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Phenolic compounds and antioxidant and antimicrobial properties of *Helichrysum* species collected from eastern Anatolia, Turkey

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Abstract: The antioxidant activity of the methanolic extracts of 4 plants from genus *Helichrysum* Mill. (Asteraceae) [*H. arenarium* (L.) Moench subsp. *erzincanicum* Davis & Kupicha, *H. arenarium* (L.) Moench subsp. *rubicundum* (C.Koch.) Davis & Kupicha, *H. armenium* DC. subsp. *araxinum* (Kirp.) Takht., and *H. plicatum* DC. subsp. *pseudoplicatum* (Nab.) Davis & Kupicha] was investigated with 2 complementary test systems, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and phosphomolybdenum assay. *H. plicatum* subsp. *pseudoplicatum* showed the highest level of total antioxidant activity (161.79 ± 0.3 mg ascorbic acid equivalent/g extract) in the phosphomolybdenum assay. *H. arenarium* subsp. *erzincanicum* showed the highest level of free radical scavenging activity, with an IC_{50} value of 23.03 µg/mL. The total phenolic contents of the extracts ranged from 71.81 to 144.50 mg gallic acid/g dry extract. Chlorogenic acid, apigenin-7-glucoside, and apigenin were found by HPLC analysis to be the main components in the extracts. Antimicrobial activity was investigated with the agar diffusion method. The methanolic extracts had antibacterial activity against *Aeromonas hydrophila*, *Bacillus brevis*, *B. cereus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* ATCC 29213. However, no activity was found against *Escherichia coli*, *Morganella morganii*, *Mycobacterium smegmatis*, *Proteus mirabilis*, *Yersinia enterocolitica*, or *Saccharomyces cerevisiae*.

Key words: *Helichrysum,* antimicrobial activity, antioxidant activity, phenolic compounds, DPPH

Türkiye, doğu Anadolu'dan toplanan *Helichrysum* türlerinin fenolik bileşik, antioksidan ve antimikrobiyal özellikleri

Özet: *Helichrysum* Mill. (Asteraceae) cinsinden dört bitkinin (*H. arenarium* (L.) Moench subsp. *erzincanicum* Davis & Kupicha, *H. arenarium* (L.) Moench subsp. *rubicundum* (C.Koch.) Davis & Kupicha, *H. armenium* DC. subsp. *araxinum* (Kirp.) Takht ve *H. plicatum* DC. subsp. *pseudoplicatum* (Nab.) Davis & Kupicha) metanollü özütlerinin antioksidan aktiviteleri DPPH (2,2-difenil-1-pikrilhidrazil) radikal süpürücü ve fosfomolibdenyum analizlerini içeren tamamlayıcı iki test sistemi tarafından araştırılmıştır. Fosfomolibdenyum analizinde *H. plicatum* subsp. *pseudoplicatum* en yüksek antioksidan aktivite (161,79 ± 0,3 mg askorbik asit eşiti/g özüt) göstermiştir. *H. arenarium* subsp. *erzincanicum* 23,03 µg/mL IC_{50} değeri ile en yüksek serbest radikal süpürücü aktivite göstermiştir. Özütlerin toplam fenolik içerikleri 71,81 ile 144,50 mg gallic acid/g kuru özüt arasındadır. HPLC analizi ile özütlerdeki başlıca bileşenlerin klorogenik asit, apigenin-7-glikozid ve apigenin olduğu bulunmuştur. Antimikrobiyal aktivite agar difüzyon analizi ile araştırılmıştır.

Metanollü özütler *A. hydrophila*, *B. brevis*, *B. cereus*, *K. pneumoniae*, *P. aeruginosa* ve *S. aureus* ATCC 29213'e karşı antibakteriyel aktiviteye sahiptir. Fakat *E. coli*, *M. morganii*, *M. smegmatis*, *P. mirabilis*, *Y. enterocolitica* ve *S. cerevisiae*'ya karşı aktiviteye sahip olmadığı bulunmuştur.

Anahtar sözcükler: *Helichrysum,* antimikrobiyal aktivite, antioksidan aktivite, fenolik bileşikler, DPPH

Introduction

Helichrysum Mill., belonging to the family Asteraceae, includes about 500 species, widespread throughout the world. This genus is represented in Turkish flora by 27 taxa, 15 of which are endemic and are widely found in Anatolia (1-4).

