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Comparative assessment of phytochemical composition, antioxidant and anticholinesterase activities of two Basidiomycota Truffle Fungi from Turkey

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ABSTRACT: The aim of the present study was to compare phytochemical composition and biological activities of various extracts of two Basidiomycota truffle collected from Turkey. Analysis of phytochemical compositions in terms of phenolic and fatty acid compositions was determined using HPLC-DAD, GC and GC/MS. Fumaric (10.62, 4.12 µg/g) and protocatechuic acids (2.71, 20.50 µg/g) were identified as the major organic and phenolic acids in *Chondrogaster pachysporus* and *Setchelliogaster tenuipes* truffles. Linoleic acid (53.03 %) for *C. pachysporus* and palmitic acid (38.60 %) for *S. tenuipes* were found to be the most dominant fatty acids. The extracts were also tested for their antioxidant and anticholinesterase activities. As for antioxidant and anticholinesterase activity results, *C. pachysporus* truffle showed better activity than *S. tenuipes*. This work presents the first phytochemical study on *C. pachysporus* and *S. tenuipes* truffles and sheds light on their potential usage as the natural source in the food, pharmaceutical, and cosmetic industries.

KEYWORDS: Anticholinesterase effect; antioxidant activity; truffle (Basidiomycota); *C. pachysporus*; *S. tenuipes*; phenolic compounds.

1. INTRODUCTION

Mushrooms are considered as valuable foods in many culture due to their unique flavor [1], taste [2, 3], nutritional properties [4, 5] and biological activities [6-8].

Truffle species are a group of hypogenous mushroom and consist of two classes: Truffles (Ascomycetes) having sac-like spore producing structures (the ascus; plural asci); The false truffles or truffle-like fungi (Basidiomycetes) containing a typical mushroom fruitbody and less mushroom-like. Most truffle-like fungi form ectomycorrhizae with plant hosts, especially in the families Pinaceae, Cistaceae, Saliaceae, Betulaceae, Fagaceae, and Myrtaceae [9]. Basidiomycetes including to mushroom species of bolete, polypores, shelf fungi, bird's nests, stinkhorns and puffballs, is the most familiar mushroom to humans. Although they are made of different types of fruitbodies, spore production style is common [10].

Truffles have been used as culinary delights for thousands of years and were known by ancient Egypt, Greek, and Roman civilizations [11]. Truffles are the most highly valued, both economically and gastronomically because of their flavor and delicious taste [12]. They are rich in protein (amino acids), minerals, fatty acids, and carbohydrates [13]. In addition to their nutritional properties, truffle species show biological properties such as antiinflammatory, antioxidant, antimicrobial, antiviral, anti-mutagenic, hepatoprotective and anticancer [11, 14-19].

Due to the nutritional and biological significance of truffle species, investigations about these species have been increased. In this study, we aimed to determine antioxidant and anticholinesterase activities of the hexane, ethyl acetate, methanol, butanol and water extracts of *C. pachysporus* and *S. tenuipes* truffles spreads under the eucalyptus trees. Also, phenolic compounds were identified by HPLC-DAD and fatty acid compositions were investigated by GC and GC/MS. According to our literature survey, there is no study about these species. So, this is the first study on chemical composition and biological activities of *C. pachysporus* and *S. tenuipes* truffles.

2. RESULTS AND DISCUSSION

2.1. Phenolic composition

Phenolic compounds are secondary metabolites capable of exhibiting antioxidant, antimicrobial, anti-inflammatory and anti-cancer activities [20]. Because of this biological

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importance of phenolic compounds, in this study, the phenolic profiles of *C. pachysporus* and *S. tenuipes* were investigated in details (Table 1).

