European Journal of Therapeutics

DOI: 10.54614/eurjther.2022.0113

Original Article

Investigation of the Antioxidant, Antimicrobial, and Cytotoxic Activities of Endemic *Marrubium rotundifolium* Boiss

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ABSTRACT

Objective: *Marrubium L.* genus (Lamiaceae), which has 40 taxa in the world, is represented by 25 taxa in Turkey. *Marrubium rotundifolium* Boiss. is an endemic species and is distributed in Aegean. This study was aimed to determine the antioxidant, antimicrobial, and cytotoxic effects of hexane, ethyl acetate, and methanol extracts of the endemic *Marrubium rotundifolium* Boiss.

Methods: Ultrasonic-assisted extraction was applied to aerial parts of the plant. The Folin-Ciocalteu and aluminum chloride/potassium acetate methods determine the extract's total phenolic and flavonoid contents. The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) radical decolorinaztion assay was used to determine the extracts' Trolox equivalent antioxidant capacity. The deoxyribose assay was carried out for determining the extracts' OH^- radical scavenging activity, and 2,2-diphenyl-1-picrylhydrazyl was used for radical scavenging assay. 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide method was used to determine the in vitro cytotoxicity of plant extracts on 3 cancer (Caco-2, SH-SY5Y, and PC-3) and 2 non-cancer cell lines (NIH-3T3 and HK-2). The antimicrobial activity was examined through the microdilution method.

Results: Phenolic contents of 2.62 ± 0.16 gallic acid equivalents µg/mL were observed in the methanol extract, while the highest flavonoid content was determined in *n*-hexane extract (168.63 ± 2.76 QE µg/mL). In the $OH^{\cdot -}$, $ABTS^{\cdot +}$, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity studies, *M. rotundifolium* methanol extract showed higher IC_{50} values at lower doses (0.277 ± 0.024, 3.21 ± 0.081, 0.033 ± 0.001 mg/mL, respectively). Concerning the cytotoxic activity, only methanol extract showed inhibition on PC-3 cells (IC_{50}: 0.173 ± 0.018 µg/mL). Minimum inhibitory concentrations of the extracts against Gram-positive bacteria were lower than Gram-negative bacteria and yeast strains.

Conclusion: This study is the first detailed study that examines antioxidant and cytotoxic properties of endemic *M. rotundifolium*.

Keywords: Marrubium, cytotoxicity, antioxidant, antimicrobial, extract

INTRODUCTION

Reactive oxygen species (ROS) are regularly produced as intermediate or end products in physiologic reactions concerning oxygen in the cell. Although ROS plays a crucial role in several vital processes (e.g., redox homeostasis, gene expression, signal transduction, enzymatic reaction, and regulation of the immune response), their uncontrolled presence in the cell poses distress linked to the high oxidant reactivity.[1,2]

Nowadays, it is well-known that ROS and oxidative stress play an essential role in the pathophysiology of various diseases like chronic inflammation, neurodegenerative, and cardiovascular diseases. Although the oxygen-free radical hypothesis has been known for over 50 years, the role of oxidative stress in the progress of diseases has been identified in the last 2 decades. Afterward, studies on antioxidants for preventing and treating these diseases have gained significant importance.[1-3]

Plant extracts are well-known sources shown to have antioxidant capacities due to the phytochemicals included. Antioxidant activities have been associated primarily with the phenolic and flavonoids contents of plants. As the most commonly applied synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are being questioned about safety, natural antioxidants take more consideration by researchers.[3]

Marrubium L. genus (Lamiaceae), 40 taxa globally, is represented by 25 taxa in Turkey. Studies show that this genus is rich in phenolic and flavonoid compounds, labdane-type diterpenes, and

Cite this article as: Aydın Köse F, Öztürk İ, Cin S, Baykan Ş. Investigation of the antioxidant, antimicrobial, and cytotoxic activities of endemic marrubium rotundifolium boiss. *Eur J Ther.* 2022;28(1):45–51.