Helichrysum species are generally known as "ölmez çiçek" or "altınotu" and are widely used for removing kidney stones in Turkey. Aerial parts of the plants have been used as a daily herbal tea for intervals of 1 week. These effects of *Helichrysum* species are due to the flavonoids that they contain (5,6). These plants have been used in folk medicine for at least 2000 years to treat gall bladder disorders, due to their regulation of bile and diuretic effects. They are used for stomachache relief and wound dressings; for their antiinfective, hepatoprotective, detoxifying, cholagogic, and choleretic effects; to stimulate the secretion of gastric juices; and in the treatment of coughs, erythema, and diabetes mellitus (7-11).

Helichrysum species are commonly used in Turkey and other parts of the world for their various biological properties, including antiinflammatory (12), antioxidant (8,13), and antimicrobial activities (14-16). Although the biological activities of many *Helichrysum* species have been investigated in different countries, there are few reports about the *Helichrysum* species belonging to Turkish flora (6,11,13,17). This work attempts to contribute to this lack of knowledge about the antioxidant and antimicrobial effects of Turkish *Helichrysum* species. Its aim is to study the phenolic compounds and antioxidant and antimicrobial activities of the methanolic extracts of 4 *Helichrysum* species collected from eastern Anatolia, Turkey.

Materials and methods

Plant materials

Collection information for the *Helichrysum* species, individually numbered, is listed below:

1. *H. arenarium* (L.) Moench subsp. *erzincanicum* Davis & Kupicha, Erzincan: Tercan, between Güzbulak and Fındıklı, 1730 m, 15 June 2007.
2. *H. arenarium* (L.) Moench subsp. *rubicundum* (K.Koch.) Davis & Kupicha, Erzurum: between Erzurum and Sarıkamış, between 38th and 44th km, 1495 m, 40°08.234N, 42°34.388E, 29 July 2007.
3. *H. armenium* DC. subsp. *araxinum* (Kirp.) Takht., Erzincan: between Erzincan and

   Kelkit, between 18th and 22nd km, 1850 m, 39°51.951N, 39°21.137E, 18 July 2006.
4. *H. plicatum* DC. subsp. *pseudoplicatum* (Nab.) Davis & Kupicha, Bitlis, 1180 m, 38°26.323N, 42°08.114E, 31 July 2007.

Voucher specimens were identified by Dr. Ahmet Aksoy and deposited at the Herbarium of the Department of Biology, Erciyes University, Kayseri, Turkey (voucher numbers: Aksoy 2094, Aksoy 2102, Aksoy 2110, and Aksoy 2103, respectively). *H. arenarium* subsp. *erzincanicum* is endemic to Anatolia (1).

Extraction

Dried aerial parts of the plants were ground to a fine powder with a grinder at room temperature. The powdered plant material (10 g) was then extracted using a Soxhlet-type extractor with 100 mL of methanol (MeOH) at 60 °C for 6 h. The extract was filtered and evaporated to dryness in a vacuum at 40 °C with a rotary evaporator. After determination of the yield, the extract was dissolved in methanol for further study (15).

Determination of total phenolics

The total phenolic content of the extracts was determined colorimetrically by the Folin-Ciocalteu method, using gallic acid as the reference compound (18). Briefly, 40 µL of the methanol solution of extract (1 mg/mL) was mixed with 2.4 mL of distilled water; then 200 µL of Folin-Ciocalteu reagent was added and

the contents of the flask were mixed thoroughly. After 1 min, 600 µL of sodium carbonate (20% Na_2CO_3) was added and the volume was increased to 4.0 mL with distilled water. After 2 h of incubation at room temperature, the absorbance was measured at 765 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

HPLC analysis of phenolic compounds in the extracts

The extracts were dissolved in methanol at a concentration of 10 mg/mL. A high performance liquid chromatograph (Shimadzu) was equipped with HPLC pumps (LC-10ADvp) and a DAD detector (278 nm). An Eclipse XDB-C18 (5 µm) column (250 × 4, 60 mm) (Shimadzu) was used. The flow rate was 0.8 mL/min and the injection volume was 20 µL. The analyses of the phenolic compounds were carried out at 30 °C using 2 linear gradients of 3% acetic acid and methanol. Detection was carried out between the wavelengths of 200 and 550 nm. Acacetin, apigenin, apigenin-7-glucoside, caffeic acid, catechin, chlorogenic acid, epicatechin, eriodictyol, ferulic acid, gallic acid, hesperidin, luteolin, naringenin, *p*-coumaric acid, *p*-hydroxybenzoic acid, quercetin, rosmarinic acid, resveratrol, rutin, and syringic acid were used as standards. Identification and quantitative analysis were done by comparison with standards.

