



Trachystemon orientalis (L.) G. Don as a Valuable Source of Rosmarinic Acid: Biological Activities and HPLC Profiles

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ABSTRACT

Objectives: *Trachystemon orientalis* (L.) G. Don, colloquially known in Türkiye as “kaldırık”, is an edible plant belonging to the Boraginaceae. This plant has been practiced in traditional medicine for many years for its various therapeutic benefits. The effectiveness and chemical composition of plants can vary depending on their parts, age, and extraction solvent. Therefore, the current study aimed to define the biological activities of various parts and extracts of *T. orientalis*, which were collected in distinct seasons as young and mature, and investigate the main component responsible for these biological effects.

Material and Methods: Plant material was collected in different seasons from the northwest of Türkiye. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activities were investigated to assess antiradical and antioxidant potential of the extracts. Anti-inflammatory activity of the extracts was also tested using human red blood cell membrane stabilizing method. Folin-Ciocalteu test was conducted to determine the total phenolic content. Reverse phase-high performance liquid chromatography-photodiode array detector (RP-HPLC-PDA) analysis was performed.

Results: Both methanol and aqueous extracts exhibited significant radical scavenging and anti-inflammatory activities compared with control ($p < 0.05$). The highest percentage of inhibition on ABTS and DPPH free radicals was obtained in aqueous extracts of the mature herbs and roots, respectively. Methanol extracts of the mature roots and herbs exhibited the strongest anti-inflammatory capacity. Rosmarinic acid possessed a much higher antioxidant and anti-inflammatory effect than the reference compounds used in each assay in our study. High rosmarinic acid content of the extracts suggests that the compound responsible for the great biological activity potential is rosmarinic acid.

Conclusion: To the best of our knowledge, the presence of rosmarinic acid in herbs and roots of *T. orientalis* was shown for the first time in our present study. Phytochemical composition and effective biological activities of *T. orientalis* explain its traditional use and indicate its significant potential in pharmaceutical industry applications.

Key words: Antioxidant activity, anti-inflammatory activity, rosmarinic acid, RP-HPLC-PDA, *Trachystemon orientalis*

INTRODUCTION

Trachystemon orientalis (L.) G. Don is a plant belonging to the Boraginaceae family that is represented by 34 genera and 325 species in Türkiye.¹ This plant is distributed in nearly all Black Sea regions in Türkiye, East Bulgaria, and West Caucasia. *T.*

orientalis is called by different names in Türkiye, such as “kaldırık, tomar, burğı, hodan, and ispit”. It is an edible and medicinal plant. The leafy and budding plant body is consumed as vegetable, while the roots and petioles are consumed as pickles in several parts of the Black Sea region.² It is used

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as an antipyretic, diaphoretic, diuretic, laxative, anticancer, analgesic, antifatulent, and antirheumatismal in medicinal treatments.³⁻⁵ Chronic and cumulative oxidative damage leads to various pathologic processes such as stimulating the genes incorporated in the inflammatory phases. The occurrence and progression of inflammation-associated conditions such as arthritis, atherosclerosis, Alzheimer's disease, autoimmune diseases, ocular diseases, diabetes, and cancer may be triggered by enhancing the number of free radicals. There has been considerable interest in the role of free radicals in chronic diseases and the protective effect of antioxidants as scavengers of free radicals.⁶⁻⁹ Inflammatory diseases are commonly treated with steroidal and non-steroidal anti-inflammatory drugs. Besides the beneficial therapeutic effects, they also have crucial side effects. Much research has been conducted to determine new, safe, pharmacologically active plants, and plant-derived compounds with lower side effects.^{10,11} Consequently, target of the current study was to analyse and determine antioxidant potentials and anti-inflammatory activities of aqueous and methanol extracts from herbs and roots of young and mature *T. orientalis*, which is the first report on relevant point for this species. Furthermore, to the best of our knowledge, this study identified rosmarinic acid as the major and possibly the most active component of the herbs and roots of this plant for the first time.