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Received: November 18, 2021 **Accepted:** December 27, 2021

lignans.[4-6] In addition, there are many pieces of research on the antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities of different *Marrubium* species.[6,7] *Marrubium rotundifolium* Boiss. is an endemic species that is distributed in Aegean region of Turkey and is known as "kalartopu."[8] It is perennial, and cauline leaves are broadly elliptical or ovate. It has been reported that the infusion prepared from the whole plant has been used as carminative and for treating intestinal spasms, dyspepsia, cold, and flu in traditional medicine.[9] However, the research on the phytochemistry and bioactivity of this species is very limited.

This study intended to ascertain the antioxidant, antibacterial, and cytotoxic effects of hexane, ethyl acetate, and methanol extracts of the endemic *M. rotundifolium* Boiss.

METHODS

Plant Materials

The aerial parts of the plant were collected from Bozdag, Izmir, in June 2016. Plats were identified by Prof. Sura Baykan. Voucher specimens were stored in the Herbarium of Ege University, Faculty of Pharmacy (IZEF: 6052).

Extraction

Aerial parts of *M. rotundifolium* (826 g) were air-dried, grinded, and then extracted with *n*-hexane, ethyl acetate, and methanol (3× 1L), by turns using an ultrasonic water bath for 24 hours, and filtered. Finally, the extracts were evaporated to dryness with a rotary evaporator at 40°C. The quantity of obtained *n*-hexane, ethyl acetate, and methanol extracts for the plant was 9.247, 10.133, and 69.652 g, respectively.

Determination of Total Phenolic Content

In order to determine the total phenolic content (TPC) of extracts, 0.5 mL of extract (0.1 mg/mL), 5 mL of Folin-Ciocalteu (10%) reagent, and 4 mL of Na_2CO_3 (1 M) were mixed in test tubes.[10] The tubes were incubated for 15 minutes at 45°C. The sample's absorbance values were measured at 765 nm by a spectrophotometer. The standard curve was established with the following gallic acid concentrations: 25, 50, 100, 150, 200, and 250 mg/mL. The TPCs of the extracts were expressed in mg gallic acid equivalents (GAE) per gram of dry weight.

Determination of Total Flavonoid Content

In order to determine the total flavonoid content (TFC) in extracts, 0.1 mL of $AlCl_3$ (10%), 0.1 mL of CH_3CO_2K (1 M), 1.5 mL methanol, 0.5 mL of extract (1 mg/mL), and 2.8 mL distilled water were added to test tubes, in the same order. Tubes were mixed and incubated for 30 minutes. Then sample's absorbance was measured at 415 nm.[11] The standard curve was established with the various quercetin concentrations (2.5, 25, 50, 75, and 100 µg/mL). Results were represented as µg quercetin equivalent (QE) per mg dry weight.

Main Points

- Antioxidant, antimicrobial, and cytotoxic effects of methanol, ethyl acetate, and hexane extracts of the endemic *Marrubium rotundifolium* Boiss. were investigated.
- The highest total phenolic content and antioxidant activities were observed in methanol extracts.
- Weak cytotoxic activity was determined on cancerous cell lines.

Assay for Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacity (TEAC) of extracts was determined by the decolorization of ABTS radical.[12] Firstly, $ABTS^{+\cdot}$ radical was prepared by mixing 1 part of $K_2S_2O_8$ (2.45 mM) with 2 parts of ABTS (7 mM) and kept in the dark for 16 hours. Then, the mixture was diluted with phosphate buffer (5 mM) until it reached an absorbance value of 0.70 ± 0.02 at 734 nm. Next, 200 µL of the diluted radical solution was mixed with 2 µL of various concentrations of extracts in a 96-well plate. The absorbance was determined at 734 nm in a microplate reader (Varioskan Multimode Flash, Thermo Scientific, Vantaa, Finland) for 0-6 minutes. Finally, the percentages of radical cation inhibition were calculated using the following equation (Eq 1):

$$ABTS^{+\cdot}\ \text{Inhibition}\% = [(A_{ABTS^{+\cdot}} - A_{6min})/A_{ABTS^{+\cdot}}] \times 100 \quad \text{(Eq 1)},$$

where $A_{ABTS^{+\cdot}}$ is the absorbance of the $ABTS^{+\cdot}$ at 734 nm and A_{6min} is the absorbance after adding the formulations to the $ABTS^{+\cdot}$.