Table 1. Composition of phenolic and organic acids in Truffle species[a]

Compounds	RT (min)	*C. pachysporus* (μg/g)	*S. tenuipes* (μg/g)
Gallic acid	4.37	nd	nd[b]
Fumaric acid	5.59	**10.62±0.002**	**4.12±0.002**
Protocatechuic acid	6.87	**2.71±0.001**	**20.50±0.002**
Catechin hydrate	8.46	0.89±0.001	nd
p-hydroxybenzoic acid	10.64	nd	0.41±0.001
6,7-dihydroxy coumarin	11.62	nd	0.48±0.001
Caffeic acid	13.13	0.08±0.001	0.11±0.001
Vanillin	14.89	nd	nd
2,4-dihydroxy benzoic acid	15.54	nd	nd
p-coumaric acid	18.74	nd	0.09±0.001
Ferulic acid	19.76	0.04±0.001	0.19±0.002
Coumarin	20.96	0.01±0.001	0.02±0.001
trans-2-hydroxy cinnamic acid	21.98	nd	nd
Ellagic acid	22.54	0.21±0.001	0.58±0.002
Rosmarinic acid	23.61	0.18±0.001	0.27±0.001
trans-cinnamic acid	24.52	0.24±0.001	0.12±0.001

[a] Values represent the means ± S.E.M. of three parallel measurements ($p<0.05$).
[b] nd: Not detected

Phenolic and organic acid compounds (expressed as μg/g extract) were identified by HPLC-DAD and results are presented in Table 1. Sixteen phenolic compounds namely, gallic acid, fumaric acid, protocatechuic acid, catechin hydrate, p-hydroxybenzoic acid, 6,7-dihydroxy coumarin, caffeic acid, vanillin, 2,4-dihydroxybenzoic acid, p-coumaric acid, ferulic acid, coumarin, *trans*-2-hydroxycinnamic acid, ellagic acid, rosmarinic acid and *trans*-cinnamic acid were analyzed. While nine of them were identified in *C. pachysporus*, eleven of them were identified in *S. tenuipes*. Fumaric acid was the predominant phenolic compound with a value of 10.62±0.002 μg/g, followed by protocatechuic acid (2.71±0.001 μg/g) and catechin hydrate (0.89±0.001 μg/g) in *C. pachysporus*. The most abundant phenolic compounds in *S. tenuipes* were identified as protocatecheuic acid (20.50±0.002 μg/g), fumaric acid (4.12±0.002 μg/g) and ellagic acid (0.58±0.002 μg/g), respectively (Table 1). There is a limited number of studies about identification and quantification of phenolic compounds of Hypogeous Ascomycota truffles in the literature. In previous studies, Villares *et al.* detected phenolic compounds in *Tuber melanosporum*, *T. aestivum* and *T. indicum* and homogentisic acid were identified as the major phenolic compound in these three truffles. Phenolic compounds of extracts of *T. aestivum* and *T. magnatum* were studied and p-hydroxybenzoic acid, protocatechuic acid, and gallic acid were found by Beara *et al.* In another research, phenolic compounds of extracts of *T. boudieri* were determined as catechin, cinnamic acid, ferulic acid and p-coumaric acid by Doğan and Aydın [22]. The obtained results are consistent with the previous studies.

2.2. Fatty acid composition

Fatty acids can regulate lipid metabolism by interacting with enzymes and nuclear transcription factors and affecting mRNA stability. Linoleic acid and linolenic acid are essential fatty acids. The deficiency of daily consumption of essential fatty acids causes in etiology and progression of diseases such as cardiovascular disease and diabetes [23, 24]. GC and GC/MS were used to analyze fatty acid compositions of *C. pachysporus* and *S. tenuipes*. The results were expressed as percentage of total fatty acids and given in Table 2.

Table 2. The fatty acid compositions of Truffle species (%)

Fatty acids	*C. pachysporus* (%)	*S. tenuipes* (%)
Caprylic acid ($C_{8:0}$)	nd	1.04
Nonanoic acid ($C_{9:0}$)	nd	6.71
Pentadecanoic acid ($C_{15:0}$)	3.22	nd
Palmitic acid ($C_{16:0}$)	**16.01**	**38.60**
Margaric acid ($C_{17:0}$)	3.78	nd
Stearic acid ($C_{18:0}$)	4.50	**12.26**
Arachidic acid ($C_{20:0}$)	nd	1.24
Σ Saturated fatty acids	27.51	59.85
Palmitoleic acid ($C_{16:1}$ ω7)	4.91	nd
Oleic acid ($C_{18:1}$ ω9)	**14.55**	**34.80**
Σ Monounsaturated fatty acids	19.46	34.80
Linoleic acid ($C_{18:2}$ ω6)	**53.03**	5.35
Σ Polyunsaturated fatty acids	53.03	5.35
Σ Unsaturated fatty acids	72.49	40.15
ω6/ω9	3.64	0.15

