ancak, bu üstünlük sadece ilacın özelliğinden dolayı olmayabilir. Bu üstün etki, aynı zamanda doz oranına da bağlı olabilir. Dolayısıyla, iki ilacın belirli bir oranda kombinasyonunu doz-etki eğrisi olan üçüncü bir ajan olarak düşünmek akılcı bir yaklaşım olacaktır. İlaç kombinasyonunun sinerjistik etkileşim gösterip göstermediğini araştırmak yerine, hangi doz oranının sinerjistik etkileşimi optimize ettiğini incelemek çok daha faydalı olabilir

[51]. Doz oranı, klinik denemelerden önce preklinik çalışmalarda belirlenmesi gerekir. Dolayısıyla, ilaç kombinasyon çalışmaları için deney tasarımı iyi planlanmalıdır. İlaçların seçilen dozlarının tüm kombinasyonlarını inceleyen tam-faktörlü tasarım (Şekil 2A) veya belirli bir sabit orana dayalı çoklu-oran tasarımı (Şekil 2B) tercih edilebilir. Farklı doz oranları, eğri-kayması analizi uygulanarak ilgili doz-etki eğrilerinin ortalaması ile karşılaştırılabilir.



Şekil 2. İlaç kombinasyon çalışmalarının tasarımı. Çoklu oran tasarımı (A), tam faktörlü tasarım (B)

Dikkat edilmesi gereken beşinci husus, ilaç kombinasyon çalışmalarında deneysel verilerin analizi için uygun metodolojilerin kullanılması gerektiğidir. Biyolojik deneysel modeller, her zaman hata barındırırlar ve sınır verileri kullanmak neredeyse imkansızdır [52]. Örneğin; 0.98 olan CI değeri rapor edilirken güven aralığı (% 95) dikkate alınmalıdır. Eğer istatistiksel analiz sonucunda elde edilen güven aralığı; 0.95-0.99 ise, kombinasyonun CI değerinin 1'den farklı olduğu çıkarımı yapılabilir. % 95 güven aralığı 0.85-1.05 ise, ilaç kombinasyon etkisi additif etkiden sapan herhangi bir etki olarak kabul edilemez [53]. HSA modeli ve yanıt-katkı yaklaşımı, önemin ölçülmesine ve kombinasyon etkisinin ilaçların bireysel maksimum etkilerine karşı istatistiksel olarak analiz edilmesine dayanır. Bliss modeli ve Loewe additif etki yaklaşımı ile hesaplanan CI değerleri, kesinliğin derecesine ilişkin değerlendirme yapılmadan rapor edilir. Dolayısıyla, bu yaklaşımlarda doğrudan istatistiksel çıkarım sağlayan bilimsel bir temel mevcut değildir. Bu teknik problem, az sayıda çalışmada ele almıştır ve kolay uygulanabilecek bir çözüm bulunmamaktadır [54-57,22].

Şunu belirtmekte fayda vardır ki, ilaç kombinasyonlarının analizi için kullanılan matematiksel ve istatistiksel metotlar karmaşıktır ve gelişmeye açık yöntemlerdir. İlaç kombinasyonu araştırma ve geliştirme süreci çoğu zaman hatalı tasarımlara ve analizlere yol açar. İstatistik uzmanlarının ilaç araştırma ve geliştirme süreçlerinin tüm aşamalarında yer alması faydalı olacaktır. Yöntemlerin çoğuna uyumlu standart yazılım ve/veya veri bankalarının geliştirilmesi, sonuçların iyileştirilmesi ve tekrarlanabilirliğin artırılması bakımından önemlidir. İlaç kombinasyonlarının analizinde özel bir yaklaşıma odaklanan örnekler mevcuttur. MixLow R paket programı, Loewe additif etki modelini önerir [58]. Çok sayıda çalışmada SAS [59] veya R kodlarının [60,61] kullanılmasından bahsedilmektedir. Ancak bu yöntemlerin nasıl uygulandığı ile ilgili literatürde bir bilgi bulunmamaktadır.

Dikkat edilmesi gereken altıncı ve son husus ise, en az üç-bileşenli ilaç kombinasyonlarının analizidir. İlaç kombinasyonlarının analizi yapılırken genellikle iki-bileşenli ilaç kombinasyonları kullanılır. Hastalıkların tedavisi için üç-bileşenli ilaç kombinasyonları da literatürde rapor edilmiştir [62]. İlaç kombinasyonlarının analizi için geliştirilen yöntemlerin çoğu, n sayıda ilacın kombinasyonlarının analizi için genişletilebilir (Şekil 3). İkili-ilaç kombinasyonlarının analizi için geliştirilmiş olan HSA yaklaşımı, Bliss yöntemi, Loewe additif etki modeli ve Chou-Talalay'ın medyan-etki denklemi üç veya daha fazla bileşenli kombinasyonların analizi için kullanılabilir. Ancak, önceki

bölümde belirtilen HSA modelinin dezavantajlarından dolayı, en az üç bileşenli ilaç kombinasyonları için deneysel tasarım ve analizin yapılması amacıyla Bliss modeli ve Chou-Talalay metodu tercih edilmelidir.

Bliss İstatiksel Bağımsızlık modeli:

$$CI = \frac{E_A + E_B + \cdots + E_N - E_AE_B - E_AE_N - E_BE_N - \cdots - E_AE_B \ldots\ldots E_N}{E_{ABC\ldots\ldots N}}$$

Chou-Talalay metodu:

$$CI = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

En Yüksek Tek Ajan (HSA) modeli:

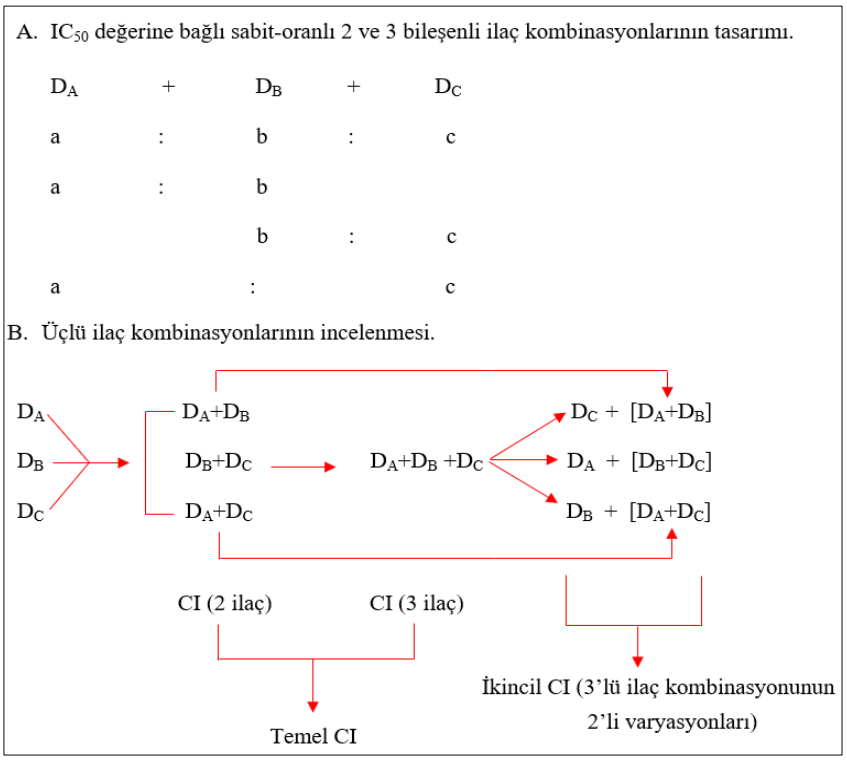
$$CI = \frac{maksimum(E_A, E_B, E_C; \ldots\ldots; E_N)}{E_{ABC\ldots\ldots N}}$$

Loewe Additif Etki modeli:

$$CI = \frac{a}{A} + \frac{b}{B} + \frac{c}{C} + \cdots + \frac{n}{N}$$

Şekil 3. n bileşenli ilaç kombinasyonlarının analizi için kullanılan matematiksel formüller

İlaç kombinasyonu üç bileşenden meydana geldiğinde, CI=1 denklemi A, B ve C ilaçlarının dozlarından geçen üçlü-koordinat eksenine karşılık gelir. Bu durum, ikili ilaç kombinasyonunda additif etki çizgisi ile gösterilir. Sinerjistik etkileşim gösteren üç ilacın (A, B ve C) kombinasyon etkisi, A ve B arasındaki sinerjistik etkileşimden kaynaklanabilir. Her ilacın kombinasyon etkisine katkısının tam olarak belirlenmesi için, tüm alt kombinasyonların da aynı deneysel koşullardaki etkilerinin değerlendirilmesi gereklidir [31]. Dr. Chou, üçlü-ilaç kombinasyonu (D_A, D_B ve D_C) için, IC_{50} oranlarının (a:b:c) kullanılmasını önermiştir [11]. D_A+D_B+D_C, (a:b:c) oranını ifade eder (Şekil 4). Aynı deneysel ortamda ilaçların iki-bileşenli kombinasyon etkilerinin (D_A+D_B (a:b), D_B+D_C (b:c) ve D_A+D_C (a:c)) de incelenmesi gereklidir.



Şekil 4. Chou-talalay yaklaşımı ile üçlü ilaç kombinasyonlarının analizi [11]

Yaklaşım, üç-bileşenli ilaç kombinasyonunun iki-bileşenli ilaç kombinasyonları halinde analiz edilmesini sağlar. Bu yaklaşım temelinde, CI değerleri ve ikincil CI değeri belirlenebilir. Deney tasarımı, aynı deneysel şartlarda bireysel ilaçlar (D_A, D_B ve D_C), D_A+D_B, D_B+D_C, D_A+D_C iki-bileşenli ilaç kombinasyonları ve üç-bileşenli ilaç kombinasyonu (D_A, D_B ve D_C) için doz-etki eğrileri elde edilecek şekilde yapılmalıdır [31]. Üç-bileşenli ilaç kombinasyonlarının iki-bileşenli ilaç kombinasyonları halinde (örneğin, D_A+D_B, orta sinerjistik, D_B+D_C antagonistik, D_A+D_C güçlü sinerjistik ve D_A+D_B+D_C sinerjistik olabilir) analizi, ilaçların farmakolojik etkileşimleri hakkında önemli bilgi sağlayabilir.

SONUÇ VE TARTIŞMA

İlaçların kombinasyon etkileri uzun yıllardır incelenmiş ve analiz edilmiştir. Kombinasyon tedavisinin avantajları yaygın olarak bilinmektedir ve bu alandaki çalışmalar, çeşitli hastalıkların fizyolojisi ve biyolojisinin anlaşılması ile önemli ölçüde artmıştır. Tıp, farmakoloji, matematik ve epidemiyoloji gibi birçok farklı disiplinden bilim insanları bir araya gelmiş ve bu alandaki araştırmalar sonucunda, biyolojik ve medikal bilimlerde çok sayıda teorik ve deneysel makale yayınlanmıştır. Bu çalışmada, ilaç kombinasyon etkilerinin, özellikle de sinerjizmin belirlenmesi için mevcut istatistiksel ve matematiksel yöntemler derlenmiştir. Bliss modeli, Loewe additif etki yaklaşımı ve Chou-Talalay'ın medyan-etki denklemi de dahil olmak üzere, ilaç kombinasyonlarının analizi için yaygın kullanılan ve çok sayıda atıf almış referans yöntemler sunulmuş ve ilişkili avantajları ve dezavantajları irdelenmiştir. Takibinde, ilaç sinerjizmini ölçmek için kullanılan güncel metodlar tanıtılmıştır. Son olarak ise, ilaç kombinasyonlarının analizinde dikkat edilmesi gereken hususlar açıklanmıştır.