Determination of antioxidant activity

Phosphomolybdenum assay

The antioxidant activity of the *Helichrysum* methanolic extracts was determined by the phosphomolybdenum method of Prieto et al. (19). First, 0.4 mL of the methanolic extract (1 mg/mL) was mixed with 4 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were then capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the green phosphomolybdenum complex was measured at 695 nm. In the case of the blank, 0.4 mL of methanol was used in place of the sample. The antioxidant activity was determined using a standard curve with ascorbic acid solutions as the standard. The mean of 3 readings was used and the reducing capacity of the extracts was expressed as milligrams of ascorbic acid equivalents (AAE) per gram of extract.

Diphenyl-2-picrylhydrazyl free radical scavenging activity

The ability of the extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was assessed spectrophotometrically (20). The 50 µL aliquots of the proper methanolic extract dilution, at a concentration range of 0.1-2 mg/mL, were mixed with 450 µL of Tris-HCL buffer (pH 7.4) and 1 mL of the methanolic DPPH solution (0.1 mM). Methanol was used as a control instead of the extract. The mixtures were left for 30 min at room temperature in the dark, and the absorbance was measured at 517 nm using methanol as a blank. IC_{50} (concentration causing 50% inhibition) values of the methanolic extracts were determined graphically. The same procedure was repeated with BHT as a positive control. The measurements were performed in triplicate and the results were averaged.

Radical scavenging activity was expressed as the percentage inhibition of DPPH radical and was calculated by following equation:

$$\text{Percentage inhibition (I\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100.$$

Determination of antimicrobial activity

The microorganism strains used in this study were *Aeromonas hydrophila* ATCC 7965, *Bacillus brevis* FMC 3, *B. cereus* RSKK 863, *B. subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* FMC 5, *Morganella morganii*, *Mycobacterium smegmatis* RUT, *Proteus mirabilis* BC 3624, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 (A), *S. aureus* ATCC 25923 (B), *Yersinia enterocolitica* ATCC 1501, *Candida albicans* ATCC 1223, and *Saccharomyces cerevisiae* BC 5461. The microorganism strains used were provided by the Department of Food Microbiology of the Engineering Faculty of Erciyes University.

The agar-well diffusion method was employed for the determination of the antimicrobial activities of the extracts (21,22). Each microorganism was suspended

in sterile nutrient broth. Test yeasts (*C. albicans* and *S. cerevisiae*) were suspended in malt extract broth, and each microorganism was diluted at approximately 10^6-10^7 colony forming units (cfu) per milliliter. Then 250 µL of each microorganism was added to a flask containing 25 mL of sterile Müller-Hinton agar or malt extract agar at 45 °C and poured into petri dishes (9 cm in diameter). The agars were allowed to solidify at 4 °C for 1 h and equidistant wells (4 mm in diameter) were cut from the agar. The extracts were prepared at 100,000, 50,000, 25,000, and 10,000 µg/mL concentrations in absolute methanol, and 40 µL of extract solutions were applied to the wells. Absolute methanol without extract was used as a control. *Y. enterocolitica*, *C. albicans*, and *S. cerevisiae* were incubated at 25 °C for 24-48 h in an inverted position. The other microorganisms were incubated at 37 °C for 18-24 h. At the end of the period, all plates were examined for any zones of growth inhibition, and the diameters of those zones were measured in millimeters. Standard antibiotics (Oxoid), namely amoxicillin (AML, 25 µg/disk), ampicillin (AMP, 10 µg/disk), carbenicillin (CAR, 100 µg/disk), chloramphenicol (C, 30 µg/disk), erythromycin (E, 15 µg/disk), gentamicin (CN, 10 µg/disk), kanamycin (K, 30 µg/disk), oxacillin (OX, 1 µg/disk), rifampicin (RD, 5 µg/disk), streptomycin (S, 10 µg/disk), tetracycline (TE, 30 µg/disk), and vancomycin (VA, 30 µg/disk), were used as positive controls. All tests were performed in duplicate and the results are presented as averages.

Statistical analysis

Data from the experiments were subjected to analysis of variance (ANOVA) using SPSS (2001) for Windows. Percentage data were transformed using arcsine √x before ANOVA. Means were separated at the 5% significance level by the least significant difference (LSD) test. Bivariate correlations were analyzed by Pearson's test using SPSS 10.0 for Windows (23).

Results and discussion

In this study, percentage extract yields, total phenolics, and antioxidant and antimicrobial activities of the methanolic extracts of 4 *Helichrysum* species collected from eastern Anatolia, Turkey, were determined. The extract yields ranged from 13.00% to 20.50% (w/w) (Table 1).