The "Trolox standard curve" was prepared for the experiment using different concentrations of Trolox solution (0.25-2.5 µmol/mL). The tested formulations' absorbance was compared to that of the Trolox standard curve, and the antioxidant value of extracts was expressed as the half-maximal effective concentration (EC_{50}) value, the concentration of antioxidants that causes a 50% decrease in the radical absorbance.

Determination of 2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was tested according to Wang et al[13] with minor modifications. Different concentrations of extracts (0, 62.5, 125, 250, 500, and 1000 µg/mL) and ascorbic acid as standards (2, 4, 8, 12, 16, 20 µg/mL) were diluted in ethanol. In a clear 96-well plate, $DPPH^{\cdot}$ solution (100 µL of 200 mmol/L) was mixed with 100 µL of extract or standard. Then the plate was incubated for 30 minutes, and the optical density value of the residual $DPPH^{\cdot}$ was determined at 517 nm wavelength in the microplate reader spectrophotometer. The inhibition percentage of radical was calculated using the following equation (Eq 2).

$$\%\ DPPH^{\cdot}\ \text{inhibition} = [(A_b - A_a)/A_b] \times 100 \quad \text{(Eq 2)},$$

where A_a is the absorbance of samples or standards and A_b is the absorbance of DPPH radical : ethanol (1 : 1) solution. Results were represented as EC_{50} value for $DPPH^{\cdot}$ scavenging.

Determination of Hydroxyl (OH^-) Radical Scavenging Activity

The hydroxyl (OH^-) radical scavenging activity of the extracts was determined by deoxyribose assay.[14] To perform the test, 100 µL of each solutions of 2-deoxy-D-ribose (3.36 mM), H_2O_2 (1 mM), $FeCl_3$ (1 mM), EDTA (1 mM), and ascorbic acid (0.1 mM) were taken and mixed with the extract samples at concentrations (0,

20, 60, 100, and 500 µg/mL). Then, 1.6 mL of phosphate buffer (20 mM, pH 7.4) was added. After 1 hour of incubation at 37°C, 1 mL of thiobarbituric acid (1% in 0.025 M NaOH) and 1 mL of trichloroacetic acid (2.8%) were added to the reaction mixture and incubated for an additional 30 minutes at 100°C. Finally, the tubes were cooled. The absorbance of the samples was measured spectrophotometrically at 532 nm. The results were represented as % inhibition of deoxyribose oxidation that was calculated using the following equation (Eq 3):

$$\text{Inhibition } \% = [(A_c - A_S)/A_c] \times 100 \text{ (Eq 3)},$$

where A_c is the absorbance of the control (α-tocopherol) and A_S is the absorbance of the sample. Results were represented as EC_{50} value for OH^- radical scavenging.

Cell Culture and Cytotoxicity Assay

The potential cytotoxic activity of extracts was tested against human colorectal adenocarcinoma (Caco-2), prostate adenocarcinoma (PC-3), neuroblastoma (SH-SY5Y), kidney tubular epithelial (HK-2), and embryonic mouse fibroblast (NIH-3T3) cell lines by colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reagent.[15] All immortalized cell lines were obtained from American Type Culture Collection (ATCC, USA).

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine supplemented at 37°C in a humidified atmosphere of 5% CO_2 air.

In order to perform cytotoxicity assays, cells were plated into 96-well plates at a density of 5×10^3 cells/well. Plates were incubated overnight. Then, the cells were treated with various concentrations of extracts (0, 0.313, 0.625, 12.5, and 50 µg/mL) for 48 hours. At the end of the incubation, the medium was replaced with 200 µL MTT (5 mg/mL) solution in DMEM. Cells were incubated for another 4 hours. Finally, DMEM was removed and 100 µL dimethyl sulfoxide (DMSO) was added to wells. The absorbance of evaluated blue formazan solution in wells was measured with a microplate reader at 570 and 620 nm wavelengths. Dimethyl sulfoxide-treated cells were used as controls.

The cell viability was calculated and revealed as a percentage (%) of control. The median inhibitory concentration (IC_{50}) was determined in GraphPad Prism 5 statistical software.

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of the samples was investigated with microdilution method.[16] *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* Refik Saydam Culture Collection (RSKK) 02021, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* RSKK 04059, *Candida albicans* ATCC 90028, and *Candida parapsilosis* RSKK 04057 strains were used in antimicrobial activity experiments.

