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Antioxidant and antibacterial activities of *Ranunculus marginatus* var. *trachycarpus* and *R. sprunerianus*

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Abstract: Hexane, ethyl acetate, methanol, and aqueous extracts of *Ranunculus marginatus* d’Urv. var. *trachycarpus* (Fisch. & Mey.) Azn. and *R. sprunerianus* Boiss. were tested in vitro for their antioxidant and antibacterial activities. Antioxidant activity of the extracts was determined by DPPH radical scavenging and Trolox equivalent antioxidant capacity assays. Methanol extracts showed the highest antioxidant activity in both assays. The total phenolics in the extracts were determined colorimetrically by using the Folin-Ciocalteau reagent. The total flavonoid content of the extracts was evaluated by a spectrophotometric method. The results obtained in the antioxidant activity tests were in positive correlation with the total phenolic and flavonoid contents of the extracts. An antibacterial activity analysis was carried out using paper disk diffusion and micro-well dilution techniques. All of the extracts displayed antibacterial activity against the tested bacteria in the disk diffusion method. The minimal inhibitory concentrations (MICs) of all the extracts of both *Ranunculus* species were found to be between 128 and 256 μg/mL.

Key words: Antibacterial activity, antioxidant activity, flavonoid, phenol, *Ranunculus marginatus* var. *trachycarpus*, *Ranunculus sprunerianus*

**Ranunculus marginatus var. trachycarpus** ve **R. sprunerianus**’un antioksidan ve antibakteriyal aktiviteleri


Anahtar sözcükler: Antibakteriyel aktivite, antioksidan aktivite, flavonoid, fenol, *Ranunculus marginatus* var. *trachycarpus*, *Ranunculus sprunerianus*
Introduction

*Ranunculus* (Ranunculaceae) is a widespread and temperate genus, represented by about 84 species in the flora of Turkey (1-3). Various parts of the plants of the *Ranunculus* species, including roots, herbs, and flowers, have been used extensively in traditional medicine in Turkey to treat a variety of illnesses, such as constipation, rheumatism, hemorroids, edema, abscesses, and jaundice (4-7). Some of the plants belonging to this genus have also been used in Turkish folk medicine for their emmenagogue, galactogogue, irritant, and wound-healing properties (4). In the northwestern part of the Black Sea region of Turkey, fresh leaves of *Ranunculus ficaria* L. subsp. *bulbifera* (Marsden-Jones) Lawalrée are consumed as a salad after being mixed with yogurt (8).

Phytochemical studies carried out on various *Ranunculus* species revealed that they produce compounds belonging to different secondary metabolite groups, including triterpene saponins (9,10), alkaloids (11,12), flavonoids (13-17), fatty acids and organic acids (18-21).

Although several plants belonging to this genus have been shown to possess important biological properties such as antibacterial (19,22), antiviral (23), antimicrobial (24,25), anti-inflammatory (26), antiprotozoal (27), xanthine oxidase inhibitory (28), and nematocidal activities (22), to our knowledge there have been no reports on the activity of *R. marginatus* d’Urv. var. *trachycarpus* (Fisch. & Mey.) Azn. and *R. sprunerianus* Boiss. Therefore, the present study aims to evaluate the antioxidant and antibacterial activities of different extracts prepared from these two *Ranunculus* species.

Materials and methods

Plant material

*Ranunculus marginatus* d’Urv. var. *trachycarpus* (Fisch. & Mey.) Azn. and *R. sprunerianus* Boiss. were collected in May 2004 from Akdağ, Karaburun, in İzmir province, Western Anatolia, and identified by Lütfi Bekat, Department of Botany, Faculty of Science, Ege University. Voucher specimens of *R. marginatus* var. *trachycarpus* (No. 1326) and *R. sprunerianus* (No. 1327) are deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Preparation of extracts

Hexane, ethyl acetate, methanol, and aqueous extracts were separately prepared from 20 g batches of the air-dried and powdered whole plants by percolation at room temperature. Then the extraction solvents were evaporated under reduced pressure to dryness (29). The yields of hexane, ethyl acetate, methanol, and aqueous extracts of *R. marginatus* var. *trachycarpus* were 1.31%, 1.96%, 11.80%, and 19.97% and of *R. sprunerianus* were 0.40%, 0.85%, 14.60%, and 25.71%, respectively. All the extracts were stored at -20 °C.

Determination of total flavonoid content

Flavonoid contents of the samples were determined spectrophotometrically by measuring the flavonoids in AlCl₃-complex form from a purified ethyl acetate phase obtained after acid hydrolysis (30,31). The results were expressed as g per 100 g of dry extract (Table 1).