The total phenolic contents of the extracts, as estimated by the Folin-Ciocalteu reagent method, ranged from 71.81 ± 1.0 to 144.50 ± 1.2 mg GAE/g extract. The statistical differences among the total phenolic contents of extracts of the 4 *Helichrysum* species were important ($P < 0.05$) (Table 1). The highest level of phenolics was found in *H. plicatum* subsp. *pseudoplicatum*, while the lowest was in *H. arenarium* subsp. *rubicundum*. No references concerning the total phenolic content of *Helichrysum* species could be found despite a thorough literature survey, except for Özkan et al. (17), who reported that the total phenolic content of the methanolic extract of *H. chasmolycicum* was 108.33 ± 0.88 mg GAE/g. Aslan et al. (11) determined that the total phenolic contents of the ethanolic and water extracts of *H. plicatum* subsp. *plicatum* collected from eastern Anatolia were 113.5 ± 8.6 and 75.9 ± 3.7 mg GAE/g extract, respectively. It has been determined that the total phenolic contents of the methanolic extracts of *H. pamphylicum*, *H. sanguineum*, and *H. chasmolycicum* were 119.85 ± 2.0, 63.8 ± 0.6, and 71.51 ± 0.5 mg GAE/g extract, respectively (24). The total phenolic content of *H. arenarium* subsp. *rubicundum* (71.81 ± 1.0 mg GAE/g extract) was very similar to that of *H. chasmolycicum* (71.51 ± 0.5 mg GAE/g extract) by the Folin-Ciocalteu method. The total phenolic contents of the other 3 species tested in this study were higher than those of *H. pamphylicum*, *H. sanguineum*, and *H. chasmolycicum*.

It is obvious that the total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quality or quantity of the phenolic constituents in the extracts (25). The phenolic acids and flavonoids were determined by the HPLC method.

The data from the qualitative and quantitative analysis of the extracts determined using HPLC, coupled with photodiode array detection (DAD), are presented in Table 2. The amount of each compound is presented as µg/g dry residue (Table 2).

The gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, rosmarinic acid, catechin,

epicatechin, rutin, resveratrol, hesperidin, apigenin-7-glucoside, eriodictyol, quercetin, naringenin, luteolin, apigenin, and acacetin were identified by comparisons to the retention times and UV spectra of authentic standards analyzed under identical analytical conditions, while the quantitative data were calculated from their respective calibration curves.

Literature surveys indicated that this study is the first research concerning the phenolic compositions of the *Helichrysum* species used here. The major phenolic component present in the extracts was identified as chlorogenic acid, followed by apigenin-7-glucoside and apigenin. Ferulic acid, *p*-hydroxybenzoic acid, syringic acid, epicatechin, and

Table 1. The yields, total phenolic components, total antioxidant activities, and IC_{50} values of *Helichrysum* methanolic extracts.

Helichrysum Species	Yield (%)	Total Phenolic Component (mg GAE/g MeOH extract)	Total Antioxidant Activity (mg AAE/g MeOH extract)	DPPH IC_{50} (µg/mL)
H. arenarium subsp. *erzincanicum*	16.00	125.57 ± 1.0 b*	132.03 ± 0.3 c	23.03 d
H. arenarium subsp. *rubicundum*	20.50	71.81 ± 1.0 d	105.86 ± 0.4 d	47.64 a
H. armenium subsp. *araxinum*	17.47	86.01 ± 0.6 c	148.97 ± 0.3 b	27.32 c
H. plicatum subsp. *pseudoplicatum*	13.00	144.50 ± 1.2 a	161.79 ± 0.3 a	38.82 b

*: Means followed by the same letter in a column are not significantly different at P = 0.05 (ANOVA followed by LSD test).
Values expressed are mean ± standard deviation of 3 experiments. Total phenolic activity is expressed as gallic acid equivalent (GAE); total antioxidant activity is expressed as ascorbic acid equivalent (AAE).

Table 2. The quantity of some phenolic compounds determined in methanolic extracts by HPLC (µg/g extract).