Serial dilutions of the extracts were performed using 96-well microplates including broth media in order to adjust the concentrations between 1 and 2048 µg/mL. Plates were incubated at 37°C for 24 hours after the addition of bacterial and fungal suspensions. The lowest concentration that inhibited the growth of the microorganisms was defined as MIC value.

Ciprofloxacin and fluconazole were used as reference molecules in the assays. The quality control ranges of the results were assessed according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST).[17,18]

Statistical Analysis

Each experiment was done in triplicate. The EC_{50} and IC_{50} values were determined with GraphPad Prism 5 software (San Diego, Calif, USA). Statistical comparisons were performed using analysis of variance followed by Tukey's post hoc test. A P value < 0.05 was recognized as statistically significant.

RESULTS

Determination of Total Phenolic Content and Total Flavonoid Content

The TPCs and TFCs of the methanol, *n*-hexane, and ethyl acetate extracts obtained from the aerial parts of the *M. rotundifolium* were determined by Folin-Ciocalteu and $AlCl_3/CH_3CO_2K$ methods, respectively. Obtained data are represented in Table 1.

As a result of the determination of TPC in the obtained extracts, only the phenolic content of the methanol extract (2.62 ± 1.6 GAE µg/mg) was determined among the 3 extract samples. There was no detected phenolic content in the other 2 extract samples.

As a result of the determination of TFC experiments performed with the same samples, the TFC of ethanol and ethyl acetate extracts were 70.76 ± 1.32 and 142.58 ± 3.45 µg QE per mg of extract, respectively. The highest TFC was found in the *n*-hexane extract (168.63 ± 2.76 µg QE per mg of extract).

Determination of Antioxidant Activities

Antioxidant activities of methanol, *n*-hexane, and ethyl acetate extracts of the *M. rotundifolium* were evaluated by TEAC, $DPPH^{\cdot}$, and OH^- radical scavenging activity. Obtained data are represented as the half-maximal effective concentration (EC_{50}) in Table 2.

Table 1. Total Phenolic and Flavonoid contents of *Marrubium rotundifolium* extracts

	Total Phenolic Content (GAE µg/mg)	Total Flavonoid Content (QE µg/mg)
Methanol	2.62 ± 0.16[a]	70.76 ± 1.32
Ethyl acetate	–	142.58 ± 3.45[b]
n–Hexane	–	168.63 ± 2.78[b,c]

Data are expressed as mean ± standard deviation. Distinct letters (a–c) indicate significant differences ($P < .05$) between extracts.
GAE, gallic acid equivalent; QE, quercetin equivalent.
[a] $P < .01$ methanol versus ethyl acetate and *n*–hexane extracts.
[b] $P < .01$ *n*–hexane and ethyl acetate versus methanol extract.
[c] $P < .01$ *n*–hexane versus methanol extracts.

Table 2. Values of Different Antioxidant Activity Assays of *Marrubium rotundifolium* Extracts (EC_{50}, mg extracts/mL)

	TEAC	DPPH Radical Scavenging Activity	OH−˙Radical Scavenging Activity
Methanol	3.21 ± 0.081[a]	0.033 ± 0.001[a]	0.277 ± 0.024[a]
Ethyl acetate	NA	0.099 ±0.001[b]	0.832 ± 0.032[b]
n–Hexane	NA	NA	1.179 ± 0.132

Data are expressed as mean ± standard deviation. Distinct letters (a–c) indicate significant differences ($P < .05$) between extracts.
TEAC, Trolox equivalent antioxidant capacity; DPPH, 2,2–diphenyl–1–picrylhydrazyl; OH, hydroxyl.
[a] $P < .01$ methanol vs. ethyl acetate and *n*–hexane extracts.
[b] $P < .01$ ethyl acetate vs. *n*–hexane methanol extract.

As can be seen from the results in Table 2, similar to TPC results, only the methanol extract has a significant TEAC value (EC_{50}: 3.21 ± 0.081 mg extract/mL) among the 3 extract samples.