İlaç keşfindeki yüksek ölçekli tarama teknolojileri ve hesaplama kapasitesindeki önemli gelişmeler sayesinde, veri eldesi ve analiz yöntemlerinde önemli ilerlemeler kaydedilmiştir. Ancak, temel metodolojik sorunlar önemli ölçüde aynı kalmıştır. Bilgi yoksunluğundan kaynaklı olarak, literatürün önemli kısmı terminoloji ve yöntemlerin yanlış anlaşılmasına yol açmaktadır. Gelecekte yapılması planlanan çalışmalarda, kombinasyon etkilerinin bilimsel ve doğru analizi için burada tartışılmış olan kavramların ve yöntemlerin daha uygun ve titiz bir şekilde uygulanması gerektiğini düşünüyoruz.

Bazı kısıtlamalarına rağmen Chou-Talalay metodu en çok atıf almış yöntemdir ve güncel olarak geniş çapta uygulama alanına sahiptir. Chou-Talalay metodundan sonra, sıklıkla tercih edilen yöntem ise Bliss modelidir. Bliss modelinin güncel olarak kullanılmasının yanı sıra, modelin modifiye versiyonları geliştirilmiş ve ilaç kombinasyonlarının analizinde kullanılmıştır [22]. Her iki metot kullanılarak n-ilaç kombinasyonları rahatlıkla tasarlanabilir ve analiz edilebilir. Dolayısıyla, ilaç kombinasyon çalışmaları ve deneysel verilerin analizi için CDI ve HSA gibi günümüzde geçerliliği olmayan ilkel yöntemler yerine Bliss modeli, Chou-Talalay metodu, ZIP modeli tercih edilmelidir. ZIP modeli kullanılarak elde edilen delta skorları, ilaç etkileşim aralığının görselleştirilmesi ve belirli doz bölgelerinde klinik-eşdeğer ilaç sinerjizminin tanımlanmasında önemli rol oynayabilir [37]. Ayrıca, CI-temelli yöntemlerin oldukça değişken ve öngörülemeyen sonuçlara yol açabileceğini düşünecek olursak, BRAID yanıt-yüzey modeli önemli bir alternatif olabilir.

Çeşitli bilgisayar yazılımlarının kullanılması, kombinasyonların analizini şüphesiz kolaylaştırır. Ancak, CompuSyn programı yalnızca Windows işletim sisteminde çalışmaktadır. Program tek seferde yalnızca bir ilaç kombinasyonu için doz-etki değerlerinin sisteme girilmesine izin verir. İlaç kombinasyonlarının analizinde CompuSyn yazılımına ek olarak, yakın zamanda geliştirilen ve güncel olarak kullanılan Combenefit [63] ve SynergyFinder [64] yazılımlarıdır. Combenefit, Loewe, HSA ve Bliss modellerinin kullanılarak analiz yapılmasını sağlar. CompuSyn ve Combenefit ücretsizdir, kullanımı kolaydır. Windows işletim sisteminde kullanılabilir ve deneysel verilerin grafikle gösterimine de olanak sağlarlar. Synerfinder 2.0 uygulaması da Loewe, HSA, Bliss ve ZIP modellerini sunar ve CI değerinin hesaplanması için farklı modeller arasında seçim yapılmasına imkan tanır. Ancak, farklı doz-etki eğrileri modeller arasında seçim yapılmasına izin vermez. Uygulama, R kod temelli paket programdır ve ücretsizdir. Ancak R kodlarının kullanımı karmaşıktır. SiCoDEA, kombinasyon verilerini analiz etmek ve seçilen modele göre farklı sonuçlar sunmak için kullanım kolaylığı olan bir araçtır. CompuSyn veya SynergyFinder 2.0 yazılımlarına alternatiftir.

İlaçların kombinasyon olarak uygulanmasındaki en önemli amaçlardan biri, terapötik etki korunurken veya sinerjistik etkileşimler ile artırılırken aynı zamanda doz ile ilişkili yan etkilerin de azaltılmasıdır. Dolayısıyla ilaç kombinasyon çalışmalarında uygulama dozu ve/veya doz aralığı belirlenirken bu durum dikkate alınmalıdır. Bu sebeple yan etkisi az, minimum çapraz dirence sahip moleküllerin kombinasyon çalışmalarında değerlendirilmesinin klinik uygulamalar ve terapötik modalitelerin geliştirilmesi bakımından hayati öneme sahip bir strateji olduğunu düşünüyoruz. İlaç kombinasyon çalışmalarında deneysel tasarım için ilaçların IC_{50} dozları ve katları kullanılarak doz-bağımlı ilaç etkileri incelenebilir. İlaç kombinasyonları düşük dozlarda ($IC_{50}/16$ veya $IC_{50}/8$ gibi) kuvvetli sinerjistik, yüksek dozlarda ($2IC_{50}$ veya $4IC_{50}$ gibi) additif hatta antagonistik etkileşimler gösterebilir [21]. Dolayısıyla, ilaç kombinasyonlarında uygulama dozu ve/veya doz aralıkları belirlenirken bu durum da göz önünde bulundurulmalıdır. Ek olarak, sabit-oranlı tasarım ve ray-tasarımı da ilaç kombinasyon çalışmalarında deney tasarımı yapılırken kullanılmalıdır [65]. Tüm deneysel koşullara uygun ideal bir referans analiz metodu olmadığı için, bu çalışmada sunulan farklı yaklaşımların birlikte kullanılması ile ilaç kombinasyonlarının analizinin kolaylaşacağını düşünüyoruz. Günümüze kadar yapılmış olan çalışmaların, klinik olarak etkili ilaç kombinasyon tedavilerinin belirlenmesi, geliştirilmesi ve optimizasyonu süreçleri için umut verici bir temel sağladığına inanıyoruz. Bu çalışmanın, süreçteki önemli hususlar için bir referans görevi göreceğini umut ediyoruz.

YAZAR KATKILARI

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ÇIKAR ÇATIŞMASI BEYANI

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REVIEW ARTICLE / DERLEME MAKALE

SELF EMULSIFYING DRUG DELIVERY SYSTEMS - AN OVERVIEW

KENDİLİĞİNDEN EMÜLSİFİYE OLABİLEN İLAÇ TAŞIYICI SİSTEMLER

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ABSTRACT

Objective: *Self-Emulsifying Drug Delivery System (SEDDS) has tremendous potential that has yet to be completely realized. They can be used in the formulation of drug compounds that have low water solubility in oral lipid administration and overcome many problems associated with these compounds. The SEDDS can increase the rate and degree of oral absorption by optimizing drug solubility in the intestinal absorption site, attributable to its small particle size, large surface area, high encapsulation efficiency, and high drug load. Furthermore, due to its lipid-based formulation, SEDDS can accelerate and increase pharmaceutical lymphatic transport, bypassing hepatic first-pass metabolism and therefore enhancing bioavailability.*
Result and Discussion: *The quantity of novel therapeutically effective lipophilic molecules that are hydrophobic has steadily increased thanks to innovative drug development approaches. The future of pharmaceutical research may not only pass through the discovery of new molecules but also better exploitation of those already known. The use of SEDDS has been proven to be quite successful in enhancing the oral bioavailability of hydrophobic and lipophilic drug molecules among the strategies to increase the oral bioavailability of these compounds.*
Keywords*:* *Drug solubility, emulsifying dosage forms, lipophilic drugs, self-emulsification, self-emulsifying delivery systems*

ÖZ

Amaç: *Kendiliğinden emülsifiye olan ilaç taşıyıcı sistemler (SEDDS), henüz tamamen aydınlatılmamış olan muazzam bir potansiyele sahiptir. Oral lipid uygulamasında suda çözünürlüğü düşük olan ilaç bileşiklerinin formülasyonunda kullanılabilirler ve bu bileşiklerle ilişkilendirilen birçok problemin üstesinden gelebilirler. SEDDS, küçük parçacık boyutuna, geniş yüzey alanına, yüksek kapsülleme etkinliğine ve yüksek ilaç yüküne atfedilebilen bağırsak emilim bölgesindeki ilaç çözünürlüğünü optimize ederek oral emilim oranını ve derecesini artırabilir. Ayrıca, lipit bazlı*

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formülasyonu nedeniyle SEDDS, hepatik ilk geçiş metabolizmasını atlayarak ve dolayısıyla biyoyararlanımı artırarak farmasötik lenfatik taşınmayı hızlandırabilir ve artırabilir.
Sonuç ve Tartışma: *Terapötik açıdan etkili olan yeni lipofilik hidrofobik moleküllerin miktarı, yenilikçi ilaç geliştirme yaklaşımları sayesinde istikrarlı bir şekilde artmıştır. Farmasötik araştırmaların geleceği, yalnızca yeni moleküllerin keşfedilmesinden değil, aynı zamanda halihazırda bilinenlerin daha iyi kullanılmasından da geçebilir. Bu bileşiklerin oral biyoyararlanımını artırma stratejilerinden hidrofobik ve lipofilik ilaç moleküllerinin oral biyoyararlanımını artırmada SEDDS kullanımının oldukça başarılı olduğu kanıtlanmıştır.*
Anahtar Kelimeler: *Emülsifiye dozaj formları, ilaç çözünürlüğü, kendiliğinden emülsifikasyon, kendiliğinden emülsifiye olabilen ilaç taşıyıcı sistemler, lipofilik ilaçlar*

INTRODUCTION

The medications are commonly administered orally; however, around 40% of new drug competitors have poor-water solvency and the oral delivery of such medications is troublesome in view of their low bioavailability, high intra- and inter-subject fluctuation, and an absence of dose proportionality [1].

The desired concentration of any drug to achieve its pharmacological response is based on its solubility majorly and thus it is very challenging for formulation scientists to maintain the pharmacological range of lipophilic drugs [1,2].

Classification of Lipid-Based Delivery Systems

The Biopharmaceutics Classification System (BCS) divides medications into four categories entrenched in their solubility and intestinal permeability, as determined by the United States Food and Drug Administration's (US FDA) statistics on intestinal drug absorption as described in table 1. In the formulation of SEDDS, class II medications that have great permeability and limited solubility are employed [3,4].

Table 1. Classification of lipid-based delivery systems

Category	Oil (lipophilic)	Surfactant (lipophilic)	Surfactant (hydrophilic)	Co-solvents	Formulations
Category I	100 %	0 %	0 %	0 %	Simple oily solutions
Category II	40-80 %	20-60 %	0 %	0 %	SEDDS
Category III	0 %	20-40 %	20-40 %	20-50 %	SMEDDS
Category IV	0 %	0-20 %	20-80 %	0-80 %	Colloidal micellar dispersion

Solubility Enhancement Techniques

To improve the therapeutic effectiveness of lipophilic drugs, researchers are working to improve their oral bioavailability.

To prevail drug solubility related problems, numerous formulation methods are practised such as the use of permeation enhancers, surfactants, lipids, salt formation, micronization, cyclodextrins, supercritical fluid process, and solid dispersions [5].

Some of the lipid-based formulations that have been utilized to increase the efficacy of weakly water-soluble bioactive compounds are liposomes, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), emulsions, and nanoemulsions. They improve water solubility by enhancing the solubilization and stability of lipid-based drugs, as well as offering a sustained, targeted, and triggered delivery mechanism [2,4]. Increased drug solubility can also be achieved using formulation approaches such as self-emulsifying formulations [6]. Some of these strategies are represented in Figure 1 [6,7].