Compounds	*Helichrysum* Species			
	H. arenarium subsp. *erzincanicum*	*H. arenarium* subsp. *rubicundum*	*H. armenium* subsp. *araxinum*	*H. plicatum* subsp. *pseudoplicatum*
Chlorogenic acid	150.30	45.20	63.70	94.00
Caffeic acid	2.00	1.43	1.68	1.19
Ferulic acid	-	-	-	0.37
p-Coumaric acid	1.56	2.70	2.54	0.91
p-Hydroxybenzoic acid	-	-	-	2.67
Syringic acid	-	-	-	2.73
Apigenin	44.20	25.70	24.70	33.20
Apigenin-7-glucoside	127.60	36.20	36.90	54.90
Epicatechin	-	-	-	2.33
Hesperidin	-	-	-	4.89
Luteolin	3.80	8.21	9.94	5.37
Naringenin	11.06	7.51	8.27	6.94
Resveratrol	0.26	0.21	0.19	-
Total	340.78	127.16	147.92	209.50

-: Not detected.

hesperidin could not be detected in the *H. arenarium* subsp. *erzincanicum*, *H. arenarium* subsp. *rubicundum*, or *H. armenium* subsp. *araxinum* extracts. Gallic acid, catechin, rutin, acacetin, eriodictyol, quercetin, and rosmarinic acid could not be detected in any of the extracts tested.

The chemical composition of the *H. plicatum* subsp. *plicatum* belonging to Turkish flora has been investigated. Several flavonoids, including apigenin, naringenin, isoastragalin, isosalipurposide, and helichrysin A and B, were isolated as the major constituents from the capitula of *H. plicatum* subsp. *plicatum* (11). Apigenin, apigenin-7-glucoside, luteolin, naringenin, and quercetin have also been isolated from the capitula of *H. compactum* and were shown to possess antioxidant activity (6).

Chlorogenic acid, naringenin glucosides, quercetin, and apigenin glucosides have been detected from the capitula of *H. stoechas* and were shown to possess antioxidant activity (26). Qualitative and quantitative characterization of the polyphenol fraction derived from *H. italicum*, which is considered responsible for its scavenger activity, was also documented (27).

Statistical differences were found among the 4 extracts for total antioxidant activity ($P < 0.05$) (Table 1). Among the 4 species tested in this study. *H. plicatum* subsp. *pseudoplicatum* (161.79 ± 0.3 mg AAE/g extract) showed the highest antioxidant activity in the phosphomolybdenum assay. The lowest antioxidant activity was found in the *H. arenarium* subsp. *rubicundum* extract, with a value of 105.86 ± 0.4 mg AAE/g extract. Results of the phosphomolybdenum assay indicate that the methanolic extracts of the *Helichrysum* species tested have strong antioxidant activities.

DPPH radical scavenging activities of the methanolic extracts are shown in the Figure. DPPH free radical scavenging activities of *Helichrysum* methanolic extracts at 0.25-2 mg/mL concentrations were compared with BHT (Figure). In the present investigation, the methanolic extracts of *H. arenarium* subsp. *erzincanicum*, *H. arenarium* subsp. *rubicundum*, *H. armenium* subsp. *araxinum*, and *H. plicatum* subsp. *pseudoplicatum* were found to be effective DPPH radical scavengers. As can be seen in the Figure, the extracts exhibited concentration-dependent DPPH scavenging activity. The free radical scavenging activities of *H. armenium* subsp. *araxinum*, *H. arenarium* subsp. *erzincanicum*, and *H. plicatum* subsp. *pseudoplicatum* (85.86%, 81.45%, and 79.59%, respectively) were near that of BHT (92.15%), used as a positive control at a concentration of 2 mg/mL. Additionally, the concentrations of the methanolic extracts required to scavenge 50% of the

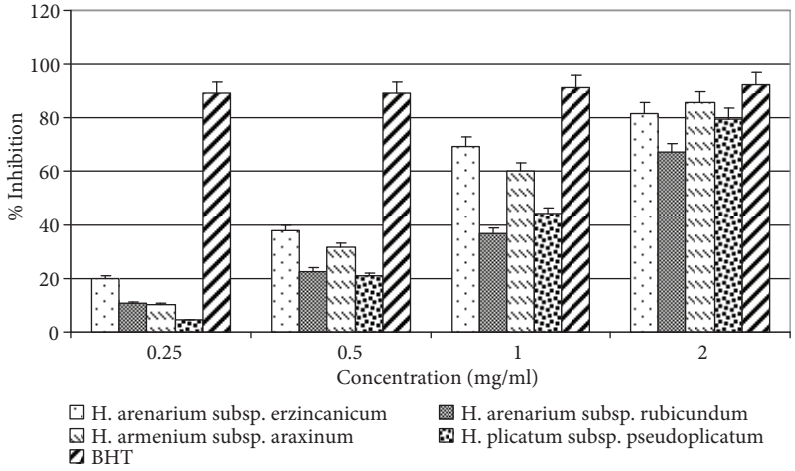

Figure. DPPH free radical scavenging activities of methanolic extracts of *Helichrysum* species tested and BHT at different concentrations (mg/mL).