Determination of total phenol content

The total phenols were determined according to the colorimetric reaction with the Folin-Ciocalteu reagent (32). Dried extracts were diluted in distilled water (1:10 v/v). As a phenolic standard, gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) was used and prepared in methanol:water (1:1, v/v) at concentrations of 50-250 mg/L. A solution of an extract (0.5 mL) of gallic acid was mixed with 5 mL of the Folin-Ciocalteu reagent (1:10; diluted in distilled water) and 4 mL of sodium carbonate (1 M). The mixtures were heated at about 45 °C in a water bath for 15 min and the total phenols were determined colorimetrically at 765 nm. Total phenol values were expressed as mg of gallic acid equivalents per g of dry extract (Table 1).

DPPH-radical scavenging activity assay (DPPH-RSC)

The capacity of the plant extracts to scavenge the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was measured as described previously (33). Extracts (0.25 mg) were dissolved in 4 mL of methanol (0.0625 mg/mL). Then it was mixed with 0.5 mL of a methanolic solution of DPPH (1 mM) and allowed to stand at room temperature for 30 min. The optical density of the mixture was measured at 517 nm.
DPPH-RSC values were expressed as a percentage of DPPH radical discolouration. The synthetic antioxidant butylated hydroxytoluene (BHT, Sigma-Aldrich Chemie, Steinheim, Germany) was used as a positive control (1 mg/mL) (Table 1).

**Trolox equivalent antioxidant capacity assay (TEAC)**

The 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical cation (ABTS•⁺) was produced by reacting ABTS aqueous solution (7 mM) with 2.45 mM of ammonium persulfate; the mixture was allowed to stand in the dark at room temperature for 12-16 h before use. The ABTS•⁺ solution was diluted with 5 mM of phosphate buffered saline (PBS) (pH 7.4) to obtain an absorbance of 0.70 (±0.02) at 734 nm. Then 1 mL of the diluted ABTS•⁺ solution was mixed with 10 μL of extract (0.1 mg/mL) or Trolox standards (0-15 μM) and the absorbance was measured. The percentage inhibition of absorbance at 734 nm was calculated and TEAC values were evaluated from the decreases in absorbance in the Trolox standard curve (34) (Table 1).

**Antibacterial activity test**

Antibacterial activity of the extracts of *R. marginatus* var. *trachycarpus* and *R. sprunerianus* was evaluated using the paper disk diffusion technique and by determining the minimal inhibitory concentration (MIC). The bacterial strains used in the antibacterial tests are listed in Tables 2 and 3. Lyophilized bacteria were obtained from the culture collection of the Department of Microbiology, Faculty of Science, Ege University.

**Disk diffusion assay**

The antibacterial activity of the crude extracts was tested by the paper disk diffusion technique (35,36). The extracts were dissolved in DMSO and then 20 μL of each extract (1024 μg/mL) of *R. marginatus* var. *trachycarpus* and *R. sprunerianus* were absorbed onto sterile 6-mm diameter filter paper disks (Schleicher and Schüll, Nr 2668, Dassel, Germany).

The bacterial strains were inoculated on Mueller-Hinton broth (Oxoid) and incubated for 24 h at 37 ± 0.1 °C. Adequate amounts of autoclaved Mueller-Hinton Agar (Oxoid) were dispensed onto sterile plates and allowed to solidify under aseptic conditions. The counts of bacterial strains were adjusted to yield approximately $1.0 \times 10^7 - 1.0 \times 10^8$ CFU/mL using the standard McFarland counting method. Then 0.1 mL of the test organisms were inoculated with a sterile swab on the surface of the appropriate solid medium in the plates.

Agar plates containing the bacteria were incubated for 1 h before placing the extract-impregnated paper disks on the plates. The sterile disks impregnated with different extracts were then placed on the agar plates and incubated at 37 ± 0.1 °C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition (in mm) against the test organisms. All experiments were done under sterile conditions in duplicate and repeated three times. Cefazidime (Fluka) and Sulbactam/Ampicillin (Oxoid) (10 mg/disk) were used as positive controls. DMSO was used as a negative control.

**Micro-well dilution assay**

The minimal inhibitory concentration (MIC) values were determined for the bacterial strains that were sensitive to *R. marginatus* var. *trachycarpus* and *R. sprunerianus* by micro-dilution assay according to the procedures developed by the National Committee of Clinical Laboratory Standards (37,38).

The bacterial strains were inoculated on Mueller-Hinton broth (Difco) and incubated for 24 h at 37 ± 0.1 °C. The inocula of the bacterial strains were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

Dilution series of the extracts were prepared in test tubes. Final concentrations of the extracts were 1024 to 0.5 μg/mL in the medium. The 96-well plates were prepared by dispensing extract into each well of broth and the inocula to obtain $1 \times 10^6$ CFU/mL. Extract prepared at the concentration of 1024 μg/mL was added into the first wells. Then its serial dilutions (512, 256, 128, 32, 16, 8, 4, 2, 1, and 0.5 μg/mL) were transferred into the consecutive wells. The final well, containing Mueller-Hinton broth without extract and the inocula on each strip, was used as a negative control. The plate was covered with a sterile plate sealer. The plates were incubated at 37 °C for 24 h.
The MIC was defined as the lowest concentration of an extract or a substance to inhibit the growth of microorganisms after 24 h. Gentamicin and Ampicillin (Sigma Aldrich Chemical Co., St. Louis, MI, USA) were used as standard antibacterial agents. Their dilutions were prepared from 128 to 0.25 μg/mL concentrations in microtiter plates. All of the assays were performed in triplicate.