MATERIALS AND METHODS

Chemicals

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetonitrile, acetylsalicylic acid (ASA), gallic acid, propyl gallate, rosmarinic acid, trifluoroacetic acid, and trolox were bought from Sigma-Aldrich (USA). Sodium carbonate, sodium chloride, potassium persulphate, sodium chloride, dimethyl sulfoxide, methanol, ethanol, and other solvents were purchased from either Merck (Germany) or Sigma-Aldrich (USA).

Plant materials

Plant material was collected in different seasons from Akçakoca town and Tahirli village in Düzce province (Türkiye), and identified by one of us (B.B.). Plant material was firstly dried at room temperature. Afterwards, a laboratory-scale mill was used to powder the material. A voucher specimen was housed in the Herbarium of Ankara University, Faculty of Pharmacy (Türkiye). Collection site, date, and herbarium number of the plant sample were noted [*T. orientalis* (L.) G. Don: A3 Düzce, Akçakoca, Tahirli Köyü, 13/02/2016-25/04/2016, AEF 26813].

Preparation of extracts

Preparation of methanol extract: Each part of plant material (5 g) was powdered and, then, the materials were macerated with 50 mL methanol three times for 8 h at 60 °C. Afterwards, they were filtered and combined. A reduced pressure was applied at 40 °C to the mixtures to concentrate them (0.73 g, 14.60% w/w young root yield, and 0.42 g, 8.4% w/w young herb

yield, and 0.66 g, 13.40% w/w mature root yield, and 0.31 g, 6.40% w/w mature herb yield).

Preparation of aqueous extract: Powdered plant material (5 g) was added to 50 mL of distilled water and boiled for 30 min. The extracts were filtered and afterwards lyophilized (1.15 g, 23.06% w/w young herb yield, and 0.73 g, 14.71% w/w young root yield, and 1.18 g, 23.70% w/w mature herb yield, and 0.92 g, 18.50% w/w mature root yield).

Antioxidant activity

Antioxidant activity of the extracts was determined by investigating their scavenging abilities against ABTS and DPPH free radicals.

ABTS free radical scavenging activity

Antioxidant activity was investigated by measuring the scavenging capacity of the samples against ABTS free radicals.¹² First, 7 mM aqueous solution of ABTS⁺ reacted with 2.45 mM potassium persulfate and then a radical cation solution of ABTS^{•+} was produced. This radical solution was maintained at room temperature overnight in dark. Dilution is applied to this dark radical solution with ethanol until obtaining absorbance of 0.700 ± 0.05 at 734 nm. Finally, a working solution was arranged (pH: 7.4). The ABTS solution was prepared freshly in each study to prevent degradation. The test sample was mixed with radical cation solution (100x). Then the mixtures were held for 6 min at room temp. The reference compound was Trolox in this assay. At the final step of the experiment, the absorbance of the samples was measured at room temperature at 734 nm. The experiments were performed at least three times. The percentage of inhibition was computed for each sample at each concentration. Then, half maximal inhibitory concentration (IC₅₀) values were computed using these values. The final results were presented as IC₅₀ \pm standard deviation (SD).

DPPH free radical scavenging activity

Both qualitative and quantitative methods were used to determine this activity.

Qualitative DPPH radical scavenging activity

Antioxidant capacity of the samples was assessed using qualitative DPPH radical scavenging assay.¹³ 0.1 mM DPPH methanol solution (1 mL) was put into the test tube, then, the extracts were inserted in different concentrations and vortexed. The mix was left to stand at room temperature for 30 min. When this DPPH solution is added to a solution containing antioxidant substance, this dark purple loses color with time.

Quantitative DPPH radical scavenging activity

DPPH free radical scavenging capacity of the extracts was examined to determine their antioxidant activity.¹⁴ DPPH stock solution was prepared first. The stock solution of each test compound in methanol was prepared (0.5 mg/mL) and then serial dilutions in equal amounts of methanol were prepared in wells. DPPH solution was added to each well to initiate the reaction. Then, these mixtures were kept in the dark for 30 min. Propyl gallate served as the reference compound.