DPPH radicals, the IC_{50} values, were calculated (Table 1). The most active radical scavenger was *H. arenarium* subsp. *erzincanicum* (IC_{50} = 23.03 µg/mL). *H. arenarium* subsp. *rubicundum* exhibited the weakest antiradical activity (IC_{50} = 47.64 µg/mL) in this study.

Bivariate correlations were analyzed by Pearson's test using SPSS 10.0 for Windows. It was observed that the total phenolic content in the extracts correlated with the total antioxidant activity (r^2 = 0.686). However, the same correlation was not observed between the total phenolic content and radical scavenging activity (r^2 = -0.320).

Antioxidant and antiradical activities of the methanolic extracts from *H. pamphylicum*, *H. sanguineum*, and *H. chasmolycicum* were recently reported from our laboratory. In our previous paper, we reported that the total antioxidant activities of *H. pamphylicum*, *H. sanguineum*, and *H. chasmolycicum* were 173.58 ± 1.1, 159.94 ± 0.3, and 147.88 ± 0.9 mg AAE/g extract, and the IC_{50} values of those species were 15.21, 12.90, and 25.33 µg/mL, respectively (24). The total antioxidant activity of *H. pamphylicum* was observed to be higher than that of the *Helichrysum* extracts tested in this study. The total antioxidant and antiradical activities of *H. armenium* subsp. *araxinum* were very similar to those of *H. chasmolycicum*.

There are many reports dealing with the antioxidant activity of different *Helichrysum* species. For instance, Özkan et al. (17) reported that the antioxidant activity of the methanolic extract of *H. chasmolycicum* was 246.83 ± 1.23 mg AAE/g. The methanolic extracts of *H. noeanum*, *H. chionophilum*, *H. plicatum* subsp. *plicatum*, and *H. arenarium* subsp. *aucheri* collected from Turkey have been reported to have antioxidant activity, according to DPPH and β-carotene linoleic acid assays (13). Our results are in agreement with those reported by Tepe et al. (13), in which the IC_{50} values of *H. chionophilum*, *H. plicatum* subsp. *plicatum*, and *H. arenarium* subsp. *aucheri* were 40.5, 48.0, and 47.6 µg/mL, respectively. Sroka et al. (28) reported that the extract obtained from *H. arenarium* inflorescence exhibited strong antiradical activity and contained caffeic acid as the major constituent.

It has been determined that the supercritical CO_2 extract of *H. italicum* was the active free radical scavenger in the DPPH and β-carotene bleaching test methods (29). Lourens et al. (30) reported that the IC_{50} values of the methanolic extracts of *H. dasyanthum*, *H. excisum*, *H. felinum*, and *H. petiolare* were 12.33, 13.67, 20.71, and 28.70 µg/mL, respectively.

Literature surveys indicated that plant phenolics constitute one of the major groups of compounds acting as primary antioxidants (31,32). Therefore, it is worthwhile to determine the phenolic compounds of the *Helichrysum* species selected for this study. Phenolic compounds are commonly found in both edible and inedible plants, and they have been reported to have multiple biological effects, including antioxidant activity (33). A strong relationship between total phenolic content and antioxidant activity in fruits, vegetables, and grain products has been reported (34). The methanolic extracts of the *Helichrysum* species tested displayed high antioxidant activity. The high antioxidant activities of these plants may be due to their flavonoid and phenolic contents. Chlorogenic acid, apigenin-7-glucoside, and apigenin, which are the major phenolic components in the extracts tested, may be responsible for their biological properties, including antioxidant and antimicrobial activity. Similarly, Czinner et al. (8) stated that the main phenolics and flavonoids of *Helichrysum* species may be responsible for their antioxidant properties.

The antimicrobial effects of the methanolic extracts against 15 microorganisms are shown in Table 3, at 100,000, 50,000, 25,000, and 10,000 µg/mL concentrations. The extracts caused different inhibition zones among the tested microorganisms. Pure methanol (control) used as a solvent had no inhibitory effects on the tested 15 microorganisms. The methanolic extracts at the lowest concentration were the least effective. No activity was found against *E. coli*, *M. morganii*, *M. smegmatis*, *P. mirabilis*, *Y. enterocolitica*, or *S. cerevisiae*. Among the tested *Helichrysum* extracts, *H. armenium* subsp. *araxinum* only had an inhibitory effect against *B. subtilis*, *S. aureus* (B), and *C. albicans*. The methanolic extracts had broader activity against *A. hydrophila*, *B. brevis*, *B. cereus*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* (A). In this study, the antimicrobial activities of the extracts were compared with standard antibiotics (Table 4).