**Results and Discussion**

The antioxidant activity of the extracts prepared from the above-mentioned *Ranunculus* species are reported in Table 1. Similar results were obtained in both of the assays employed for the determination of the antioxidant activity. The strongest activity was detected in the methanol extracts of the tested *Ranunculus* species. A positive correlation between the total phenolic and flavonoid contents of the extracts and the antioxidant activity was observed. It is already well known that polyphenols are the major plant compounds with antioxidant activity and that flavonoids are among the important polyphenolic components detected in plant extracts (39-41). Moreover, in a study by Mantle et al. (42), the antioxidant activity of methanol-water (8:2) extracts prepared from the leaves and flowers of *R. repens* L. were reported as 0.13 ± 0.01 and 0.12 ± 0.01 mM TE/g dry weight, respectively. In another study, it has been reported that flavonoid or phenolic components played a significant role in the free radical scavenging capacity of *R. sardous* Crantz. pollen (43). Since flavonoids are among the major constituents isolated from the *Ranunculus* species and have also been considered as good taxonomic markers in this genus (14,16), they may very well be the main components that contribute to the antioxidant activity observed in the present study.

The antibacterial activity levels of the extracts of *R. marginatus* var. *trachycarpus* and *R. sprunerianus*, evaluated by the disk diffusion and micro-well dilution techniques, are reported in Tables 2 and 3, respectively. In the disk diffusion assay (Table 2), the maximal inhibition zones ranged between 7 and 12 mm, and, in the micro-well dilution assay (Table 3), the MIC values of the extracts were between 128 and 256 μg/mL, which indicate that all of the extracts of *R. marginatus* var. *trachycarpus* and *R. sprunerianus* showed significant activity against the tested bacterial species. The highest susceptibility to the tested extracts was displayed by the gram-negative bacterium *Enterobacter aerogenes*, with an inhibition zone ranging between 10 and 12 mm and a MIC value of 128 μg/mL. Moreover, all of the plant extracts showed significant activity against another gram-negative bacterium, *Escherichia coli*, with an inhibition zone of 9-10 mm and an MIC value of 128 μg/mL. Interestingly, besides these two gram-negative bacteria, *Pseudomonas aeruginosa*, which is also a gram-negative bacterium, turned out to be the least susceptible among those tested with an inhibition zone of 7-8 mm and a MIC value of 256 μg/mL.

### Table 1. Total phenolic, total flavonoid contents, and antioxidant activity of *Ranunculus marginatus* var. *trachycarpus* and *R. sprunerianus.*

<table>
<thead>
<tr>
<th>Extract</th>
<th><em>R. marginatus</em> var. <em>trachycarpus</em></th>
<th><em>R. sprunerianus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Phenol (mg/g dry mass)</td>
<td>Total Flavonoid (%)</td>
</tr>
<tr>
<td>hexane</td>
<td>122.80 ± 3.95</td>
<td>0.149 ± 0.01</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>131.72 ± 4.20</td>
<td>0.155 ± 0.01</td>
</tr>
<tr>
<td>methanol</td>
<td>694 ± 30.70</td>
<td>0.709 ± 0.03</td>
</tr>
<tr>
<td>aqueous</td>
<td>331.67 ± 3.02</td>
<td>0.405 ± 0.05</td>
</tr>
</tbody>
</table>

Results are mean ± SD of three replicate analysis.

DPPH-RSC of synthetic antioxidant BHT (1 mg/mL) was determined as 86.54 ± 2.4%.
Table 2. Antibacterial activity of *Ranunculus marginatus* var. *trachycarpus* and *R. sprunerianus* by the disk diffusion method.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th><em>R. marginatus</em> var. <em>trachycarpus</em></th>
<th><em>R. sprunerianus</em></th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hexane extract</td>
<td>EtOAc extract</td>
<td>MeOH extract</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>ATCC 8043</td>
<td>G(+)</td>
<td>11</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 6538/P</td>
<td>G(+)</td>
<td>8</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>ATCC 12228</td>
<td>G(+)</td>
<td>11</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>ATCC 6633</td>
<td>G(+)</td>
<td>10</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>CCM 5445</td>
<td>G(-)</td>
<td>10</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 27853</td>
<td>G(-)</td>
<td>8</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>ATCC 13048</td>
<td>G(-)</td>
<td>12</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 29998</td>
<td>G(-)</td>
<td>9</td>
</tr>
</tbody>
</table>

CF20: Ceftazidime (20 mg); SAM20: Sulbactam (10 mg)/Ampicillin (10 mg).