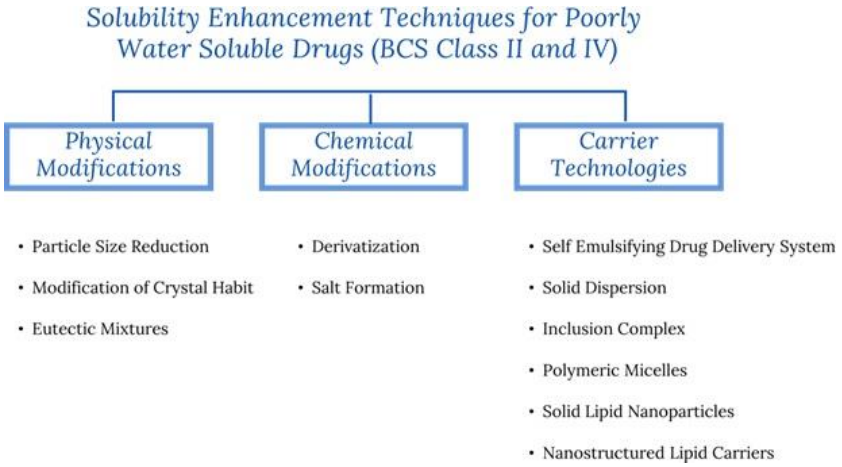

Figure 1. Several solubility improvement techniques for weakly water-soluble drugs

Self-Emulsifying Drug Delivery Systems (SEDDS)

"SEDDS are isotropic and thermodynamically stable systems consisting of oil, surfactant, co-solvent/co-surfactant, and drug components, which can form an oil/water microemulsion when mixed with water at low speed" [8].

The spontaneous formation of the emulsion in the gastrointestinal tract with slight agitation produced by stomach motility is the principle underlying SEDDS improving dissolving rate. The drug is delivered in a solubilized state, and the droplet's small size offers a large interfacial surface area for drug absorption [8,9].

The SEDDS can affect drug absorption in a variety of ways, including enhancing drug solubility, permeability, and lymphatic uptake [8,10]. When a SEDDS is taken orally (Figure 2), it is released into the GIT's (gastrointestinal tract) lumen and interacts with the GI fluid to generate micro or nano-emulsions. Oil droplets can quickly move through the stomach, increasing drug distribution throughout the GIT, and absorption by lymphatic way permits the drug to evade first-pass metabolism, enhancing drug oral bioavailability [10].

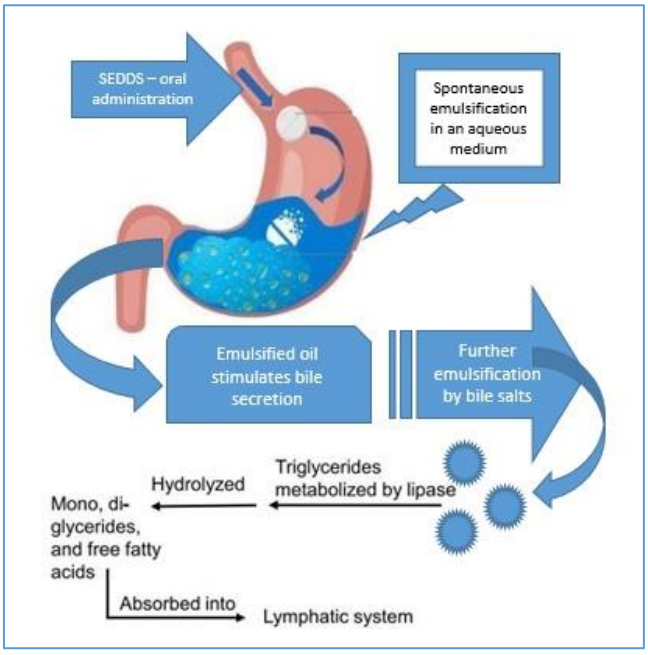

Figure 2. Oral absorption procedure of SEDDS

Self Nano-Emulsifying Drug Delivery System (SNEDDS)

SNEDDs are heterogeneous dispersions of two immiscible liquids with mean droplet sizes in the nanometer range (less than 100nm). Regardless of the method used to prepare it, this is especially important for medications that increase solubility, such as simvastatin and atorvastatin [11].

Self Micro-Emulsifying Drug Delivery System (SMEDDS)

When they encounter water, they make microemulsions (Figure 3). SMEDDS' emulsions have a mean droplet size that ranges from 100-250nm on a micrometric range. The fundamental distinction between traditional emulsions and microemulsions is the mean droplet size. Thermodynamically, SMEDDS are stable [3,8,11]. They produce optically clear emulsions. The surface area for absorption and dispersion is considerably improved due to the very small droplet size, and it quickly penetrates the gastrointestinal system and could be absorbed [3].

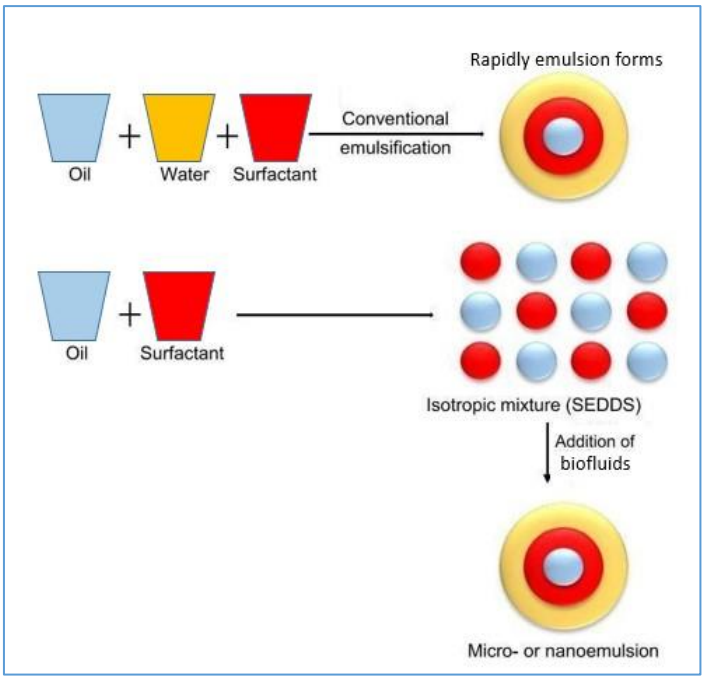

Figure 3. Illustration of the self-emulsification mechanism

Self-emulsification is a complicated process that is still being studied. On the other hand, as attested by some theories, self-emulsification happens at the time of the entropy shift, it promotes dispersion that is larger than the requisite energy to expand the dispersion surface area. The free energy of a traditional emulsion formation is proportional to the energy needed to produce a new surface between the two phases, and can be expressed using the following equation:

$$\Delta G = \sum N i \pi r 2 \, \sigma$$

Where ΔG represents the process's free energy (reckon without the mixing free energy), N indicates the number of droplets of Radius. The interfacial energy is represented by r and σ.

Throughout time intervals, separation of the emulsion's two phases will occur, lowering the area of the interface, so that, as a result, the system's free energy. Consequently, traditional emulsifying agents stabilize aqueous dilution emulsions by forming emulsion droplets surrounded by monolayer. As a result, the energy of the interface is lowered, and coalescence is prevented. Before the emulsification occurs spontaneously, in self-emulsifying systems, the free energy necessary to generate the emulsion is either extremely low but also positive or quite low and negative [12].

Application of SEDDS/SMEDDS Formulation

Improvement of Oral Absorption:

SEDDS partially eliminates the need for a second drug dissolving stage before absorption in the GIT. They improve drug absorption by increasing the quantity of solubilized drug in the intestinal liquids. Furthermore, incorporating lipid-based ingredients in the formulation may aid in medication absorption [9,13].

Retardation of Gastric Emptying Time:

Surfactants are thought to slow down gastric transit time, allowing the active pharmaceutical ingredient to be dissolved and absorbed more quickly. Surfactants can pace down gastric emptying for a while in the intestinal and gastric lumen by forming a viscous mass and enhancing the bioavailability of an investigational drug.

Increase in Effective Drug Solubility in Lumen:

Oral lipid-based formulations' biopharmaceutical characteristics and effective preparation development are strongly dependent on the lipidic transport route from the GI lumen to the systemic circulation. SEDDS passes through the digestive, absorptive, and circulatory stages after being taken orally [11,13].

Lymphatic Pathway:

Most of the drug delivery of SEDDS are absorbed systematically via the portal vein. The lymphatic system is a massive drainage system that runs across the whole body. It is located behind the blood flow system and is accountable for returning liquid that has seeped into the tissue space to the blood. Intestinal lymphatics are especially important for the absorption of lipid-digested substances, like longchain fatty acids and lipid-soluble vitamins [7,13].

Effect of P-glycoprotein (P-gp) Inhibition:

Reduction in P-gp drug efflux might contribute to an increase in the intake of SEDDS from the GIT. Moreover, to be a multidrug efflux pump, phase I metabolism by intestine cytochrome P450s has a substantial influence over oral bioavailability. In some cases, excipients in SEDDS/SMEDDS have been shown to block both pre-systemic drug metabolism and P-gp-mediated intestinal efflux, resulting in enhanced oral absorption of cytotoxic drugs [13].

Properties of SEDDS

SEDDS has the following properties:

1. They can self-emulsify fast in GI biofluids and create a fine o/w emulsion under theinfluence of peristaltic and other GIT motions [1,5,14].
2. Drugs (hydrophobic or hydrophilic) can be efficiently incorporated into the oilsurfactant mixture.
3. Suspension, emulsion, pills, pellets, and suppositories are all examples of liquid andsolid dose forms of SEDDs.
4. In comparison to traditional dosage forms, they require a lower drug dose [11-15].

Advantages

- The principal advantage is improving the oral bioavailability of lipid soluble medicines.
- They enable more steady drug absorption patterns, selective drug targeting to a particular absorption window in the GIT, and drug protection from the gastrointestinal tracts' hostile conditions, as well as protection of sensitive drugs.
- SEDDS' fine oil droplets are likely to move quickly and stimulate extensive drug dispersion down through GIT, reducing the discomfort commonly seen during prolonged contact between the gut wall and bulk medicine material.
- Variability reduction, including dietary effects.

- SEDDS has a greater drug loading capability than other lipid/oil-based formulations.
- SEDDS is unaffected by the lipid digesting process.
- The method is simple to manufacture and scale up.
- SEDDS can be applicable for both liquid and solid dosage types.
- SEDDS has a rapid onset of effect.
- Dose reduction by the ability to improve the solubility [9,14-17].

Disadvantages

- Traditional dissolution methods do not work because these formulations are potentially dependent on digestion prior to the release of the drug.
- This *in vitro* model needs further development and validation before its strength can be evaluated.
- Further development will be based on *in vitro – in vivo* correlations and therefore different prototype lipid-based formulations needs to be developed and tested *in vivo* in a suitable animal model.
- Chemical instabilities of drugs and high surfactant concentrations in formulations (approximately 30-60%) [13-17].

Composition of Self-Emulsifying Drug Delivery Systems

When selecting excipients, it is critical to evaluate the pharmacological acceptance of the excipientsas well as the toxicological problems of the ingredients.

Self-emulsification is based on:

- The type of oil and surfactant used
- The quantity of surfactant used
- The temperature at which self-emulsification occurs [17].

Components of SEDDS are depicted in Figure 4.

Active pharmaceutical ingredient (API)

They have the following characteristics:

- Drugs undergo significant hepatic metabolism.
- Drugs with sufficient half-life.
- Low dosage.
- A greater log P number shows that the drug has a high lipophilicity.
- Drugs in the BCS Class II (poor water solubility).
- The drug's bioavailability should be limited [6,11,17].