ω6/ω9: linoleic/oleic acid ratio; nd: not detected

The major fatty acid was found as linoleic acid (53.03%), followed by palmitic (16.01%) and oleic acids (14.55%) in *C. pachysporus*. Palmitic acid (38.60%) was the most abundant fatty acid, followed by oleic (34.80%) and stearic acids (12.26%) in *S. tenuipes* as seen in Table 2. In earlier studies, fatty acid compositions of *Tuber claveryi*, *T. nivea*, *T. pinoyi*, *T.boudieri*, *T. texense* and *Picoa juniperi* truffles were investigated and linoleic (4.13-53.2 %), oleic (3.75-45.90%), palmitic (8.0-27.87%) and margaric acids (7.71-18.0%) were found as the most abundant fatty acids [13, 25-28]. Our findings are similar to previous studies.

2.3. Antioxidant activity

Many studies report that daily antioxidant consumption protects against reactive oxygen species (ROS). Fresh fruits, vegetables, teas and especially edible wild, commercial and culture mushrooms contain the natural antioxidants such as flavonoids, phenolic acids, vitamins, anthocyanins and other phenolic compounds. So they have a protective effect against oxidative damage and diseases by reacting with ROS. In addition to their potential beneficial effects, there has been an increasing demand in pharmaceutical, cosmetic and food industries for the discovery of natural antioxidants compared to synthetic ones such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertbutyl hydroquinone (TBHQ) and gallate (PG), owing to their harmful effects to human health, resulting in carcinogenesis and liver damage [29, 30].

The antioxidant activities of the hexane, ethyl acetate, methanol, butanol and water extracts of *C. pachysporus* and *S. tenuipes* were tested by β-carotene-linoleic acid, DPPH radical scavenging, ABTS cation radical scavenging, cupric-reducing antioxidant capacity (CUPRAC) and metal chelating activity assays. The obtained results and the comparison with the standard compounds i.e. BHA, α-tocopherol, and EDTA are shown in Table 3. The extracts were tested at different concentrations and IC_{50} values determined. Results were found to be statistically significant ($p<0.05$) when compared with those of controls in each test.

β-carotene-linoleic acid assay was used to test the antioxidant effect of the extracts of *C. pachysporus* and *S. tenuipes*. In this test system, peroxy radicals formed from linoleic acid autoxidation cause oxidation and decolourisation of β-carotene. The presence of the antioxidant in this reaction mixture scavenges radicals and prevents the formation of oxidation [31]. As shown in Table 3, except the hexane extract, all extracts of *C. pachysporus* exhibited higher lipid peroxidation inhibitory activity than α-tocopherol. Also, the butanol and water extracts of *C. pachysporus* exhibited higher lipid peroxidation inhibitory activity than BHA. The butanol extract of *S. tenuipes* showed the highest lipid peroxidation inhibition activity with an IC_{50} value of 6.25±0.21 μg/mL, followed by methanol (IC_{50}: 7.57±00.04 μg/mL) and ethyl acetate extracts (IC_{50}: 8.77±0.17 μg/mL). In general, butanol extracts of truffles showed the highest antioxidant activity among the other extracts.

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals were used to measure free radical scavenging activity of the extracts of truffles. The $DPPH^{\bullet}$, a purple stable free radical, enters the reaction with radicals, electrons or hydrogen atoms and loses color at 515 nm. The $ABTS^{\bullet+}$ is formed using a potassium persulphate as an oxidizing agent and loses its color in the presence of radical antioxidant [32]. The radical scavenging activity results are presented in Table 3, In DPPH• assay, all extracts of *C. pachysporus* showed high activity while all extract of *S. tenuipes* displayed low

Table 3. Antioxidant activity of the extracts of Truffle species by β-Carotene-linoleic acid, DPPH, ABTS, CUPRAC and metal chelating assays[a]