Absorbance was measured at room temperature at 517 nm. The experiments were performed at least three times. The percentage of inhibition was computed for each concentration of each sample. IC_{50} values were then computed using these values. The final results were presented as $IC_{50} \pm SD$.

Anti-inflammatory activity

Anti-inflammatory activities of the samples were assessed by human red blood cell membrane stabilizing method.^{15,16} The protocol was confirmed by the Human Research Ethics Committees of Ankara University, Faculty of Medicine with 14.05.2020/I5-273-20. Fresh human blood was taken from the healthy volunteers. The volunteers without any chronic disease had not taken any medicine for up to 15 days prior to the test (especially steroidal and anti-inflammatory drugs). Then, the centrifuge process was applied at 3000 rpm for 10 min. The packed cells were isolated and washed with 0.85% isosaline at least three times (pH: 7.2). Then, a 10% v/v cell suspension was arranged. An equal volume of this cell suspension was added to the tubes consisting of the test samples, and then incubation was applied at 56 °C for 30 min. Afterwards, the tubes were left to cool and centrifuge process was applied at 2500 rpm for 5 min. Then the absorbance was determined at 560 nm. ASA served as the standard compound. The tests were conducted in triplicates. The percentage of inhibition of hemolysis was computed for each concentration of each sample. Then, IC_{50} values were computed and the outcomes were presented as $IC_{50} \pm SD$.

Phytochemical analysis

Total phenolic content quantification assay

Total phenolic contents of the extracts were examined by the Folin-Ciocalteu method.¹⁷ Folin-Ciocalteu's reagent, 20% (w/v) aqueous Na_2CO_3 and samples were mixed and the volume was completed with distilled water. The solution was kept at room temperature for 30 min. The absorbance was then determined at room temperature at 765 nm. The same method was applied for gallic acid with the samples and gallic acid calibration curve was drawn. The results were served as gallic acid equivalent (GAE) (mg GAE/g extract dry weight). All experiments were performed at least three times.

Qualitative and quantitative analysis of rosmarinic acid using reverse phase-high performance liquid chromatography-photodiode array detector (RP-HPLC-PDA)

Qualitative and quantitative analyzes of rosmarinic acid (536954, Sigma-Aldrich) in mature leaf and root methanol extracts were assessed using a previously validated method.¹⁸ HPLC system was Agilent 1260 series. The system is equipped with a quaternary pump, an auto-sampler, a column oven, and a PDA detector. Agilent ChemStation software was used for data analysis. The separation was performed on an ACE 5 C18 (250 × 4.60 mm) column with a mobile phase of a mix of trifluoroacetic acid (HPLC grade, ≥99.0%) 0.1% in water (solution A), trifluoroacetic acid 0.1% in methanol (HPLC grade, ≥99.9%) (solution B), and trifluoroacetic acid 0.1% in acetonitrile (HPLC grade, ≥99.9%) (solution C). Gradient profile was (A: B:

C), 80:12:8 at 0 min, 75:15:10 at 8 min, 70:18:12 at 16 min, 65:20:15 at 24 min, 50:35:15 at 32 min, 25:60:15 at 40 min, and 80:12:8 at 45 min. The period between each run was arranged as 2 min. 10 µL was applied for injection. Detection ultraviolet (UV) wavelength was regulated at 330 nm. Column temperature was controlled and arranged to 30 °C. All quantification and validation parameters for rosmarinic acid were given in our previous study.¹⁸

Statistical analysis

All experiments were assessed at least three times for all test samples. SPSS 23.0 was used to examine the results. Data were tested for significant differences by One-Way ANOVA. Then, *post-hoc* Tukey test was performed. A *p* value below 0.05 was set statistically significant.