Moreover, as a result of the determination of the DPPH assay, the EC_{50} value of methanol extract (0.033 ± 0.001 mg extract/mL) was found significantly higher than ethyl acetate extract (0.099 ± 0.001 mg extract/mL). Nevertheless, no DPPH radical scavenging activity was observed in the ethyl acetate extract (Table 2).

In the $OH^{\cdot -}$ radical scavenging activity, the lowest EC_{50} value was determined in the methanol extract (0.277 ± 0.024 mg/mL), while the EC_{50} values of ethyl acetate and *n*-hexane extract were 0.832 ± 0.032 and 1.179 ± 0.132 mg/mL, respectively.

Antimicrobial Activity

The results of the microdilution method are presented in Table 3. Dimethyl sulfoxide was ineffective against bacterial and fungal strains at studied concentrations, and the MIC values of the ciprofloxacin and fluconazole were within the limit according to the EUCAST criteria. According to the results of the microdilution method, ethyl acetate extract inhibited the growth of *S. aureus* and *E. faecalis* at 128 µg/mL concentrations and *n*-hexane extract inhibited the growth of *E. faecalis* and *B. subtilis* at similar concentrations. Moreover, 128 µg/mL methanol extract also inhibited the growth of *B. subtilis*. Additionally, the MIC values of ethyl acetate and n-hexane extract were similar as 256 µg/mL against *S. aureus*. Ethyl acetate extract also inhibited the growth of Gram-negative bacteria and yeast strains at the concentration of 512 µg/mL.

Determination of Cytotoxic Activities

The cytotoxic effects of the aerial parts of *M. rotundifolium* methanol, *n*-hexane, and ethyl acetate extracts were tested against 3 cancerous (Caco-2, PC-3, and SH-SY5Y) and 2 non-cancerous (NIH-3T3 and HK-2) cell lines. For this, cells were treated with increasing concentrations (5-100 µg/mL) of *M. rotundifolium* extracts for 48 hours. The potential cytotoxic activity of extracts on cell line were determined by MTT assay (Table 4).

In the cytotoxicity studies performed on cancerous cells, it was determined that methanol extract of *M. rotundifolium* (100 µg/mL) caused a significant decrease in PC-3 cell viability compared to the control cells (40.37 ± 1.32%, IC_{50}: 0.173 ± 0.018 µg/mL) ($P < .01$). However, it was determined that the other extracts did not have a significant cytotoxic effect on any of the tested cancerous cell lines at the tested concentrations ($IC_{50} > 200$ µg/mL).

It was determined that the application of methanol, *n*-hexane, and ethyl acetate extracts led to a significant decrease in NIH-3T3 cell viability compared to the control (40.90 ± 0.361%, 44.50 ± 1.04%, and 46.99 ± 0.652%, respectively) ($P < .01$). In addition, as a result of the calculations made with the data obtained, the IC_{50} values of the *n*-hexane and ethyl acetate extracts

Table 3. Minimum Inhibitory Concentration Values of *Marrubium rotundifolium* Extracts (µg/mL)

		Extracts		
Strains		**Methanol**	**Ethyl Acetate**	***n*–Hexane**
Gram (+) bacteria	*Staphylococcus aureus*	128	256	256
	Enterococcus faecalis	128	256	128
	Bacillus subtilis	256	128	128
Gram (–) bacteria	*Escherichia coli*	512	1024	1024
	Salmonella enterica	512	512	1024
	Pseudomonas aeruginosa	512	1024	1024
Fungi	*Candida albicans*	512	1024	1024
	Candida parapsilosis	512	512	512

Table 4. Cytotoxic activities of *Marrubium rotundifolium* Extracts (IC_{50}, µg/mL)

Extract	Caco-2	PC-3	SH-SY5Y	NIH-3T3	HK-2
Methanol	0.778 ± 0.048	0.173 ± 0.018	NA	0.750 ±0.003	0.119 ±0.002
Ethyl acetate	0.262 ± 0.009	NA	NA	0.139 ± 0.001	0.156 ±0.011
n-hexane	0.388 ± 0.031	0.243 ± 0.033	NA	0.145 ± 0.009	0.159 ±0.017

Values are represented as mean ± standard deviation.
NA, not active at 100 µg/mL concentration.

were determined as 0.145 ± 0.009 and 0.139 ± 0.001 µg/mL, respectively.