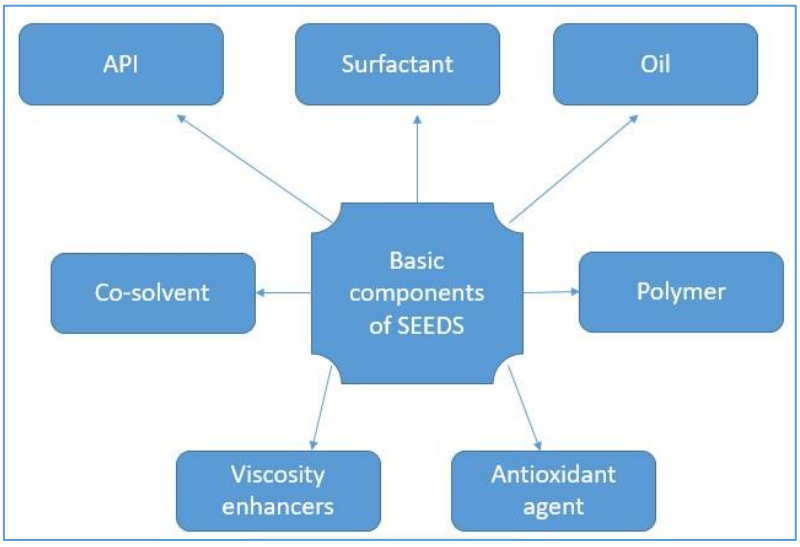

Figure 4. Basic components of SEDDS

Oil

Since oil solubilizes the needed amount of the lipophilic drug, it is the most critical excipient in SEDDS composition. It also serves to make self-emulsification easier. SEDDS are also known as self-emulsifying oil formulations due to the role of oil in them. In a SEDDS, natural or synthetic oils can be employed.

Oils improve lymphatic permeability in the intestines, solubility in the stomach and liquids in the intestines, shield the medicine from biotransformation, and increase the dissolution rate, these together enhance the bioavailability of oral lipophilic drugs [3,8,18].

Surfactants

"In the composition of SEDDS, nonionic surfactants having large HLB value are employed. Because non-ionic surfactants are not toxic, they are recommended over cationic and anionic surfactants in SEDDS" [3].

Many compounds with surfactant qualities may be useful in the development of SEDDSs, but the options are restricted since only surfactants appropriate for oral administration are applicable. One of the most crucial aspects while choosing a surfactant seems to be safety. Natural emulsifiers are favored over synthetic surfactants whereas they are thought to be safer. Non-ionic surfactants having a HLB (e.g., Tween, Labrasol, Labrafac CM 10, Cremophore, etc.) are the most widely recommended [19].

For the development of stable SEDDS, the surfactant concentration normally varies between 30-60% w/w of the formula's composition. This high concentration of surfactants causes gastrointestinal distress, which is a disadvantage of SEDDS.

Surfactants included in the SEDDS formulation enhance bioavailability through a variety of methods, including increased intestinal epithelial permeability, decreased or blocked drug efflux of p-glycoprotein, improved active pharmaceutical ingredient solubility.

Co-solvent

Co-solvents aid in the dissolution of phases that are immiscible in a formulation (o/w). In the oil phase, co-solvents dissolve significant quantities of hydrophilic surfactants or hydrophobic drug. One or more hydrophilic solvents can be employed.

In order to develop an efficient self-emulsifying system, comparatively large concentrations of surfactants (typically bigger than 30% w/w) are needed, which tends to produce GI irritation [16]. As a result, co-solvents are utilized to decrease the surfactant amount. When combined with surfactants, they lower interfacial tension to an extravagantly small, even temporarily negative value. When this number is reached, the interface extends to form finely dispersed droplets, which absorb more surfactant and surfactant with co-surfactant until their bulk state is diminished sufficiently to reestablish positive interfacial tension [11,15]. This is known as spontaneous emulsification, and it is what inevitably leads to emulsions.

Polymers

In SEDDS, around 5-40% w/w Polymer matrix (inert) is found, which is not ionizable at biological pH and may be used to produce matrix. They inhibit precipitation when added to SEDDS formulation [20,21].

Antioxidant agents

Lipophilic antioxidants help to keep SEDDS formulations oil part stable [19].

Diagrammatic representation of SEDDS and commonly used excipients in SEDDS formulation is presented in Figure 5.

Dosage Forms of SEDDS

The dosage forms of SEDDS are summarized in Figure 6.

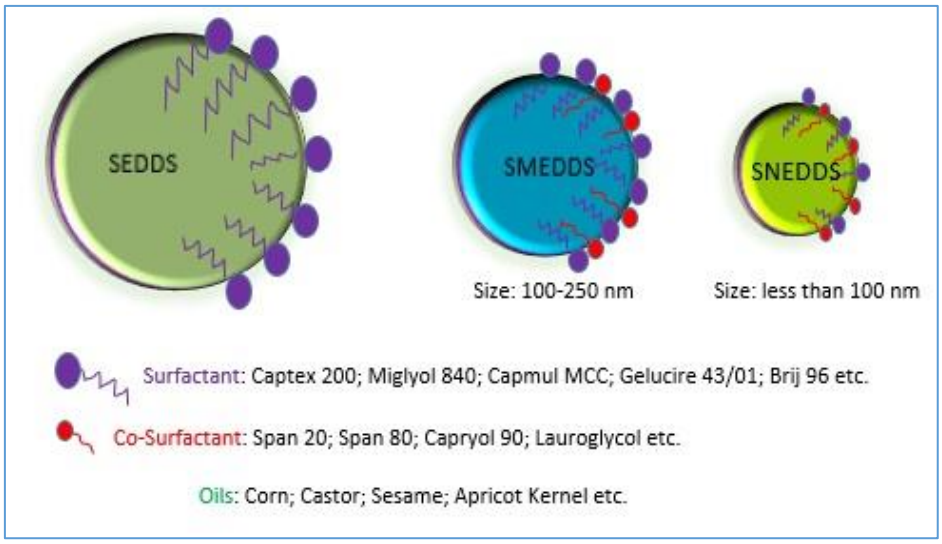

Figure 5. SEDDS, SMEDDS, and SNEDDS are depicted in a diagram

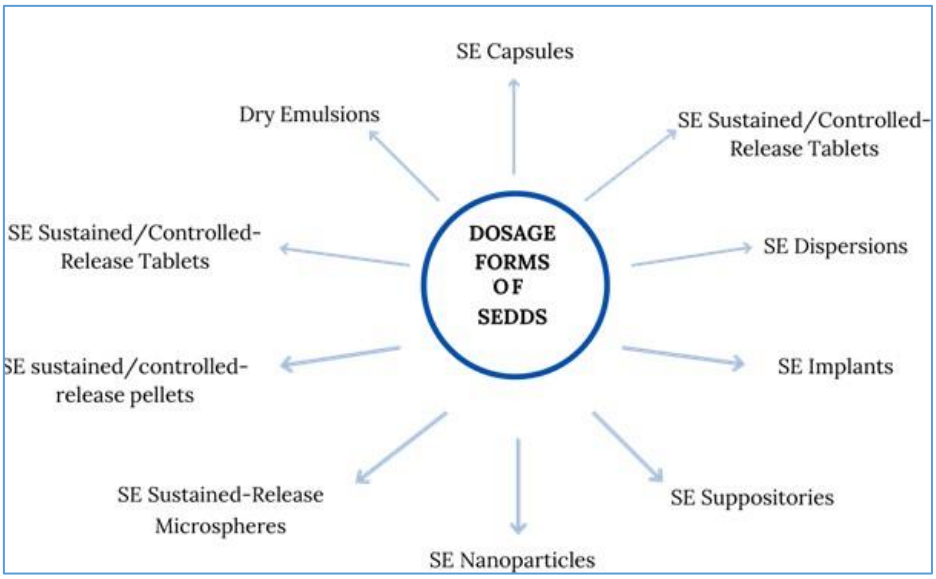

Figure 6. Diagram of SEDDS dosage forms

Dry Emulsions

Dry emulsions are powders that emulsify spontaneously when introduced to an aqueous medium or when introduced to an aqueous medium. They can be utilized in the production of capsules and tablets. Dry emulsion preparations are commonly produced via spray drying, freeze-drying and rotary evaporation in the aqueous phase from (O/W) emulsions including a solid carrier (maltodextrin lactose, and so on). This technique eliminates some of the issues associated with traditional emulsions during storage (phase separation, microbe contamination, etc) [21,22].

Self-emulsifying Capsules

For providing suitable single-unit dose preparations, lipophilic active pharmaceutical ingredients could be dissolved in SEDDS and encapsulated in soft or hard gelatin capsules [21,23]. When traditional liquid SE preparations are administered in capsule form, microemulsion droplets develop and scatter throughout the GIT to reach absorption areas. However, if the micro emulsion's phase separation is

permanent, no enhancement in medication absorption may be predicted; Sodium dodecyl sulphate was included in the SE composition to address this issue [21,24].

Self-emulsifying Sustained/controlled-release Tablets

SE tablets are frequently produced because they're more stable than other dosing types. A polymeric method can be used to provide sustained action [25]. SE tablets are made up of compressed or molded liquid SEDDS that have been solidified. These preparations have a number of benefits, including the ability to liquefy at body temperature under agitation owing to the peristalsis of the GIT, which decreases the liquidation period, which causes quicker emulsification and higher drug plasma concentration (shown in Figure 7). A gelled SEDDS has been created to minimize the quantity of solidifying agents needed for the conversion of SEDDS into solid dosage forms [21-25].

The SE osmotic pump tablets are the most recent innovation of SE tablets research. This approach offers several advantages, including steady plasma concentrations, a controlled release rate, and increased drug absorption [26,27].

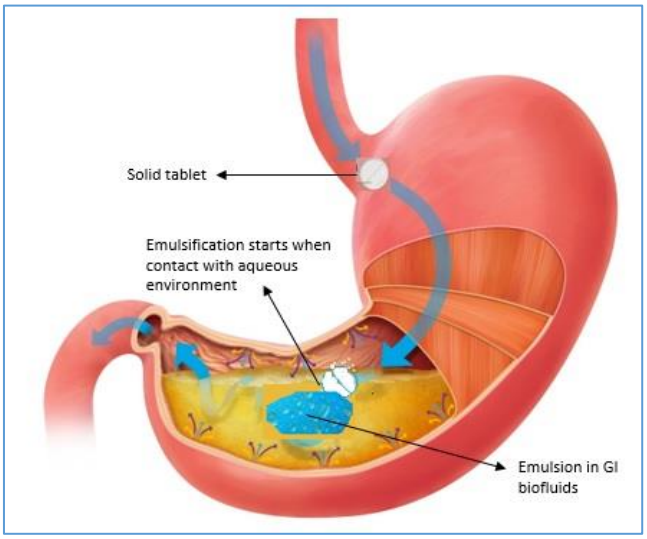

Figure 7. Mechanism of action of solid self-emulsifying tablet

Self-emulsifying Controlled/sustained Release Pellets

Even though the use of SEDDS is mainly designed to increase the absorption of lipophilic medications, it would also be desired to offer sustained release action in the case of pharmaceuticals with a short biological half-life and frequent dosing. As a result, researchers had the idea of combining SEDDS with control release agents in terms of designing matrix-type controlled release solid SEDDS. Solid SEDDS matrix-type spherical pellets have been designed [28].

Self-emulsifying Beads

It was theorized that by utilizing capillary forces, SEDDS might be designed to convert a liquid SE formulation into a solid form with little solidifying aids while avoiding the leakage and leaching issues that face conventional liquid SE formulations [16].

Self-emulsifying Sustained-release Microspheres

ZTO (a conventional drug used in China) has a wide range of pharmacological effects, such as tumor suppressive, antimicrobial, and antithrombotic properties. In one of the preparations of solid SE sustained release microspheres, ZTO was used as the oil phase. When compared to standard liquid SEDDS, following oral delivery of these microspheres, the concentration of plasma was attained with a 135.6% enhancement in the bioavailability [21,25].