Table 3. Antimicrobial activity of *Helichrysum* species (100,000, 50,000, 25,000, and 10,000 µg/mL concentrations) as mean of inhibition diameter zone (mm).

	Plant Names (%)															
	H. arenarium subsp. *erzincanicum*				*H. arenarium* subsp. *rubicundum*				*H. armenium* subsp. *araxinum*				*H. plicatum* subsp. *pseudoplicatum*			
Microorganisms	100 000	50 000	25 000	10 000	100 000	50 000	25 000	10 000	100 000	50 000	25 000	10 000	100 000	50 000	25 000	10 000
A. hydrophila	28.0 ± 0.0*	26.5 ± 0.7	24.0 ± 1.4	22.0 ± 1.4	25.0 ± 0.7	23.0 ± 0.0	21.0 ± 0.7	18.0 ± 0.0	14.0 ± 1.4	10.5 ± 0.7	8.5 ± 0.7	7.5 ± 0.7	28.0 ± 0.0	25.5 ± 0.7	24.0 ± 1.4	20.5 ± 0.7
B. brevis	22.5 ± 0.7	20.0 ± 1.4	18.0 ± 1.4	15.5 ± 0.7	21.0 ± 0.7	20.0 ± 0.7	18.0 ± 1.4	17.0 ± 0.7	16.0 ± 0.0	15.0 ± 0.0	14.0 ± 0.0	11.0 ± 0.0	23.0 ± 0.0	20.0 ± 1.4	19.0 ± 1.4	15.0 ± 1.4
B. cereus	22.5 ± 0.7	21.5 ± 0.7	20.0 ± 0.0	17.0 ± 1.4	22.0 ± 1.4	21.0 ± 1.4	20.0 ± 1.4	17.0 ± 1.4	21.5 ± 0.7	18.0 ± 0.0	13.5 ± 0.7	12.0 ± 1.4	20.5 ± 0.7	20.0 ± 0.0	19.0 ± 0.0	17.5 ± 0.7
B. subtilis	-	-	-	-	-	-	-	-	11.5 ± 0.7	9.5 ± 0.7	8.5 ± 0.7	7.0 ± 0.0	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K. pneumoniae	11.0 ± 0.0	10.0 ± 0.0	-	-	17.0 ± 1.4	16.0 ± 1.4	15.0 ± 1.4	12.0 ± 0.7	9.0 ± 0.0	8.0 ± 0.0	6.5 ± 0.7	-	18.0 ± 1.4	17.0 ± 1.4	16.0 ± 1.4	13.5 ± 0.7
M. morganii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. smegmatis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. mirabilis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	25.0 ± 0.0	23.0 ± 0.0	22.0 ± 1.4	19.0 ± 1.4	28.0 ± 0.0	26.0 ± 0.0	25.0 ± 0.0	21.0 ± 1.4	11.5 ± 0.7	9.5 ± 0.7	8.5 ± 0.7	7.5 ± 0.7	25.0 ± 1.4	24.0 ± 1.4	22.0 ± 1.4	20.0 ± 1.4
S. aureus (A)	23.5 ± 0.7	22.0 ± 0.0	19.5 ± 0.7	14.5 ± 0.7	17.0 ± 0.0	16.0 ± 0.0	16.0 ± 0.0	12.0 ± 0.7	9.0 ± 0.0	8.0 ± 0.0	7.0 ± 0.0	6.5 ± 0.7	21.5 ± 0.7	20.5 ± 0.7	19.5 ± 0.7	16.5 ± 0.7
S. aureus (B)	-	-	-	-	-	-	-	-	10.0 ± 1.4	8.5 ± 0.7	7.0 ± 0.0	6.0 ± 0.0	-	-	-	-
Y. enterocolitica	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	18.0 ± 0.0	17.0 ± 0.0	16.5 ± 0.0	12.5 ± 0.0	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*: Values expressed are mean ± standard deviation of 2 experiments; inhibition zones include diameter of hole (4 mm); sample amount: 40 µL.
-: Not detected.

Table 4. Antibacterial activities of standard antibiotics (inhibition zone, mm).