In HK-2 cells, the treatment with 100 µg/mL of *M. rotundifolium* methanol, *n*-hexane, and ethyl acetate extracts for 48 hours caused a significant decrease in cell viability compared to control cells by 47.85 ± 0.724%, 40.54 ± 1.356% and 43.67 ± 0.872%, respectively ($P < .01$). As a result of the calculations made with the findings obtained, the IC_{50} values of the methanol, *n*-hexane, and ethyl acetate extracts were determined as 0.119 ± 0.002, 0.159 ± 0.017 and 0.156 ± 0.011 µg/mL, respectively.

DISCUSSION

This research investigates the antioxidant capacities and potential cytotoxic effects of different polarity extracts of *M. rotundifolium*. Our study is the first report that investigates the antioxidant capacity of the plant in detail.

In previous reports concerning the antioxidant activities of *Marrubium* species, the DPPH radical scavenging activity test was widely used among the antioxidant activity assays. Also, it is reported many times that methanol/ethanol extracts have more antioxidant activity than essential oils and other apolar extract types such as ethyl acetate, chloroform, and acetone extracts.[19-21] Similarly, in our $OH^{\cdot -}$, ABTS, and DPPH radical scavenging effect studies, *M. rotundifolium* methanol extract showed higher IC_{50} values at lower doses (0.277 ± 0.024, 3.21 ± 0.081, 0.033 ± 0.001 mg/mL, respectively). The radical scavenging effects are thought to be due to the high phenolic content in methanol extracts. Five different extracts (acetone, methanol, petroleum ether, ethyl acetate, and sodium hydrogen carbonate) were prepared from the aerial parts of *Marrubium peregrinum* with the study carried out by Stankovic in Serbia.[21] The activity of the extracts was expressed as percent inhibition of DPPH radicals and IC_{50} values (µg/mL). Percentage values range from 27.26% to 89.78%. The largest capacity to neutralize DPPH radicals was found for methanol extract, which neutralized 50% of free radicals at the concentration of 187.41 µg/mL.[21] Furthermore, in 2010, *n*-hexane and methanol extracts were prepared from the leaves of the *M. parviflorum* collected from Gaziantep to study the in vitro antioxidant activity by Yumrutas and Saygideger.[22] The extracts' TPC and TFC were investigated by β-carotene/linoleic acid, DPPH, ABTS, potency reduction, and metal chelation tests. Since methanol extracts of these plants contain a high amount of phenolic compounds, they exhibited more significant antioxidant activity than *n*-hexane extract. *M. parviflorum* methanol extract has been reported to have the following antioxidant potentials: DPPH: 22.72 ± 0.11 mmol TE/g dw, ABTS: 34.10 ± 1.80 mmol TE/g dw, power reduction test: 46.34 ± 2.43 mmol AAE/g dw, and metal chelation test: 11.47 ± 0.81 mmol EDTAE/g dw.[22]

According to our results obtained from determining the TPC by the Folin-Ciocalteu method, no phenolic content was observed in the *n*-hexane and ethyl acetate extract, while a phenolic content of 2.62 ± 0.16 GAE µg/mL was observed in the methanol extract. In a previous study conducted in Muğla, the TPC of the *M. globosum* subsp. *globosum* was found to be the highest in the polar subfractions (25.60 ± 0.74 µg/mL).[23] Likewise, in another study, among the petroleum ether, ethyl acetate, chloroform, butanol, and methanol extracts obtained from the leaves of the *Marrubium deserti*, the TPC was found to be higher in the methanol extract by the Folin-Ciocalteu colorimetric method.[24] As a result, when we search the studies conducted in this field with *Marrubium* species, it is generally seen that the TPC is higher in polar extracts in parallel with the results we obtained.