Self-emulsifying Nanoparticles

Nanoparticle methods are used in the manufacture of self-emulsifying nanoparticles. A solvent injection is one of these methods. In this technique, the main components are melted all together and then injected dropwise inside a stirred nonsolvent. The self-emulsifying nanoparticles are filtered and then dried. Nanoparticles (approx. 100 nm) are produced with a 74% drug loading efficiency by this method [17].

Self-emulsifying Suppositories

Solid SEDDS has been shown to improve gastrointestinal adsorption and also rectal adsorption in addition to vaginal adsorption. Glycyrrhizin, which hardly reaches therapeutic plasma concentrations whenever administered orally, may be reached at therapeutic quantities for chronic hepatic disorders via vaginal or rectal self-emulsifying suppositories [17,21].

Self-emulsifying Implants

Solid SEDDS have progressed thanks to self-emulsifying implants. Self-emulsifying implants are made from co-polymers with a hydrophilic area and about two cross linkable functional groups. These co-polymers function as sealants.

Carmustine is a short-acting drug employed to treat brain tumors. Compression molding was used to develop wafer-like implants from self-emulsifying carmustine, also known as Bis chloroethyl nitrosourea [3,25,27]. All types of SEDDS dosage forms are summarized in Table 2.

Table 2. Case studies on various types of dose formulations generated via solidifying SEDDS

Product Type	Formulation/ Strategy / Sample	Outcome
SE Beads	Self-microemulsifying system for nutraceuticals	Good stability in SGF as well as storage stability was achieved
SE Controlled release pellet	Solid self-microemulsifying pellets for curcumin	Bioavailability was found to be improved in comparison to drug suspension
SE Solid dispersion	Combined Self Nanoemulsifying and Solid Dispersion Systems	Enhanced dissolution was observed at an elevated pH
SE Implant	SEDDS of 1,3-bis(2-choloroethyl)-1-nittosourea-loaded PLGA wafer	First order release and good penetration depth was achieved by developing the SE wafer
SE Microsphere	Zedoary turmeric oil microspheres with SE ability	Bioavailability was found to be improved in comparison to conventional SE systems
SE Nanoparticle	Natural antiproliferative agent loaded self micro-emulsifying nanoparticle	Significant inhibition in the growth of carcinoma cells was observed with piperine loaded SE nanoparticles
SE Suppositories	Self-microemulsifying suppository formulation of β-artemether	Improved pharmacodynamics activity was observed compaired to PEG based suppositories

Bioavailability Enhancement Properties of SEDDS

When a drug is introduced into SEEDS, the solubility is increased since the dissolving stage is bypassed in the event of a Class-II drug (low solubility/high permeability). Parameters affecting the bioavailability of pharmaceuticals are listed in Figure 8.

Drug samples and the enhanced level of bioavailability by using SEDDs are given in Table 3. For example, Ketoprofen, a lipophilic nonsteroidal anti-inflammatory medication (NSAID), is a preferred agent for sustained release formulations, although it has a significant risk of gastritis in long-term treatment. Ketoprofen also demonstrates partial release from sustained release formulations due to its limited solubility. The lipid matrix of SEEDS rapidly interacts with water, resulting in a fine particle

oil-in-water (o/w) emulsion. The drug will be delivered to the gastrointestinal mucosa in a dissolved condition, making it readily available for absorption. As a result, several drugs exhibit a rise in AUC (bioavailability) and Cmax when delivered in SEEDS [16,29,30].

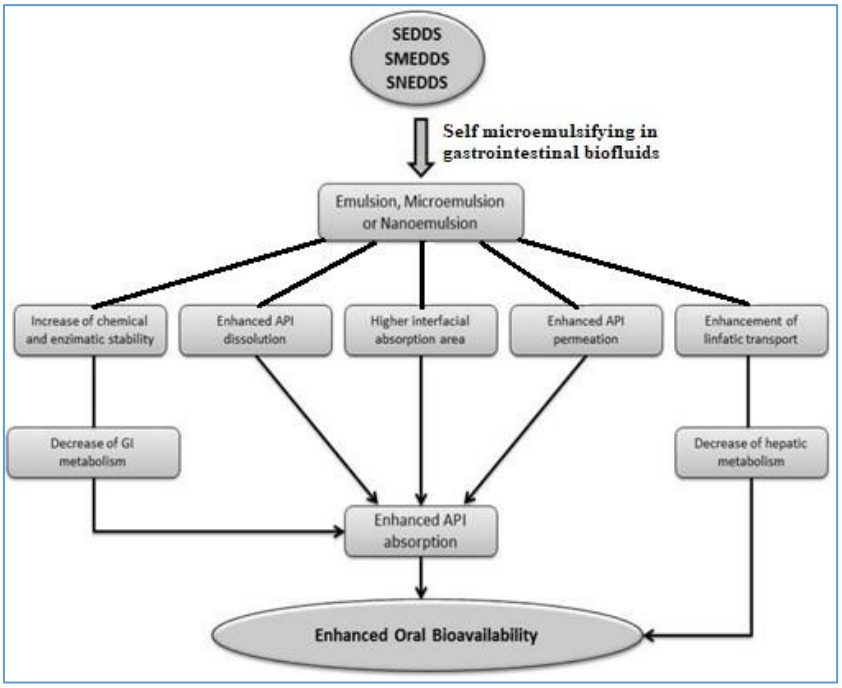

Figure 8. Parameters affecting the bioavailability of pharmaceuticals produced in SEDDS, SMEDDS, orSNEDDS

Table 3. Updated research on several claims of SEDDS enhancing bioavailability

Drug	Enhancement	With reference to	Species
Acyclovir	3.5 fold	Solution	Male albino rats
Anethole trihione	2.5 fold	Tablets	Rabbits
Atorvastatine	1.5 fold	Tablets	Beagle dogs
Bicalutamide	2 fold	Suspension	Rats
Carvedilol	4.13 fold	Tablets	Beagle dogs
Fenofibrate	1.075 fold	Tablets	Human
Gentamycin	5 fold	*iv* Saline	Beagle dogs
Insulin	1.15 fold	sc injection	Beagle dogs
Itraconazole	1.9-2.5 fold	Capsules	Human
Ketoconazole	2 fold	API	Rats
Ketoprofen	1.13 fold	API	Human
Mitotane	3.4 fold	Tablets	Rabbits
Nimedipine	2.6-6.6 fold	Tablets	Rabbits
Nitredipine	1.6 fold	Tablets	Beagle dogs
Slymarin	3.6 fold	Capsules	Rats
Oleanolic acid	2.4 fold	Tablets	Rats
Simvastatin	1.5 fold	Tablets	Beagle dogs
Tritionin	1.67 fold	Capsules	Beagle dogs

RESULT AND DISCUSSION

The quantity of novel therapeutically effective lipophilic molecules that are hydrophobic has steadily increased thanks to innovative drug development approaches. Transforming such compounds into oral delivery formulations having acceptable bioavailability is a huge issue for pharmaceutical experts. The use of SEDDS has been proven to be quite successful in enhancing the oral bioavailability of hydrophobic and lipophilic drug molecules among the strategies to increase oral bioavailability of these compounds.

The two major problems of a formulation intended for the oral route are the aqueous solubilization and the intestinal permeability of the molecule of interest. In the case of a BCS class II compound, characterized by low aqueous solubility and high intestinal permeability, dissolution in the digestive environment is the rate-limiting step for absorption.

The use of SEDDS during the step of pre-formulation makes it possible to solubilize the active principle BCS class II, thus authorizing their passage in sufficient quantity through the intestinal membrane. Furthermore, they make it possible to encapsulate molecules with different hydrophilic/lipophilic balances in the same system. They also allow the reduction of the volume of the vehicle, which minimizes the toxic side effects. In addition, these pharmaceutical forms make it possible to improve the bioavailability of the compound as well as to reduce its inter and intra- individual variability, and obtain a better dose/exposure linearity. This translates into better job security, especially since this molecule will have a narrow therapeutic window. They also limit the effect of food on the absorption of compounds.

The future of pharmaceutical research may not only pass through the discovery of new molecules but also better exploitation of those already known. Indeed, better absorption, a reduction in inter-individual variability, easier administration, and a reduction or even elimination of the side effects of these molecules would be of great benefit to patients.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

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Yayım Koşulları

1. Ankara Üniversitesi Eczacılık Fakültesi Dergisi (Ankara Ecz. Fak. Derg. – J. Fac. Pharm. Ankara) yılda üç kez (Ocak-Mayıs-Eylül) yayımlanır.

2. Dergiye Eczacılığın her alanında daha önce hiç bir yerde yayınlanmamış, Türkçe veya yabancı dilde (İngilizce, Fransızca, İspanyolca ve Almanca) olarak hazırlanmış makaleler kabul edilir. Deneylerde, insan için "the Declaration of Helsinki" ve hayvan için "European Community Guidlines"'a bağlı kalınmalıdır.

3. Yayın Komisyonuna gelen makaleler en az 2 danışmana gönderilir.

4. Makaleler yayına kabul ediliş sırasına göre yayınlanır.

5. Danışmanlar tarafından önerilen düzeltmelerin yapılması için yazar/ yazarlara geri gönderilen makaleler, düzeltilip yayınlanmak üzere 3 ay içinde tekrar yayın kuruluna gönderilmezse, yeni başvuru olarak işlem görür. Makale yayımlandan önce yazarların yayımcıya makalenin "Copyright Transfer Form'unu doldurarak telif hakkını göndermesi gerekmektedir.

6. Yayımlarda intihal olup olmadığı kontrol edilmelidir.

7. Dergimize aşağıdaki makale türleri kabul edilir:

    a) **Araştırma makalesi**: Türkçe veya ingilizce hazırlanmış, şekiller ve tablolar dahil tamamı en çok 20 A4 kağıdı sayfası olan, orjinal araştırmaların bulgu ve sonuçlarını açıklayan makalelerdir.
    b) **Derleme:** Türkçe veya ingilizce hazırlanmış, şekil ve tablolar dahil tamamı en çok 25 A4 kağıdı sayfası olan, yeterli sayıda bilimsel makale taranarak, o güne kadarki gelişmeleri özetleyerek ortaya koyan ve sonuçlarını yorumlayarak değerlendiren makalelerdir.
    c) **Önbilgiler:** Devam etmekte olan bir çalışmanın bulgularını zaman kaybetmeden duyurmak için Türkçe veya ingilizce yazılan en çok 5 A4 kağıdı sayfası olan makalelerdir.

