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ORIGINAL ARTICLE / ÖZGÜN MAKALE

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

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

Esra KÖNGÜL ŞAFAK[1], Selen İLGÜN[2]*, Kübra Nur ÇOBAN[1], Sena AKÇAKAYA MUTLU[2], Halil YILMAZ[3], Gökçe ŞEKER KARATOPRAK[1]

[1]Erciyes University, Faculty of Pharmacy, Department of Pharmacognosy, 38039, Kayseri, Türkiye

[2]Erciyes University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 38039, Kayseri, Türkiye

[3]Ordu University, Faculty of Medicine, Department of Anatomy, 52200, Ordu, Türkiye

ABSTRACT

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

ÖZ

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

* **Corresponding Author / Sorumlu Yazar:** Selen İlgün
e-mail / e-posta: serturk@erciyes.edu.tr, **Phone / Tel.:** +903524380486

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

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

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

INTRODUCTION

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

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

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

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

MATERIAL AND METHOD

Plant Material and Extraction Procedure

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

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

Antioxidant Activity

DPPH• Radical Scavenging Activity

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

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

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

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

Enzyme Inhibition Tests

Lipoxygenase Inhibitory Activity

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

α-Glucosidase Inhibitory Activity

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

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

Cell Culture

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

Cytotoxic Activity

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

Statistical Analysis

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

RESULT AND DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

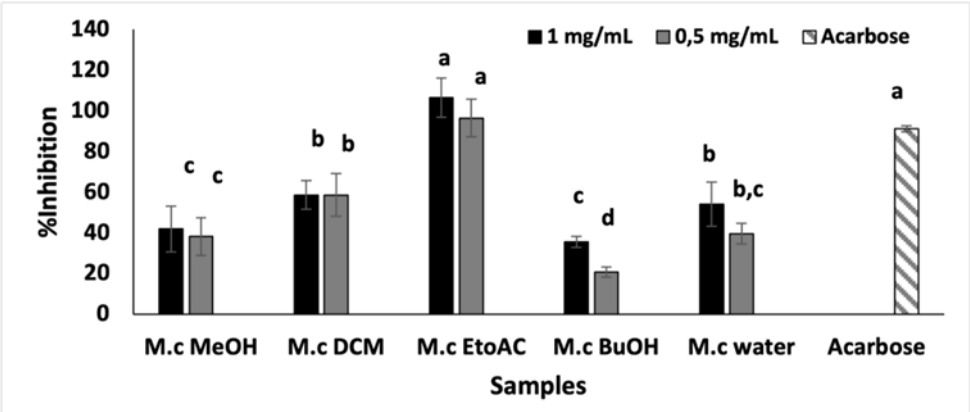

Figure 1. α-Glucosidase enzyme inhibition activities of *M. communis* extracts. M.c MeOH: *M. communis* methanol extract, M.c DCM: *M communis* dichloromethane fraction, M.c EtOAc: *M. communis* ethyl acetate fractions, M.c BuOH: *M. communis* butanol fractions, M.c water: *M. communis* residual water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

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

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

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

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

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

AUTHOR CONTRIBUTIONS

Concept: E.K.Ş., S.İ., G.Ş.K.; Design: E.K.Ş., S.İ., G.Ş.K.; Control: S.A.M., G.Ş.K.; Sources: S.A.M., G.Ş.K.; Materials: E.K.Ş., S.İ., K.N.Ç., S.A.M., H.Y., G.Ş.K.; Data Collection and/or Processing: E.K.Ş., S.İ., K.N.Ç., S.A.M., H.Y., G.Ş.K.; Analysis and/or Interpretation: E.K.Ş., S.İ., K.N.Ç., S.A.M., H.Y., G.Ş.K.; Literature Review: G.Ş.K.; Manuscript Writing: K.N.Ç., S.A.M., G.Ş.K.; Critical Review: E.K.Ş., S.İ., S.A.M., G.Ş.K.; Other -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

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

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