Values (mean of three replicates) indicate zone of inhibition in mm and include filter paper disk diameter (6 mm); G: gram reaction; "-": no inhibition

Table 3. The MIC values (μg/mL) of *Ranunculus marginatus* var. *trachycarpus* and *R. sprunerianus* against bacteria tested in the micro-well dilution assay.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th><em>R. marginatus</em> var. <em>trachycarpus</em></th>
<th><em>R. sprunerianus</em></th>
<th>Standard</th>
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<td>MeOH extract</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>ATCC 8043</td>
<td>G(+)</td>
<td>128</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 6538/P</td>
<td>G(+)</td>
<td>256</td>
</tr>
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<td><em>Staphylococcus epidermidis</em></td>
<td>ATCC 12228</td>
<td>G(+)</td>
<td>128</td>
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<td>G(-)</td>
<td>128</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 27853</td>
<td>G(-)</td>
<td>256</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>ATCC 13048</td>
<td>G(-)</td>
<td>128</td>
</tr>
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<td><em>Escherichia coli</em></td>
<td>ATCC 29998</td>
<td>G(-)</td>
<td>128</td>
</tr>
</tbody>
</table>

G: gram reaction; GN: Gentamicin; AMP: Ampicillin.
Several studies have been carried out to determine the antimicrobial activity of extracts, essential oils, and compounds isolated from various *Ranunculus* species. Previously, Barbour et al. (24) investigated the antimicrobial activity of methanol and aqueous extracts of the flowers of *R. cuneatus* Boiss. and the whole plants of *R. myosuroides* Boiss. & Kotschy, together with several other plant species collected from Lebanon, by the disk diffusion and minimal inhibitory concentration methods. They found that aqueous extracts of these *Ranunculus* plant parts did not result in the inhibition of the test organisms, namely, *Escherichia coli*, *Proteus* sp., *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Salmonella enteridis*, *Staphylococcus faecalis*, and *Candida albicans*. They also reported that the methanol extracts of whole plants of *R. myosuroudes* and flowers of *R. cuneatus* showed an efficacious inhibitory effect against 88.8% and 77.7% of the tested microorganisms, respectively. The MIC value for the methanol extract of *R. myosuroudes* was also determined and found consistent at 1/2.5 (herbal weight/methanol volume ratio) for all test microorganisms, with the exception of *Streptococcus faecalis*. They detected no efficacy against this bacterium at any serial dilution of the extract.

Quave et al. (44) tested the effect of the ethanol extract of the leaves, stems, and flowers of *R. acris* L. on the planktonic growth and biofilm formation of methicillin-resistant *Staphylococcus aureus* (ATCC 33593), a common cause of skin and soft tissue infection, together with some other plant species. There was no growth detected within the MIC range of 8-512 μg/mL, and no IC₅₀ was identified for biofilm formation within the concentration range of 4-128 μg/mL.

In a recent study, antimicrobial and essential oil compositions of *Ranunculus constantinopolitanus* (DC.) d’Urv. and *R. arvensis* L. were reported. The essential oils of these plants were tested against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus cereus*, and *Candida albicans* using the micro-well dilution method. The essential oil of *R. constantinopolitanus* showed activity against *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*, whereas the essential oil of *R. arvensis* displayed activity against *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*. It was indicated that the tested extracts showed stronger antimicrobial activity against the gram-positive bacteria when compared to the gram-negative bacteria (25).

In another study, by Noor et al. (19), six compounds, including R(+)-dalbergiphenol, R(+)-4-methoxydalbergione, methyl-3,4,5-trihydroxy-benzoate, 4-hydroxy-2-benzoi acid, rho-hydroxy cinnamic acid, and beta-sitosterol, were isolated and their antibacterial activity was evaluated.

It has been documented in the literature that major groups of antimicrobial metabolites from plants include phenolics and polyphenols (simple phenols and phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, and coumarins), terpenoids, essential oils, alkaloids, lectins, and polypeptides (45). Some of this group of metabolites, such as flavonoids (14-16), phenolic acids (19), alkaloids (11), and essential oils (25), have been found to be synthesized by several *Ranunculus* species. In conclusion, the data obtained in previous studies indicate that *Ranunculus* species possess antioxidant (42,43) and antimicrobial (19,22,24,25) properties. The findings in our study also demonstrate that *R. marginatus* var. *trachycarpus* and *R. sprunerianus* have significant antioxidant and antibacterial activities. Further investigations are necessary to identify the compounds responsible for the activity of the screened efficacious extracts.

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References


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