Yazım Kuralları

1. Metinler, A4 normunda (21 x 29,7 cm) yazılmış olmalıdır.
2. Metinler A4 normundaki sayfanın sağ ve sol tarafından 2,5 cm., üst ve alt kenarlarından 3 cm. boşluk bırakılarak 1 satır aralıkla yazılmalıdır. Yayımı kabul edilen makaleler doğrudan "Microsoft Word" dosyası halinde çevrim içi olarak sisteme yüklenecektir (online submission). Ana metin yazı karakteri "**Times New Roman**" ve **11 punto** olmalıdır.
3. Sayfa numaraları makalede **belirtilmemelidir**.
4. Paragraf başları **1 cm içeriden** başlamalıdır. Paragraflar arası ilave boşluk bırakılmamalıdır.
5. Başlık sayfasında yayın adı, yazar/yazarların adları ve yazışma yapılacak yazarın açık adresi, telefon ve e-mail adresi belirtilmeli ve ortalı yazılmalıdır. İlk sayfada başlıktan önce yukarıdan 5 satır aralığı bırakılmalıdır. Başlık ile Öz/Abstract arası 1.5 satır aralıkla yazılmalıdır. Sorumlu yazarın soyadının üstüne (*) işareti konularak belirtilmelidir. Bu kişinin Adı Soyadı, açık adresi, telefon numarası ve e-mail adresi başlık sayfasının en altında belirtilmelidir.
6. **Yazar Adı** (ilk harfi büyük diğerleri **küçük harf**) ve **SOYADI** (tamamı **büyük harf**) **koyu** olarak başlığın altına bir satır aralık verildikten sonra altına unvan belirtmeden yazılmalıdır. Birden çok yazar varsa virgülle ayrılıp bir boşluk bırakılarak yazılmalıdır. Yazarların soyadları üzerine konulacak rakamlarla hemen isimlerin altındaki satıra kurum adları ve posta adresleri (Örneğin: Ankara Üniversitesi Eczacılık Fakültesi, Farmasötik Kimya Anabilim Dalı, 06560, Ankara, Türkiye) açıkça yazılmalıdır.
    - **Tüm yazarlar için ORCID numarası** mutlaka beyan edilmelidir. Yazarların ORCID ID'leri ilgili logoya köprü oluşturularak URL linklerinin eklenmesiyle gerçekleştirilmelidir.
7. Uluslararası kısaltmalar kullanılabilir. Metin içinde mililitre için ml; dakika için dak. olarak belirtilen şekliyle yazılmalıdır.
8. Birimler metrik sistemi kullanılarak ifade edilmelidir.
9. Bütün tablo ve şekiller metin içindeki yerlerine yazım alanından taşmadan yerleştirilmiş olmalıdır.
10. Tablolar üstlerine, şekiller (formül, grafik, şema, spektrum, kromatogram, fotoğraf vb.) de altlarına arabik rakamlarla (**Şekil 1**., **Tablo 2**.,) numaralandırılmalı ve metin içinde yer verilmelidir. "Tablo", "Şekil" sözcükleri ile bunlara ait numaralar koyu yazılmalı ve 11 punto olmalıdır. Şekil/Resim (**JPEG formatında**) makale içinde yerleşmiş ve **resimler 300 dpi veya daha yüksek çözünürlükte** olmalıdır. Üzerinde oynanmış (parlaklık, kontrast, gama ayarı vb.) şekillerde şekil altı metninde yapılan ayarlar belirtilmelidir. **Yazarlar, önceki makalelerinden alıntılanmış olsalar bile, diğer kaynaklardan herhangi bir görüntüyü çoğaltmak için ilgili yayıncılardan yazılı izin almalıdır**.
11. **Tablo** başlıkları Tabloların üstüne ve iki yana yaslı ve bunların genişliğini aşmayacak şekilde 11 punto ve bir satır aralıkta yazılmalıdır. Tabloya ait açıklama varsa tablonun altına 9 punto ile yazılmalıdır. Tablo içindeki metin 8-11 punto arasında yazılabilir. **Şekil** başlıkları ise şekillerin altına birer satır aralıkla ortalı ve 11 punto yazılmalıdır. Şekil başlığı ve şekil arasında 6 nk aralık olmalıdır. Tablo ve Şekiller metin içine yerleştirilirken metin ile aralarında 18 nk aralık olmalıdır.

    Örnek tablolar için bakınız.
    - Tüm satır ve sütun çizgileri yer almalı.
    - Tablo tasarımı tüm makalede tek tip ve düz olmalı, herhangi bir renklendirme/gölgelendirme kullanılmamalıdır.
    - Tablo içinde yer alan başlıklar **bold/koyu** renkte yazılmalıdır. Tablo başlığı ve tablo arasında 6 nk aralık olmalıdır.

Tablo 1. Türlere ait morfolojik özellikler

Bitki kısmı*	***C. nummularia***	***C. integerrimus***
Yaprak	Genişçe eliptik-orbikular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	Orbikulardan ovata kadar farklı şekillerde, 1.2-(4-5) x 0.9-3 cm
Tohum	3.5-4 x 1-2 mm, koyu kahverengi	3-4 x 1.5-2 mm, açık kahverengi

*Açıklama: 9 punto, 1 aralık olmalı.

Tablo 2. Hastaların özellikleri

Demografik bilgiler	**A grubu***	**B grubu**	**C grubu**
Erkek cinsiyet	10 (%30)	20 (%60)	10 (% 30)
Sigara kullanımı	20 (%60)	10 (%30)	20 (%60)

*Açıklama: 9 punto yazılmalıdır.

Örnek şekil;

Şekil 1. *C. nummularia*'nın genel görünüşü (Yazı karakteri "Times New Roman" ve 11 punto, "1" aralık, ortalı)

12. Makalelerin bölümleri **BAŞLIK** (Türkçe ve İngilizce), **ÖZ**, **ABSTRACT**, **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA, TEŞEKKÜR** (varsa eklenmeli), **YAZAR KATKILARI**, **ÇIKAR ÇATIŞMASI**, **ETİK KURUL ONAYI** (varsa eklenmeli) ve **KAYNAKLAR** sırasına uygun olarak hazırlanmalıdır. Bu bölümleri ifade eden başlıklar (Makalenin ilk başlığı hariç) **12 punto ile koyu olarak büyük harflerle ve sayfanın solundan başlanarak** yazılmalıdır. **GİRİŞ**'ten önce ve sonra sırasıyla 24 nk ve 6 nk aralık bırakılmalıdır. Diğer ana başlıklardan önce ve sonra sırasıyla 12 nk ve 6 nk aralık olmalıdır. Bölüm başlıkları ile metin arasında belirtilenin dışında ayrıca aralık **bırakılmamalıdır.**

- **BAŞLIK:** Türkçe ve İngilizce olarak büyük harf ve **ilk başlık** (Türkçe makalelerde Türkçe başlık, İngilizce makalelerde İngilizce başlık ilk başlıktır) **14 punto, koyu** ve ikinci başlık 12 punto, *italik* olarak yazılmalıdır. Başlık metine uygun, kısa, çalışmayı tanıtıcı ve açık ifadeli olmalıdır.

- **ÖZ** ve **ABSTRACT:** Türkçe (**ÖZ**) ve İngilizce (**ABSTRACT**) olarak makalelerin başında **200**'er kelimeyi geçmeyecek şekilde 10 punto ile *italik* olarak yazılmalıdır. Yabancı dilde yazılmış makalelerde önce **ABSTRACT** daha sonra mutlaka Türkçe olarak **ÖZ** bulunmalıdır. **ÖZ** ve **ABSTRACT** başlıkları 12 punto ve koyu yazılıp kendi içlerinde alt başlıklar (aşağıda görüldüğü gibi) halinde makalenin özeti sunulmalıdır. Her bir alt başlık 10 punto, koyu, normal yazılmalıdır. Alt başlıkların içeriğindeki metinler *italik* yazılmalıdır. **ÖZ ve ABSTRACT metni blok halinde sağdan ve soldan 1 cm boşluk bırakılarak yazılmalıdır.**

Özgün makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Gereç ve Yöntem: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Material and Method: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

Derleme makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

- **Anahtar Kelimeler (Keywords):** En fazla 5 sözcükten oluşmalı, ilgili dilde alfabetik, *italik* olarak, yalnızca ilk anahtar sözcüğün ilk harfi büyük olacak şekilde (büyük harf kullanılarak yapılan kısaltmalar hariç) aralara virgül konularak yazılmalı son anahtar sözcükten sonra ise bir imla işareti **kullanılmamalıdır.**

- **METİN:** Orijinal Türkçe makalede metin kısmı **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA** olmak üzere 3 ana başlıktan oluşmalıdır. Bu ana başlıkların tamamı 12 punto, **büyük harflerle** ve koyu olacak şekilde yazılmalıdır. Derleme makalelerde ise **GİRİŞ** ile **SONUÇ VE TARTIŞMA ana başlıkları olmalı,** diğer başlıklar yazarın belirleyeceği şekilde **her kelimenin ilk harfi büyük diğerleri küçük ve koyu** olacak şekilde yazılmalıdır. Alt başlıklar 11 punto, 1satır aralık, **bold/koyu** yazılmalı ve sola dayalı olmalıdır Alt başlıklarda numaralandırma sistemi **kullanılmamalıdır**. Alt başlıklardan önce ve sonra 6 nk aralık olmalıdır.

- **GİRİŞ:** Araştırmanın amacı ve konuyla ilgili çalışmaların yer aldığı bölüm olmalıdır.

- **GEREÇ VE YÖNTEM**: Kullanılan gereç belirtilerek, uygulanan yöntem hakkında gerekli bilgiler açıkça ifade edilmelidir. **Bileşiklerin karakterizasyonu** ayrı bir paragraf ile gösterilmeli ve yeni bileşiklerin saflıkları ve yapı aydınlatılmaları sağlanmalıdır. Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırıcılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul onayı alındığına dair ifadenin çalışma içinde yer almasını sağlamalıdırlar. Etik Kurul onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem kısmında belirtilmelidir. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiği ve onaylandığı, makalenin gereç ve yöntem bölümüne eklenmelidir. Detaylı bilgi için lütfen http://journal.pharmacy.ankara.edu.tr/en/ethical-principles-and-publication-policy/ web sayfasını ziyaret ediniz.

- **SONUÇ VE TARTIŞMA:** Bulguların verilerek değerlendirildiği bölümdür.
  - Metinde sonuçlar (conclusions) başlığı yer **almamalıdır.**

- **TEŞEKKÜR:** Varsa araştırmayı destekleyen kuruluşa ve katkısı olan kişilere Yazarların Katkısından önce yer alan bu bölümde kısaca teşekkür edilebilir.

- **YAZAR KATKILARI:** Makalede yer alan yazarların katkısı yazarlar tarafından imzalanan Telif Hakkı Devir Sözleşmesi (*Copyright Transfer Agreement*) uyarınca, çıkar çatışması bildiriminden hemen önce, makalede yer alan isim sırası gözetilerek yazılmalıdır. Lütfen bu bildirim için açık ad ve soyad yerine aşağıdaki örnekte olduğu gibi yazarların baş harflerini kullanınız. Yazar katkısı belirtilmeyecek alanlar için - işareti konulmalıdır.

  Örnek:

  YAZAR KATKILARI

  Kavram: İ.Y., M.M.H., C.H., K.B.; Tasarım: İ.Y., C.H., I.Ö.G., Ö.Ü.; Denetim: C.H., I.Ö.G., M.M.H., K.B.; Kaynaklar: Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.; Malzemeler: I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.; Veri Toplama ve/veya İşleme: A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.; Analiz ve/veya Yorumlama: Ö.Ü., B.G., T.C.Ş.T., E.K.S.; Literatür Taraması: B.K., D.Ç.P, B.G., B.E.; Makalenin Yazılması: A.K., İ.A., T.C.Ş.T.; Kritik İnceleme: İ.Y., B.G., Ö.Ü., İ.A.; Diğer: -

- **ÇIKAR ÇATIŞMASI BEYANI**

  Çıkar çatışması varsa ne şekilde olduğu açıkça beyan edilmelidir. Eğer yok ise "Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler." ifadesini kullanmalıdırlar.

- **ETİK KURUL ONAYI**

  Çalışmanın sonunda kaynaklardan önce etik kurul onayı alınmışsa hangi kurumdan ve ne zaman alındığı onay numarası ile mutlaka belirtilmeli ve Etik Kurul Onayını makale gönderim sırasında yüklemelidir. Etik kurul onayına gerek olmayan çalışmalarda aşağıdaki cümle yazılmalıdır.

  "Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir."

- **KAYNAKLAR:** Kaynak yazım stili Amerikan Psikoloji Derneği'ne (APA) göredir. Yazı karakteri "Times New Roman" ve 10 punto, "1" aralık, iki yana yaslı. Metinde, geçiş sırasına göre köşeli parantez içinde, örneğin: [1,6,9], [5-7] gibi numaralandırılmalı ve metin sonunda bu numaralara göre sıralanmalıdır. Alt başlıkların yanına kaynak belirtilmemelidir. Tablo içinde kaynak bildirilmesi gerekiyorsa metin içinde verildiği gibi belirtilmelidir.