Microorganisms	Standard Antibiotics (µg)											
	AML	AMP	K	C	CAR	CN	E	OX	RD	S	TE	VA
A. hydrophila ATCC 7965	33.0*	27.0	13.0	18.0	35.0	8.5	20.0	15.0	17.0	12.0	28.0	18.0
B. brevis FMC 3	12.0	8.0	20.0	20.0	9.0	16.0	22.0	-	10.0	20.0	23.0	27.0
B. cereus RSKK 863	34.0	31.0	15.0	21.0	38.0	11.0	18.0	20.0	17.0	15.0	25.0	19.0
B. subtilis ATCC 6633	25.0	24.0	15.0	25.0	24.0	12.0	20.0	19.0	18.0	16.0	27.0	20.0
E. coli ATCC 25922	12.0	6.5	7.0	17.0	6.5	9.0	-	-	10.0	11.0	21.0	7.0
K. pneumoniae FMC 5	16.0	14.0	11.0	13.0	12.0	6.5	11.0	-	11.0	8.0	15.0	17.0
M. morganii	-	-	6.5	11.0	18.0	-	-	-	10.0	6.5	6.5	7.0
M. smegmatis RUT	29.0	25.0	13.0	17.0	27.0	8.5	-	-	11.0	8.0	8.0	6.5
P. mirabilis BC 3624	31.0	26.0	13.0	19.0	30.0	8.0	-	-	10.0	8.5	7.0	-
P. aeruginosa ATCC 27853	30.0	25.0	12.0	15.0	31.0	12.0	-	-	13.0	12.0	7.0	6.5
S. aureus ATCC 29213 (A)	17.0	16.0	12.0	15.0	13.0	7.0	12.0	-	11.0	8.0	15.0	13.0
S. aureus ATCC 25923 (B)	6.5	20.0	12.0	16.0	14.0	10.0	15.0	21.0	18.0	11.0	20.0	14.0
Y. enterocolitica ATCC 1501	13.0	8.0	14.0	17.0	9.0	9.0	7.0	-	12.0	13.0	22.0	8.5
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-

Amoxicillin (AML, 25 µg/disk), ampicillin (AMP, 10 µg/disk), carbenicillin (CAR, 100 µg/disk), chloramphenicol (C, 30 µg/disk), erythromycin (E, 15 µg/disk), gentamicin (CN, 10 µg/disk), kanamycin (K, 30 µg/disk), oxacillin (OX, 1 µg/disk), rifampicin (RD, 5 µg/disk), streptomycin (S, 10 µg/disk), tetracycline (TE, 30 µg/disk), and vancomycin (VA, 30 µg/disk).
*: Inhibition zones include diameter of disc (6 mm).
-: Not detected.

The antimicrobial activities of *Helichrysum* species have been reported elsewhere (16,17,24,35,36). Özkan et al. (17) determined that the methanolic extract of *H. chasmolycicum* demonstrated antibacterial activity and that *Y. enterocolitica* was the most resistant bacteria. It was previously reported that the methanolic extract of *H. compactum* exhibited antibacterial activity against 15 lactobacilli, 16 *S. aureus* strains, *E. coli* ATCC 25922, and *Y. enterocolitica* ATCC 1501 (16). It was also determined that the acetone extract of *H. dasyanthum* was active against *S. aureus* (MIC = 15.63 µg/mL) (30). Nostro et al. (35) showed that *H. italicum* diethyl ether extract inhibited staphylococcal growth and some of its enzymes. Cushine and Lamb (36) also showed that *H. aureonitens* demonstrated antibacterial activity against *S. aureus*. The antimicrobial activity of the methanolic extract of *H. aureonitens* against *B. cereus* was demonstrated by Meyer and Afolayan (37). Steenkamp et al. (38) reported that the methanolic extract of *H. foetidum* inhibited the growth of *S. aureus*, *Streptococcus pyogenes*, *E. coli*, and *P. aeruginosa* (MIC > 4 mg/mL). Van Vuuren et al. (39) showed that the acetone extract of *H. cymosum* subsp. *cymosum* was active against *Enterococcus faecalis*, *B. cereus*, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *Y. enterocolitica*, *K. pneumoniae*, *Cryptococcus neoformans*, and *C. albicans* (MIC = 0.078-0.313 mg/mL). In this study, the findings were similar to the observations of previous studies. However, it is difficult to compare the results of different studies because of the different *Helichrysum* species used and/or the different methods used for evaluation of antimicrobial activities.

The results show that the 4 selected *Helichrysum* species had significant antioxidant and antimicrobial activity in all tests performed in this study. The results reported here can be considered as the first information on the phenolic compositions and antimicrobial and antioxidant activities of *H. arenarium* subsp. *erzincanicum*, *H. arenarium* subsp. *rubicundum*, *H. armenium* subsp. *araxinum*, and *H. plicatum* subsp. *pseudoplicatum*. Further studies

should be carried out for the isolation and identification of individual phenolic compounds, and in vivo studies are also needed for an understanding of their mechanisms of action as antioxidant and antimicrobial agents.

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