RESULTS

Antioxidant activity

ABTS free radical scavenging activity

Antioxidant activity of the samples was determined by measuring their radical inhibitory capacity on ABTS. The results were presented in Table 1. All extracts exhibited significant radical scavenging effect in a concentration-dependent manner compared to the control ($p < 0.05$). No significant differences were found according to the age of plant. Both mature and young plants were found to be reactive toward ABTS free radical and had a considerable reducing ability. Generally, aqueous extracts exhibited higher radical scavenging activity than those of methanol extracts. The greatest percentage of inhibition was observed in aqueous extracts of mature herbs among all extracts (IC_{50} : 21.86 ± 1.63 µg/mL). Rosmarinic acid was found to be almost 20 fold more effective than trolox, which was used as a standard compound for this assay. IC_{50} value of rosmarinic acid was 0.57 ± 0.02 µg/mL, while trolox was 9.88 ± 0.02 µg/mL.

DPPH free radical scavenging activity

Qualitative DPPH analysis

The outcomes of qualitative DPPH analysis demonstrated that all extracts showed high antioxidant activities according to inhibition zones (Figure 1).

Quantitative DPPH analysis

DPPH free radical scavenging activity of the extracts was assigned as a mark of antioxidant capacity. DPPH inhibition profiles of the extracts are displayed in Table 2. All extracts exhibited a significant DPPH radical scavenging effect compared to control ($p < 0.05$). No significant differences between young and mature plants were noted regarding this effect. Both groups were found to be effective in inhibiting DPPH. Generally, aqueous extracts exhibited better DPPH free radical scavenging potential than the methanol extracts, correlatively to the outcomes of other radical assay (ABTS). Aqueous extracts of the mature root displayed the highest inhibitory effect on DPPH free radicals, followed by aqueous extracts of the young herbs (IC_{50} : 3.50 ± 0.04 and 3.87 ± 0.04 µg/mL, respectively).

Anti-inflammatory activity

Anti-inflammatory effect of the extracts was determined by measuring their protection capacity on human erythrocyte membrane as shown in Table 3. In general, the methanol extracts

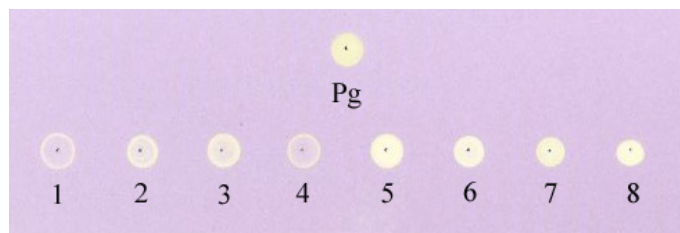


Figure 1. Reactions of extracts with DPPH

1: Young herb methanol, 2: Young root methanol, 3: Mature herb methanol, 4: Mature root methanol, 5: Young herb aqueous, 6: Young root aqueous, 7: Mature herb aqueous, 8: Mature root aqueous, Pg: Propyl gallate, DPPH: 2,2-Diphenyl-1-picrylhydrazyl

showed stronger protective effects than those of aqueous extracts and mature plants were more effective than young ones. Methanol extracts of mature roots and herbs exhibited the highest protective effect (IC_{50} : 0.30 ± 0.01 mg/mL) that was similar to the reference compound. Rosmarinic acid was found almost 4 fold more effective in this assay with IC_{50} value of 0.07 ± 0.02 mg/mL than that of the reference compound (IC_{50} : 0.27 ± 0.05 mg/mL).

Phytochemical analysis

Total phenolic content quantification

Total phenolic content of the extracts was evaluated by the Folin-Ciocalteu method. Gallic acid calibration curves including gallic acid concentration and absorbance values are shown in Figure 2.