  - **Makale için:** Yazarın soyadı, adının baş harfleri (Birden fazla adı olan yazarın her bir isminin baş harfinden sonra nokta konmalı ve arada boşluk bırakılmamalıdır. Birden fazla yazarların arasında virgül yer almalıdır. **Son yazar ile bir önceki yazar arasında "ve" kelimesi veya "&" sembolü kullanılmamalıdır**.), makalenin tam başlığı, derginin adı, cilt no, varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa numarası, yıl yazar isimlerinden sonra (parantez içinde) yazılmalıdır. **Birden fazla yazar varsa hepsi yazılmalıdır.** Makalenin adı yazılırken ilk kelimenin ilk harfi büyük diğer kelimelerin ilk harfi küçük yazılmalıdır. Kaynaklarda verilen **dergi adları kısaltma yapılmadan açık olarak yazılmalıdır.**

Her bir referansın sonuna [CrossRef] **ekleyerek aşağıdaki formatta DOI numarasını köprü olarak giriniz. Lütfen https://www.crossref.org/'da yer almayan makaleleri** [CrossRef] şeklinde **belirtmeyiniz.**
https://doi.org/10.1016/0006-2952(89)90403-6

Örnekler:

1. Martinez, M.J.A., Del Olmo, L.M.B., Benito, P.B. (2005). Antiviral activities of polysaccharides from natural sources. Studies in Natural Products Chemistry, 30, 393-418. [CrossRef]
2. Bahiense, J.B., Marques, F.M., Figueira, M.M., Vargasa, T.S., Kondratyuk, T.P., Endringer, D.C., Scherer, R., Fronzaa, M. (2017). Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. Pharmaceutical Biology, 55(1), 991-997. [CrossRef]

- **Elektronik Makale için:**

Örnek:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. British Medical Journal, 317, from http://www.bmj.com/cgi/content/full/317/7150/ Erişim tarihi: 14.03.2021

- **Web sitesi için:**

Örnek:
Clinical Pharmacology Web site. (2001). Erişim adresi http://cpip.gsm.com/ Erişim tarihi: 14.03.2021.

- **Kitap için:** Yazarın soyadı, adının baş harfleri, kitabın adı, cilt no (varsa), kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

- **Kitap bölümü için:** Yazarın soyadı, adının baş harfleri, bölümün başlığı, editör/editörlerin soyadı, adının baş harfleri, (Ed./Eds.) ibaresi, kitabın adı, varsa cilt no, kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Weinberg, E.D. (1979). Antifungal Agents. In: M.E. Wolff and S.E. Smith (Eds.), Burger's Medicinal Chemistry, (pp. 531-537). New York: John Wiley and Sons.

- **Tez için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Ne tür tez olduğu belirtildikten sonra tezin başlığı, nerde yapıldığı yazılmalıdır.

Örnek:
Ahmed, J. (2008). PhD Thesis. Pharmaceutical Botany investigations on *Prangos* Lindl. (Umbelliferae) growing in Konya province. Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, Turkey.

- **Patent için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Patent başlığı ve patent numarası yazılmalıdır.

Örnek:
Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

Etik İlkeler ve Yayın Politikası

Editörün Sorumlulukları

Yayın politikası:

Ankara Üniversitesi Eczacılık Fakültesi Dergisi, açık erişimli, hakemli bir dergi olup Türkçe veya İngilizce olarak farmasötik bilimler alanındaki önemli gelişmeleri içeren orijinal araştırmalar, derlemeler ve kısa bildiriler için uluslararası bir yayım ortamıdır.

Editör, dergiye gönderilen makalelerden hangilerinin yayınlanması gerektiği kararından tek başına sorumludur. Editörün kararı, derginin editör kurulunun prensipleri doğrultusunda olabileceği gibi, onur kırıcı yayın yapmak, telif hakkı ihlali ve intihal gibi konularla ilgili olarak yürürlükte olan yasal gereklilikler ile sınırlandırılmıştır. Editör, karar verirken editör kurulunun üyelerine ya da hakemlere danışabilir. Makale yayımlanmadan önce yazarların yayımcıya makalenin "*Copyright Transfer Form*"unu doldurarak telif hakkını göndermesi gerekmektedir. Dergiye gönderilen makaleler editör ve editör yardımcıları tarafından biçimsel olarak incelenir. Dergi kurallarına göre hazırlanmamış makaleler kesinlikle değerlendirmeye alınmayarak reddedilmektedir.

Yayın değerlendirmesi:

Editör, yayın değerlendirme sürecinin adil, tarafsız ve zamanına uygun şekilde gerçekleştiğini garanti altına almaktadır. Araştırma makaleleri genel olarak dışardan ve bağımsız en az iki hakem ile değerlendirilmekte ve gerek olması durumunda editör üçüncü bir hakemden ek görüş istemektedir. Editör, gerekli, kapsayıcı ve çeşitli düzeylerde değerlendirmelere ihtiyaç duyulacağını göz önüne alarak, ilgili alanda uygun uzmanlığı olan hakemler seçmektedir. Editör, olası çıkar çatışmaları için yapılan açıklamaları, hakemler tarafından yapılan "*self-citation*" önerilerini ve herhangi bir taraflılık olasılığını değerlendirmek ve karar vermek için dikkatli bir şekilde gözden geçirmektedir.

Editör, bir makaledeki hataları yayınlanmadan önce tespit ederse düzeltmeli, daha sonra tespit edilmesi durumunda ise düzeltmeleri yayınlamalıdır. Tüm düzeltme veya geri çekme bildirimleri, dergide belirgin bir şekilde yayınlanmalı ve orijinal makaleye veya özete tam bibliyografik referansı içermelidir. Ayrıca içindekiler sayfasında listelenmeli ve belirgin bir şekilde etiketlenmelidir (ör. Yazım hatası, geri çekme veya özür dileme).

Adil değerlendirme:

Editör, makalaleri yazarların ırk, cinsiyet, cinsel eğilim, inanç, etnik köken, vatandaşlık ya da politik görüşlerine bakmaksızın bilimsel içeriklerine göre değerlendirmektedir. Derginin editöryal prensipleri şeffaf ve tümüyle dürüst değerlendirmeyi desteklemektedir. Editör, hakemlerin ve yazarların kendilerinden bekleneni tam olarak anladıklarından emin olmalıdır. Editör, dergi ile ilgili tüm iletişimini derginin elektronik başvuru sisteminden yapmaktadır. Editör, editöryal kararlara itiraz durumunda şeffaf bir mekanizma işletmektedir.

Gizlilik ilkesi:

Editör, dergiye yapılan başvurudaki tüm materyallerin ve hakemlerle yapılan tüm iletişimin gizliliğini (ilgili yazar ve hakemlerle aksi onaylanmadığı sürece) korumakla yükümlüdür. Hakemler isimlerinin açıklamasını kabul etmediği sürece, editör, hakemlerin kimliklerini korumaktadır. Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan editörün kendi çalışmaları/araştırmaları için kullanılmamalıdır. Değerlendirme sürecinde edinilen ayrıcalıklı bilgi ya da fikirler gizli tutulmalı ve kişisel amaçlar için kullanılmamalıdır.

Hakemlerin Sorumlulukları

Editöryal kararlara katkı:

Etik ile ilgili sorumluluklara ek olarak, hakemlerin yazarlara ve sundukları çalışmalara uygun şekilde davranmaları ve iyi bir değerlendirme performansı göstermeleri istenmektedir. Makalede yer alan araştırmayı değerlendirmeye yetkin olmadığını düşünen ya da verilen sürede değerlendirmeyi yapamayacağını farkeden hakemin, editörü bilgilendirmesi ve değerlendirme sürecinden çıkartılması gerekmektedir.

Gizlilik:

Hakemler makale hakkındaki değerlendirmelerini ya da bilgilerini hiç kimseyle paylaşmamalı, editörden izin almadan yazar ile iletişime geçmemelidirler. Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan hakemin kendi çalışmaları/araştırmaları için

kullanılmamalıdır. Değerlendirme sürecinde edinilen bilgi ya da fikirler gizli tutulmalı ve kişisel amaçlar için kullanılmamalıdır.

Etik sorunları farketme:

Hakem, makalede yer alan olası etik sorunları farketmeli ve editörün dikkatine sunmalıdır. Bahsedilen olası sorunlar içinde, değerlendirmede olan makale ile daha önce basılmış bir makale arasındaki önemli ölçüdeki benzerlik ya da örtüşme de bulunmaktadır. Daha önce yayınlanmış olan herhangi bir gözlem ve/veya argüman, ilgili kaynak/referans ile birlikte verilmelidir.

Tarafsızlık ve rekabet standartları:

Hakemler tarafsız olarak değerlendirmelerini yapmalıdırlar. Hakemler, makale değerlendirmesi sırasında önyargılı olabilecekleri konusunda farkında olmalıdırlar. Yazarın kişi olarak eleştirilmesi uygun değildir. Hakemler görüşlerini destekleyici argümanlarla ifade etmelidirler. Hakemler makaleyi değerlendirmeyi kabul etmeden önce olası çıkar çatışması durumuna karşı (makale ile ilişkili yazar, şirket ya da kurum ile rekabetçi, işbirliği içinde olan ya da diğer ilişkiler/bağlantılar için) editöre bilgi vermelidirler. Hakem, yazarın hakemin (ya da hakemle birlikte çalışan kişilerin) çalışmalarının kaynak olarak alındığını ileri sürerse, gerçek bilimsel gerekçeler sunulmalı, bu durumun hakemin kaynak gösterilme sayısını ya da çalışmalarının görünürlüğünü arttırmaya yönelik bir girişim olmamasına özen gösterilmelidir.

Yazarın Sorumlulukları

Bildirim standartları:

Orijinal araştırmanın makalesini hazırlayan yazarlar, çalışmanın önemine ilişkin tarafsız bir tartışma ile gerçekleştirilen araştırmayı net bir şekilde sunmalıdırlar. Makalede veri açık bir şekilde sunulmalıdır. Çalışma, diğer araştırmacıların çalışmayı tekrar edebilmesine izin verecek şekilde yeterli detay ve kaynak içermelidir. Yanıltıcı ya da bilinçli olarak net olmayan ifadelerin verilmesi etik olmayan davranış olarak görülür ve kabul edilmemektedir.

Veri ulaşımı ve saklama:

Yazarlardan editöryal değerlendirme için makalelerini destekleyici araştırma verisi istenebilir. Yazarların belirtilen veriyi erişime açmaları, yayınlandığı tarihten belli bir süre sonra da gerekli görülmesi durumunda belirtilen veriyi sunabilmeleri gerekmektedir.

Orijinallik, intihal ve kaynakların belirtilmesi:

İntihal, yazarın başka bir makaleyi kendi çalışması olarak göstermesi, kaynak göstermeden başka birine ait çalışmanın belli bölümlerinin kopyalanması ya da başka sözcüklerle anlatılması veya başkaları tarafından yapılan çalışmanın sonuçlarının alınarak sunulması şeklinde olabilir. İntihalin her biçimi etik olmayan davranıştır ve kesinlikle kabul edilmemektedir. Yazarlar çalışmalarının tümüyle orijinal olduğunu garanti etmelidirler. Eğer yazarlar başkalarına ait iş ve/veya metinleri kullanıyorlarsa, mutlaka uygun şekilde kaynak ya da alıntı gösterilmeli ve gerekliyse izin alınmalıdır. Yazarlar kendilerine ait olan çalışmayı etkileyen ve çalışmaya ait uygun içeriğin oluşturulmasında katkısı olan tüm yayınları veya eserleri kaynak olarak göstermelidirler. Özel olarak (görüşme, yazışma ya da üçüncü taraflar ile tartışma) ile elde edilen bilgiler kullanılmamalı ya da kullanılacaksa izin alınarak bildirilmelidir. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderilen tüm makaleler, yazarlar tarafından, intihal tarama programları ile taranmış olmalıdır.