Antioxidant Activity						
		β-Carotene-linoleic acid assay	$DPPH^{\bullet}$ assay	$ABTS^{\bullet+}$ assay	CUPRAC assay	Metal chelating assay
Truffle species	**Extract**	IC_{50} (μg /mL)	IC_{50} (μg /mL)	IC_{50} (μg/mL)	$A_{0.50}$ (μg/mL)[c]	Inhibition %[b]
C. pachysporus	Hexane	2.55±0.12	52.63±0.01	37.51±0.43	38.75±0.31	21.49±0.09
	Ethyl acetate	2.08±0.56	53.54±0.06	35.79±0.22	23.21±0.07	42.27±0.68
	Methanol	1.77±0.23	70.92±0.41	37.99±0.09	61.68±0.14	25.08±0.59
	Butanol	0.51±0.05	61.28±0.53	38.50±0.18	53.75±0.61	19.85±0.86
	Water	0.48±0.10	58.51±0.29	34.52±0.37	155.55±0.22	35.72±0.85
S. tenuipes	Hexane	12.83±0.83[b]	2.56±0.31[b]	9.26±0.74[b]	286.51±0.33	78.62±0.15
	Ethyl acetate	8.77±0.17	5.39±0.0.24[b]	28.31±0.45[b]	227.41±0.75	6.06±0.31
	Methanol	7.57±0.04	12.55±0.48[b]	170.93±0.49	263.55±0.12	42.76±0.18
	Butanol	6.25±0.21	17.14±0.18[b]	62.75±0.31	181.57±0.09	37.53±0.34
	Water	13.54±0.32	41.03±0.88[b]	40.22±0.26	242.50±0.47	73.81±0.37
Standards	α-Tocopherol[d]	2.10±0.08	37.20±0.41	38.51±0.54	66.72±0.81	NT[e]
	BHA[d]	1.34±0.04	19.80±0.36	11.82±0.09	24.40±0.69	NT[e]
	EDTA[d]	NT[e]	NT[e]	NT[e]	NT[e]	94.21±0.41

[a]: IC_{50} values represent the means ± SEM of three parallel measurements ($p< 0.05$).
[b]: % inhibition of 200 μg/mL concentration of truffle extracts.
[c]: $A_{0.50}$ values represent the means ± SEM of three parallel measurements ($p< 0.05$).
[d]:Reference compounds.
[e]:NT: not tested.

activity (Table 3). In the $ABTS^{\bullet+}$ assay, all studied extracts of *C. pachysporus* displayed higher radical scavenging activity than α-tocopherol (IC_{50}: 38.51±0.54 μg/mL) used as a standard. The water extract of *S. tenuipes* exhibited the highest activity with the IC_{50} value of 40.22±0.26 μg/mL and followed by methanol (IC_{50}: 62.75±0.31μg/mL). In both studied truffles, water extracts showed the highest radical scavenging activity. The water of truffles is important in treatment of eyes infections without side-effects as Prophet Muhammed (Peace Be Upon Him) mentioned in the prophetic medicine [33].

The reducing ability of the extracts of truffles was determined by CUPRAC assay. In this assay, the extracts or standard antioxidants are mixed with $CuSO_4$ and neocuproine, the absorbance is measured at 450 nm. Cu (II) is reduced to Cu (I) with the electron-donating antioxidants. The reducing power of the extracts of *C. pachysporus* were decreased in the order of ethyl acetate>hexane>butanol>methanol>water. Even as the ethyl acetate extract ($A_{0.50}$: 23.21±0.07 μg/mL) showed higher reducing power than α-tocopherol and BHA, all extracts of except water extract exhibited higher reducing power than α-tocopherol. All extracts of *S. tenuipes* showed less activity than standard antioxidants (Table 3).

Electron donating activities of the truffles extracts were measured by reduction of iron ions. The yellow color of the solution turns into blue color at a rate varying according to the reduction effect of the sample. Antioxidants reduce Fe^{3+}/ Ferric cyanide complex to ferrous form and Perl's Prussian blue color of the Fe^{2+} complex is measured by spectrophotometrically [34]. As seen in Table 3, the results are given as inhibition % of 200 μg/mL concentration of truffle extracts and compared with EDTA as a standard. The highest metal chelating activity was observed in ethyl acetate (42.27±0.68%) and water extracts (35.72±0.85%) of *C. pachysporus* among all extracts. The hexane and water extracts of *S. tenuipes* showed the highest metal chelating activity with inhibition values of 78.62±0.15% and 73.81±0.37%, respectively.