All examined extracts had significant amounts of phenolic compounds. Total amount of phenolic compounds of the samples displayed a narrow range from 462.22 ± 8.64 and 578.33 ± 3.14 mg GAE/g extract (dw) (Table 4). In general, aqueous extracts

Table 1. ABTS free radical scavenging effect of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	IC_{50} (μ g/mL)
<i>T. orientalis</i>	Mature	Herbs	Methanol	$30.67 \pm 1.51^*$
			Aqueous	$21.86 \pm 1.63^*$
		Root	Methanol	$31.22 \pm 0.48^*$
			Aqueous	$27.51 \pm 0.73^*$
	Young	Herbs	Methanol	$29.84 \pm 0.21^*$
			Aqueous	$29.33 \pm 0.54^*$
		Root	Methanol	$40.29 \pm 0.24^*$
			Aqueous	$25.82 \pm 0.61^*$
Trolox				$9.88 \pm 0.02^*$
Rosmarinic acid				$0.57 \pm 0.02^*$

* $p < 0.05$; compared with the control, statistically significant. Each value represents mean \pm SD (independently replicated three times)

Table 2. DPPH free radical scavenging effect of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	IC_{50} (μ g/mL)
<i>T. orientalis</i>	Mature	Herbs	Methanol	$4.31 \pm 0.04^*$
			Aqueous	$4.33 \pm 0.04^*$
		Root	Methanol	$6.12 \pm 0.06^*$
			Aqueous	$3.50 \pm 0.04^*$
	Young	Herbs	Methanol	$6.24 \pm 0.06^*$
			Aqueous	$3.87 \pm 0.04^*$
		Root	Methanol	$4.79 \pm 0.05^*$
			Aqueous	$4.14 \pm 0.04^*$
Propyl gallate				$1.73 \pm 0.02^*$

* $p < 0.05$; compared with the control, statistically significant. Each value represents mean \pm SD (independently replicated three times)
SD: Standard deviation, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, IC: Inhibitory concentration

contain higher total phenolic content than methanol extracts. Maximum phenolic content was detected in aqueous extracts of the young herbs followed by methanol extracts of the young roots.

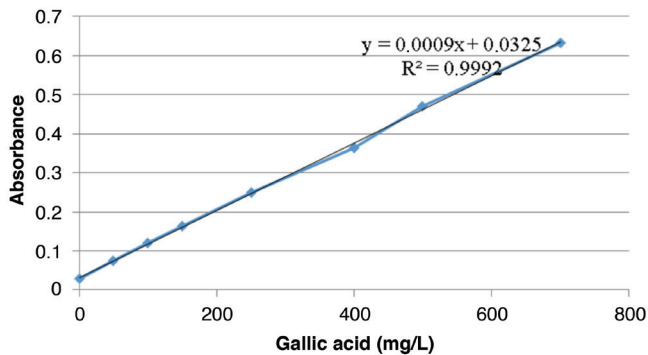


Figure 2. Gallic acid calibration curve

HPLC analysis of rosmarinic acid in *T. orientalis* extracts

Based on the HPLC analysis results, rosmarinic acid was detected as the major compound in both methanol extracts of mature herbs and roots with contents of 74.56 ± 0.03 mg/g and 77.03 ± 0.01 mg/g, respectively. HPLC chromatograms of *T. orientalis* herb and root extracts were presented in Figure 3. Also, the overlaid UV spectra of standard rosmarinic acid and rosmarinic acid in the extracts were presented in Figure 4.

DISCUSSION

There is a positive correlation between an antioxidant-rich diet and reduction of oxidative damage and inflammation. This antioxidant-rich healthy diet includes a range of plant foods, fruits, and vegetables. In addition, not only for nutrition, but also for oxidative stress and inflammation process, natural compounds and trace elements with antioxidant properties can be a solution in regulating key points.¹⁹⁻²³ Many plants consist of a range of radical scavenging molecules, such as phenolics, flavonoids, anthocyanins, coumarins, alkaloids, and carotenoids. Several studies have indicated that natural phenolic compounds and anthocyanins have antioxidant, anti-inflammatory,

Table 3. Anti-inflammatory activity of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	IC ₅₀ (mg/mL)
<i>T. orientalis</i>	Mature	Herbs	Methanol	$0.30 \pm 0.01^*$
			Aqueous	$0.45 \pm 0.05^*$
		Root	Methanol	$0.30 \pm 0.01^*$
			Aqueous	$0.47 \pm 0.03^*$
	Young	Herbs	Methanol	$0.34 \pm 0.01^*$
			Aqueous	$0.45 \pm 0.02^*$
		Root	Methanol	$0.32 \pm 0.01^*$
			Aqueous	$0.51 \pm 0.02^*$
ASA				$0.27 \pm 0.05^*$
Rosmarinic acid				$0.07 \pm 0.02^*$