Çoklu, gereksiz ve tekrar yayınlama:

Aynı makale ile birden fazla dergiye başvuruda bulunmak etik olmayan bir davranıştır ve asla kabul edilmemektedir. Genel olarak, yazar daha önce basılmış bir yayını, özet formunda ya da yayınlanmış bir ders, akademik tez ya da elektronik ön baskının bir parçası olması dışında, değerlendirme için başka bir dergiye göndermemelidir.

Yazar katkıları:

Yazar katkıları, çalışmanın konseptine, tasarımına, gerçekleştirilmesine ya da yorumlanmasına önemli katkı sağlayan kişiler ile sınırlandırılmalıdır. Belirtilen süreçlere önemli katkı sağlayan kişiler eşyazar (co-author) olarak belirtilmelidir. Çalışmaya başka açılardan (dil düzenlemesi ya da tıbbi yazım gibi) katkı sunan kişiler "Teşekkür" (Acknowledgement) bölümünde yer almalıdırlar. Sorumlu yazar, tüm eşyazarların çalışmada uygun şekilde yer aldığına, tüm eşyazarların çalışmayı görüp onayladıklarına ve yayınlanmak üzere başvuru yapılmasına dair verdikleri onaya ilişkin sorumluluğu üstlenmelidir. Yazarlar, makale başvurusu yapmadan önce yazar listesini ve sıralamasını dikkatli bir şekilde değerlendirmeli ve orijinal başvuru sırasında kesin bir yazar listesi sunmalıdırlar. Çalışmanın başvurusu

tamamlandıktan sonra, sadece istisna durumlarda, editör yazar listesinde ekleme, silme ya da yeniden düzenleme yapabilir. Tüm yazarlar bu şekilde yapılacak ekleme, silme ve yeniden düzenleme konusunda fikir birliği içinde olmalıdırlar. Tüm yazarlar çalışmanın ortak sorumluluğunu aldıklarını kabul ederler. Her yazar, uygun şekilde araştırılan ve karara bağlanan çalışmanın kesinliği ve bütünlüğü ile ilişkili sorulardan sorumludur. Ankara Üniversitesi Eczacılık Fakültesi Dergisine makale ile birlikte "Yazar Katkı Formu"nun da doldurulup gönderilmesi gerekmektedir.

Çıkar çatışması beyanı:

Tüm yazarlar, çalışmalarını uygunsuz bir şekilde etkileyebilecek olarak gördükleri diğer kişi veya organizasyonlarla her türlü maddi ve kişisel ilişkilerini beyan etmelidirler. Araştırmanın yürütülmesinde ve/veya makalenin hazırlanmasında maddi destek sağlayan tüm kaynaklar ile çalışmanın planlanması, verinin toplanması, analizi ve yorumlanması, raporlanması ve makalenin yayın için başvurma kararının alınmasında rol oynayan kişi ve/veya kurumlar belirtilmelidir. Fon kaynaklarının belirtilen şekilde bir katkısı olmamışsa, bu durum da ifade edilmelidir. Açıklanması gereken potansiyel çıkar çatışmalarına örnek olarak istihdam, danışmanlıklar, hisse sahipliği, ücret, ücretli uzman tanıklığı, patent başvuruları/tescilleri ve hibeler veya diğer fonlar sayılabilir. Olası çıkar çatışmaları mümkün olan en erken aşamada açıklanmalıdır.

Temel hataların bildirimi:

Yazar, yayınlanmış olan bir çalışmasında önemli bir hata ya da eksiklik farkettiğinde, acil olarak dergi editörüne/yayınevine bildirmek ve editör tarafından gerekli görülmesi durumunda makaleyi geri çekmek veya düzeltmek için editörle işbirliği yapmak ile yükümlüdür. Editör/yayınevi yayınlanmış olan makalenin bir hata içerdiğini üçüncü bir taraftan öğrenirse, editör ile işbirliği yapmak ve gerektiğinde destekleyici kanıt sağlamak, yazarın yükümlülüğüdür.

Olası riskler ve insan veya hayvan konuları:

Eğer kullanımları sırasında olağandışı risk yaratan kimyasallar, işlemler ya da malzemeler çalışmada yer alıyorsa, yazar bu durumu metin içinde açıkça belirtmelidir. Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırıcılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul onayı alındığına dair ifadenin çalışma içinde yer almasını sağlamalıdırlar. Etik Kurul onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem kısmında belirtilmelidir. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiği ve onaylandığı, makalenin gereç ve yöntem bölümüne eklenmelidir. Etik kurul raporu alınması gerektiği halde, etik kurul raporu olmayan çalışmalar reddedilecektir. İnsanlar veya insandan elde edilen örnekler üzerinde yapılan klinik araştırmalarda bilgilendirilmiş onam formu mutlaka alınmış olmalıdır ve gereç ve yöntem kısmında belirtilmelidir. İnsan gönüllüleri ile yapılan araştırmalar için araştırma protokolüne uygun olarak hazırlanmış yazılı bilgilendirilmiş gönüllü onam formu alınmalıdır. İnsanlar üzerinde gerçekleştirilen tüm araştırmalar Helsinki Bildirgesi ilkelerine göre yapılmalıdır (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). Tüm hayvan çalışmaları ARRIVE kılavuzuna uygun olmalı (Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines) ve "Bilimsel Amaçlı Kullanılan Hayvanların Korunmasına İlişkin Konsey Direktifi"ne (EU Directive 2010/63/EU for animal experiments), "Birleşik Krallık Hayvan Yasası"na (The U.K. Animals (Scientific Procedures) Act 1986) ve/veya "*U.S. İnsan Bakımı ve Laboratuvar Hayvanlarının Kullanımına İlişkin Halk Sağlığı Hizmeti Politikası"* rehberine (U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals) uygun şekilde yürütülmelidir. Bitkiler ile ilgili tüm deneysel araştırmalar, uluslararası yönergelere uygun olmalıdır.

Guide for Authors

1. The Journal of Faculty of Pharmacy of Ankara University (J. Fac. Pharm. Ankara) is published three times (January-May-September) a year.

2. The Journal of Faculty of Pharmacy of Ankara University publishes articles in every field of Pharmaceutical Sciences. The manuscript to the journal should not be published previously as a whole or in part and not be submitted elsewhere. Manuscript should be written in Turkish or in a foreign language (English, French, Spanish or German). The experiments used have to be adhered to the Declaration of Helsinki for humans and European Community Guidlines for animals.

3. All manuscripts will be submitted to a review process by the editors and by qualified at least 2 outside reviewers.

4. Manuscripts are published in order of final acceptance after review and revision.

5. If a manuscript returned to the authors for revision is not received back to the editor within 3 months it will be treated as a new article. When the article is published, the by authors are considered to transfer all rights of the manuscript to the Publisher.

6. Manuscript will be controlled using plagiarism checker.

7. Manuscripts with the following charactheristics are accepted:

    a) **Research article**: Articles written in English or Turkish in scientific format presenting original research. Articles should be printed on A4 size papers not exceeding 20 pages (including tables and figures).
    b) **Review:** An updated comprehensive review of scientific works on a particular subject. Articles written in English or Turkish should be printed on A4 size papers not exceeding 25 pages (including tables and figures).
    c) **Rapid communication:** Rapid announcement of the results of a continuing research written in English or Turkish, no longer than 5, A4 size pages.

Preparation of Manuscript

1. Texts must be written in A4 norm (21 x 29.7 cm).
2. Texts should be written with 1 line spacing, with 2.5 cm margins on the left and right sides of the A4 norm page, 3 cm margins each from the top and bottom edges (5 line spacing from the top on the first page). Articles accepted for publication will be directly uploaded to the system as a "Microsoft Word" file (online submission). The main text font should be **"Times New Roman"** and **11 pt**.
3. Page numbers **should not be specified** in the article.
4. Paragraph headings must **begin 1 cm inside**. Additional spaces should not be left between paragraphs.
5. On the title page, the title of the manuscript the name/s and the full address/es of the author/s, and the full address, telephone number, e-mail address of the corresponding author should be written and all should be centered in the text. It should be indicated by placing (*) above the surname of the corresponding author. Name, surname, full address, telephone number and e-mail address of this person should be specified at the bottom of the title page.
6. **Author's Name** (**first letter capital, others lowercase**) and **SURNAME** (**all capital letters**) should be written in bold, three lines spaced under the title, and without a title underneath. If there is more than one author, they should be written by separating them with a comma and leaving a space. The numbers to be placed on the surnames of the authors and the institution names and postal addresses (For example: Ankara University Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06560, Ankara, Turkey) should be clearly written on the line just below the names.
   - **ORCID ID number must be declared for all authors**. ORCID IDs of the authors should be created by creating a hyperlink to the relevant logo and adding URL links.
7. International abbreviations may be used. ml for milliliter in the text; min. for minutes It should be written as specified.
8. Units should be expressed using the metric system.
9. All tables and figures should be placed in their places in the text without exceeding the writing area.
10. Tables should be numbered on the top, figures (formula, graph, chart, spectrum, chromatogram, photograph, etc.) should be numbered below with Arabic numbers (**Figure 1., Table 2.)** and should be included in the text. The words "Table", "Figure" and their numbers should be written in bold and in 11 pt. Figure/Picture (**in JPEG format**) must be placed in the article and pictures must be at least **300 dpi or in higher resolution**. Authors must obtain written permission to reproduce any images from other sources.
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    See for below examples for tables:
    - All row and column lines should be included.
    - Table design should be uniform and straight throughout the article, no coloring / shading should be used.
    - Headings in the table should be written in **bold**. There must be **6 nk** space between the table and table title.

Table 1. Morphological characteristics of the species

Plant part*	***C. nummularia***	***C. integerrimus***
Leaf	Broadly elliptical-orbicular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	From orbicular to ovate, 1.2-(4-5) x 0.9-3 cm,
Seed	3.5-4 x 1-2 mm, dark brown	3-4 x 1.5-2 mm, light brown

* Explanation should be 9 font size, 1 range.

Table 2. Patient demographics

Demographics	**Group A***	**Group B**	**Group C**
Male gender	10 (%30)	20 (%60)	10 (% 30)
Cigarette consumption	20 (%60)	10 (%30)	20 (%60)

* Explanation should be 9 font size, 1 range.

Example for figure:

Figure 1. General view of *C. Nummularia* (The font size must be 11 pt with 1 line spacing and "Times New Roman" font, and must be centered in the text)

12. The sections of the articles should be prepared in accordance with the **TITLE** (Turkish and English), **ABSTRACT, INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION, ACKNOWLEDGEMENTS** (if available), **AUTHOR CONTRIBUTIONS, CONFLICT OF INTEREST, ETHICS COMMITTEE APPROVAL** (if available) and **REFERENCES**. Titles expressing these sections (except the first title of the article) should be written in **12 pt**, **bold capital letters and starting from the left of the page**. **There should be 24 nk space before and 6 nk space after the INTRODUCTION.** For, there should be 12 nk space before and 6 nk space after the other titles. Between the chapter titles and the text, a separate space **should not be left** other than the specified in this document.

- **TITLE:** Capital letters and **first title** in Turkish and English (Turkish title is the first title in Turkish articles, English title is the first title in English articles), **14 pt, bold** and the second title should be written in 12 pt, *italic*. The title should be appropriate to the text, short, introducing the work and clearly worded.

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Result and Discussion: *Text should be written in italic.*
Keywords:

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Anahtar Kelimeler: *Text should be written in italic.*

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Objective: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler:

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