The obtained results support the previous studies about antioxidant activity of truffles [18, 19, 22, 28, 35].

2.4. Anticholinesterase activity

Alzheimer's disease (AD) is a neurodegenerative disorder associated with progressive age-related and causing loss of memory and cognitive abilities. Using of inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is the most commonly used method for the treatment of AD. Researchers show great interest in investigating useful phytochemicals from natural sources with low toxicity that inhibits AChE and BChE. Table 4 summaries the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the extracts of *C. pachysporus* and *S. tenuipes*. Against AChE enzyme, all of the extracts of *C. pachysporus* indicated low activity, as for *S. tenuipes*, except for hexane extract; other extracts were found to be inactive. The hexane (IC_{50}: 10.55±0.13μg/mL), methanol (IC_{50}: 13.21±0.50 μg/mL) and water extracts (IC_{50}: 20.48±0.27 μg/mL) of *C. pachysporus* showed higher inhibitory activity against BChE enzyme when compared with galantamine (IC_{50}: 50.80±0.93 μg/mL) used as a standard. The hexane, ethyl acetate and methanol extracts of *S. tenuipes* exhibited moderate inhibitory activity against BChE enzyme.

Table 4. Anticholinesterase activities of the extracts of Truffle species[a]

Truffle species	Extract	AChE assay IC_{50} (μg/mL)	BChE assay IC_{50} (μg/mL)
	Hexane	94.75±0.63	10.55±0.13
C. pachysporus	Ethyl acetate	6.90±0.21[b]	66.01±0.98
	Methanol	4.05±0.04[b]	13.21±0.50
	Butanol	2.29±0.31[b]	99.92±0.15
	Water	22.88±0.09[b]	20.48±0.27
	Hexane	118.85±0.11	84.55±0.41
S. tenuipes	Ethyl acetate	NA[c]	188.25±0.99
	Methanol	NA[c]	131.16±0.28
	Butanol	NA[c]	>200
	Water	NA[c]	NA[c]
Standards	Galantamine[d]	5.00±0.13	50.80±0.93

[a] :IC_{50} values represent the means ± standard deviation of three parallel measurements (p<0.05).
[b]: % inhibition of 200 μg/mL concentration of truffle extracts.
[c]: NA: not active
[d]: Reference compounds.

3. CONCLUSION

This is the first comprehensive study related to antioxidant and anticholinesterase activities of *C. pachysporus* and *S. tenuipes* with phenolic and fatty acid compositions. Both studied truffles were found to be rich in phenolic and fatty acids. All studied extracts of *C. pachysporus* displayed higher antioxidant activity than standard, α-tocopherol in β-carotene-linoleic acid, ABTS cation radical scavenging and cupric-reducing antioxidant capacity (CUPRAC) assays. In anticholinesterase activity, the hexane, methanol and water extracts of *C. pachysporus* exhibited higher inhibitory activity against BChE enzyme when compared that of galantamine.

According to the results of our study, truffle extracts can be a new source of antioxidant and anticholinesterase drugs. Further investigation about the isolation and identification of the bioactive compounds of *C. pachysporus* and *S. tenuipes* are important to explore potential therapeutic effects in more details.

4. MATERIALS AND METHODS

4.1. Truffle materials and Extraction

C. pachysporus and *S. tenuipes* were collected from Muğla, Turkey in 2015. Voucher specimens were deposited at the herbarium of Natural Products Laboratory of Muğla Sıtkı Koçman University. Each Truffle species were extracted separately with different solvents (2.5 L) i.e. hexane, ethyl acetate, methanol, butanol, and water at room temperature for 24 h and four times. Solvents were evaporated on a rotary evaporator to obtain hexane, ethyl acetate, methanol, butanol, and water extracts. All extracts were stored at +4°C until analysis.