* $p < 0.05$; compared with the control, statistically significant. Each value represents mean \pm SD (independently replicated three times)

ASA: Acetylsalicylic acid

Table 4. Total amounts of phenolic compounds of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	mg GAE/g extract (dw)
<i>T. orientalis</i>	Mature	Herbs	Methanol	472.22 ± 5.50
			Aqueous	530.56 ± 3.14
		Root	Methanol	462.22 ± 8.64
			Aqueous	559.44 ± 6.29
	Young	Herbs	Methanol	532.22 ± 2.36
			Aqueous	566.11 ± 4.71
		Root	Methanol	570.00 ± 8.64
			Aqueous	578.33 ± 3.14

Each value represents mean \pm SD (independently replicated three times)

antidiabetic, and antiproliferative effects.^{11,24-27} Rosmarinic acid, one of these phenolic compounds, is an antioxidant found in the structure of many plants from the Boraginaceae family. Some experimental studies have also revealed that rosmarinic acid exhibits inhibitory effects on oxidation and inflammation. Rosmarinic acid represents antioxidant, anti-inflammatory, and hepatoprotective activities and alleviates the impacts of inflammatory diseases including inflammatory bowel syndrome and rheumatoid arthritis.^{28,29} Rosmarinic acid has been reported to inhibit colon inflammation *via* binary reduction of NF- κ B and STAT3 activation in dextran sulfate sodium-induced mice.³⁰

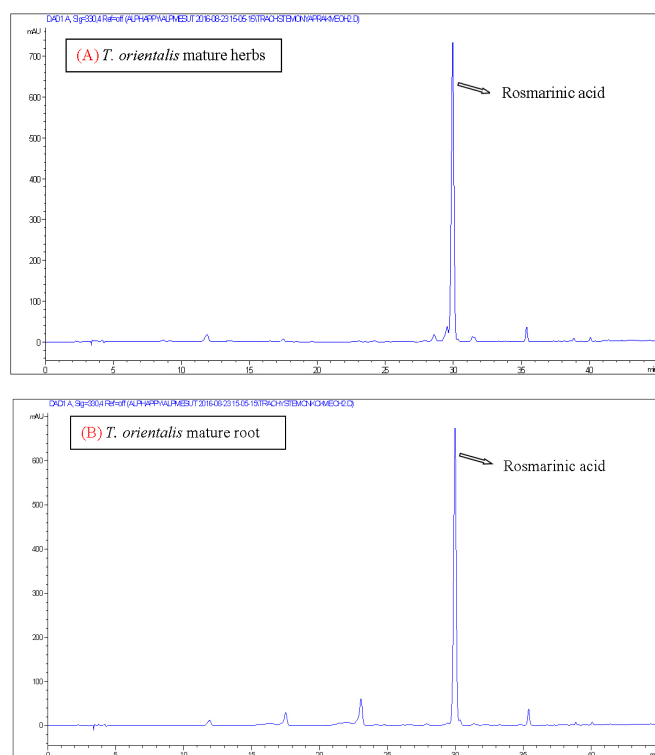


Figure 3. HPLC chromatograms of herbs and roots of *Trachystemon orientalis*. HPLC analysis of rosmarinic acid in *T. orientalis*. (A) *T. orientalis* mature herbs, (B) *T. orientalis* mature roots