Determining the in vitro cytotoxicity of plant extracts is the first step toward investigating the anticancer effects of compounds derived from natural sources. With this in mind, the cytotoxic effects of methanol, ethyl acetate, and hexane extracts from the aerial parts of *M. rotundifolium* were investigated on 3 cancerous (Caco-2, SH-SY5Y, and PC-3) and 2 non-cancer cell lines (NIH-3T3 and HK-2) by the MTT method. No significant results were found in the extracts generally. Only methanol extract of *M. rotundifolium* (100 µg/mL) caused a significant decrease in PC-3 cell viability compared to the control cells (40.37 ± 1.32%, IC_{50}: 0.173 ± 0.018 µg/mL) ($P < .01$). When we look at the other studies in this field, the cytostatic effects and cell growth inhibitory activities of the methanol extract obtained from the aerial parts of the *Marrubium thessalum* against HeLa, MCF-7, FM3, and HCT-116 cancer cells lines were demonstrated by MTT assay. It has been reported to exhibit potent cytotoxic activity against cell lines.[25] In another study, the cytotoxic effects of *Marrubium crassidens* methanol extract against MCF-7 (breast cancer) cell line were demonstrated by the MTT method, too.[26] In previous studies with other *Marrubium* species, the cell lines we tested were not used.

According to the results of antimicrobial activity experiments, the extracts inhibited the growth of 3 Gram-positive bacteria at the concentration of 128 or 256 µg/mL. When the MIC values were compared, the extracts were found to be more effective against Gram-positive strains than Gram-negative bacteria and

yeasts. In parallel to our findings, Zarai et al[27] showed that the *Marrubium vulgare* essential oil had antimicrobial activity against Gram-positive bacteria including *S. aureus*, *E. faecalis*, and *B. subtilis* strains.[27] In another study, Ulukanlı and Akkaya[28] reported that the hexane extracts of *Marrubium catariifolium* and *Phlomis pungens* var. *hirta* compared to the acetone and methanol extracts exhibited antibacterial activity against Gram-positive bacteria such as *S. aureus*, *Staphylococcus epidermidis*, and *B. subtilis* except *E. faecalis*.[28] In our studies, it was demonstrated that the MIC value of the ethyl acetate extract was 128 µg/mL against *S. aureus* and *E. faecalis*. Ethyl acetate extract inhibited the growth of Gram-negative bacteria and yeast strains at 512 or 1024 µg/mL concentrations. In addition to the inhibitory effects against Gram-positive bacteria, methanol and *n*-hexane extracts also inhibited the growth of Gram-negative bacteria and yeast at 512 or 1024 µg/mL concentrations.

Although the rich traditional uses are high in a number of Anatolian *Marrubium* species, the knowledge about the phytochemical and bioactivity is still limited. Many *Marrubium* taxons are used as an antipyretic, analgesic, diuretic, and against the cold in Anatolian folk medicine.[29-31] However, all the uses are associated with the plants' antioxidant and antimicrobial potentials, and more research is needed with *Marrubium* species. Also, *M. rotundifolium* is a much districted studied plant, and further detailed phytochemical studies and more bioactivity studies should be performed to understand the causality of traditional uses of this plant.

CONCLUSION

Marrubium species attract significant attention in the world. Although the gene center is accepted as Turkey due to the high rate of endemism, and it shows a dense distribution area, scientific studies on *Marrubium* species in Anatolia are limited. This study is the first detailed study that examines antioxidant, antimicrobial, and cytotoxic properties of endemic *M. rotundifolium*. Although there is a potential for an antioxidant and antimicrobial effect in general, weak cytotoxic effects were observed. Further detailed phytochemical studies are needed to explain the causality of the activities.

Ethics Committee Approval: The manuscript does not contain any studies with human participants or animals.

Informed Consent: The manuscript does not contain any studies with human participants or animals.

Peer-review: Externally peer-reviewed.

Acknowledgments: The authors thank all study participants for their cooperation and institutions for their support.

Author Contributions: Concept – F.K., Ş.B.; Design – F.K., Ş.B., S.C.; Supervision – Ş.B.; Resources – F.K., S.C., İ.Ö., Ş.B.; Materials – F.K, Ş.B.; Data Collection and/or Processing – XXXX; Analysis and/or Interpretation – F.K., İ.Ö., S.C.; Literature Search – F.A.K., S.C., İ.Ö.; Writing Manuscript – F.A.K., S.C., Ş.B.; Critical Review – F.A.K., Ş.B.; Other – F.A.K., İ.Ö., S.C., Ş.B.

Declaration of Interests: The authors have no conflicts of interest to declare.

Funding: This study is supported by Ege University Scientific Research Projects Coordination Unit. Project Number: 07ECZ011.

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