4.2. Analysis of phenolic compounds

The phenolic compounds analysis was performed by the method of Barros *et al.* [36] with slight modification. The truffle samples (3 g) were extracted with acetone: water (80:20 v/v; 30 mL) at – 18 °C for 24 h. After ultrasonic bath for 15 min, the truffle extract was centrifuged at 4000 rpm for 10 min and filtered through Whatman no. 4 paper. The residue was then re-extracted by two additional 30 mL of the acetone: water. The combined extracts were evaporated at 40 °C under reduced pressure to remove acetone. The obtained extract solved in water: methanol (80:20) and filtered through a 0.20 μm disposable LC filter disk for HPLC-DAD. All analysis conditions were identical to those described in the recent publication [8]. The phenolic compounds were characterized according to their retention times, and UV data were compared with commercial standards. Three parallel analyses were performed. For the quantitative analysis of phenolic compounds, calibration curves were obtained via the injection of known concentrations (0.0, 0.00782, 0.01563, 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 ppm) of different standard compounds i.e. gallic acid, fumaric acid, protocatechuic acid, catechin hydrate, p-hydroxybenzoic acid, 6,7-dihydroxy coumarin, caffeic acid, vanilin, 2,4-dihydroxy benzoic acid, p-coumaric acid, ferulic acid, coumarin, *trans*-2-hydroxy cinnamic acid, ellagic acid, rosmarinic acid, *trans*-cinnamic acid. The results were expressed as μg per g of dry weight (dw).

4.3. Analysis of fatty acids

The n-hexane extract was dissolved in 0.5 M NaOH (2 mL) in a 25 mL flask. After the flask was heated in a water bath (50°C), then 2 mL BF_3:MeOH was added. The mixture was boiled for 2 minutes, and then the mixture was left until it cooled down, and then the volume was completed to 25 mL with saturated NaCl solution. Esters were extracted with n-hexane; thus, the organic layer was separated. The hexane layer was washed with a potassium bicarbonate solution (4 mL, 2%) and dried with anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure by a rotary evaporator to give methyl esters of fatty acid. Derivatization of methyl esters of fatty acids was performed according to the previous method [37]. Qualitative and quantitative analysis of the fatty acid esters were carried out by GC and GC/MSD as reported previously [38].

4.4. Antioxidant activity

The total antioxidant activity was evaluated using β-carotene-linoleic acid test system as previously reported in the literature [39]. The free radical scavenging activity was determined spectrophotometrically by the $DPPH^•$ assay described by Blois [40] with slight modifications [39]. The spectrophotometric analysis of $ABTS^{•+}$ scavenging activity was performed according to the method of Re *et al.[41]* with slight modifications [39]. The cupric reducing antioxidant capacity was determined according to the method of Apak *et al.* [42] with slight modifications [39]. The metal chelating activity of the extracts on Fe^{2+} was measured using the method described in the literature [43].

4.5. Anticholinesterase activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activities were measured by the spectrophotometric method developed by Ellman *et al.* [43] with slight modifications [39]. Galantamine was used as reference compound. The IC_{50} values of the extracts were calculated using a program developed from the graph of anticholinesterase inhibitory activity percentages (Inhibition %) against sample concentrations (μg/mL).

4.6. Determination of IC50 values and A0.50 values

The results are given as 50% inhibition concentration (IC_{50}) in β-carotene-linoleic acid, $DPPH^•$ and $ABTS^{•+}$. Sample concentrations that showed 50% activity (IC_{50}) were calculated from the plot % activity vs sample concentration. The sample concentration having 0.50 absorbance ($A_{0.50}$) was calculated from the plot of CUPRAC absorbance against sample concentration.

4.7. Statistical analysis

All data on antioxidant and anticholinesterase activity tests were the average of triplicate analyses. Data were recorded as mean ± S.E.M. Significant differences between means were determined by student's-t test, p values <0.05 were regarded as significant.

Authorship statement

Author contributions: Concept –G.TÇ., M.E.D.; Design –G.TÇ., M.E.D.; Supervision –G.TÇ., M.E.D.; Resource

– G.TÇ., M.E.D.; Materials – G.TÇ., F.Ç., M.E.D.; Data Collection and/or Processing –G.TÇ., E.D., F.Ç.; Analysis and/or Interpretation –G.TÇ., E.D., F.Ç.; Literature Search –G.TÇ., E.D., F.Ç.; Writing –G.TÇ., E.D.; Critical Reviews –G.TÇ., E.D., F.Ç., M.E.D.

Conflict of interest statement

The authors declared no conflict of interest in the manuscript.

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