HPLC: High performance liquid chromatography

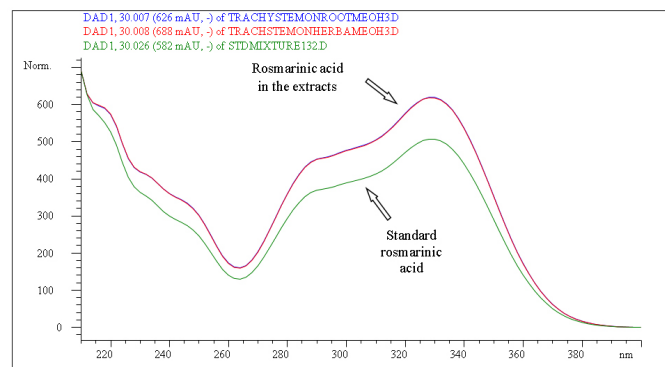


Figure 4. The overlaid ultraviolet spectra of standard rosmarinic acid and rosmarinic acid in the extracts

Anthocyanin-rich extracts and rosmarinic acid, in combination, reduced the symptoms of inflammatory bowel disease.²⁴ *T. orientalis* has been reported to consist of phenolic compounds, anthocyanins, tannins, essential oils, saponins, and resins and exhibits significant antioxidant activity.³¹⁻³³

Chemical composition of the extracts varies depending on the growing location, environmental conditions, parts, and age of the plant as well as extraction solvent. Thus, different extracts are expected to elicit various clinical responses. For this reason, *in vitro* antioxidant and anti-inflammatory activities as well as phytochemical contents of several parts of *T. orientalis* gathered in different seasons were determined in this study. The results of our study indicated that all parts of *T. orientalis* exhibited significant antioxidant activity regardless of age. Generally, the aqueous extracts exhibited better free radical scavenging activities than the methanol extracts. All extracts offered a substantial anti-inflammatory activity compared with the control. The methanol extracts exhibited better anti-inflammatory activity than aqueous extracts. Both antioxidant and anti-inflammatory potentials of the extracts were increased dose-dependently. The strong antioxidant and anti-inflammatory effects of the extracts are most often correlated with the high content of total phenols. In our study the total phenolics of the extracts was found to be high which supports this information. In addition, rosmarinic acid, a very effective phenolic compound, was found as the main ingredient in our extracts. After obtaining this information, the antioxidant and anti-inflammatory efficacy of not only the extracts but also rosmarinic acid was evaluated. Rosmarinic acid exhibited higher activity than reference compounds tested in all methods. The antioxidant activity of rosmarinic acid was ratified and found to be correlated with the anti-inflammatory effect in our study. Inhibition of free radical production also protects cell membranes against oxidative stress and oxidative damage.^{34,35} This explains the link between rosmarinic acid and *T. orientalis* extracts showing both high free radical scavenging and cell membrane stabilizing effect at the same time. Presence of rosmarinic acid in the herbs and roots of *T. orientalis* was shown for the first time in the current study. Moreover, antioxidant and anti-inflammatory effects of *T. orientalis*, which gathered in both young and mature periods, were presented for the first time as well. Our data supply evidence that *T. orientalis* herbs and roots can be used as potential sources of rosmarinic acid.

CONCLUSION

To the best of our knowledge, this is the first report to assess the presence of rosmarinic acid in herbs and roots of *T. orientalis*. Moreover, there are no adequate data comparable to the results obtained in our current study. *T. orientalis* has substantial antioxidant and anti-inflammatory characteristics and rosmarinic acid is probably the main responsible compound contributing to these biological activities. Phytochemical composition and biological activities of *T. orientalis* explain its traditional use and indicate its potential applications in pharmaceutical and cosmetic industries.

Ethics

Ethics Committee Approval: The protocol was confirmed by the Human Research Ethics Committees of Ankara University, Faculty of Medicine with 14.05.2020/I5-273-20.

Informed Consent: Informed consent was obtained from all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: B.B., S.Y.S., A.G., T.Ç., M.C., Design: B.B., S.Y.S., A.G., T.Ç., M.C., Data Collection or Processing: B.B., S.Y.S., A.G., T.Ç., M.C., Analysis or Interpretation: B.B., S.Y.S., A.G., Literature Search: B.B., S.Y.S., A.G., T.Ç., M.C., Writing: B.B., S.Y.S., A.G., T.Ç., M.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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