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Original Article

Antimicrobial Activity of Five Endemic *Asperula* Species from Turkey

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Abstract

In this study, methanol and ether extracts of five endemic *Asperula* species (*Rubiaceae*) from Turkey (*A. antalyensis*, *A. brevifolia*, *A. pseudochlorantha*, *A. purpurea* subsp. *apiculata* and *A. serotina*), used in the traditional system of medicine, were tested for antimicrobial activity by the agar well diffusion method and the broth dilution method. The most active species were *Asperula brevifolia* and *A. serotina* which showed broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and maximum inhibition was shown by methanol extract of *A. antalyensis* against *Candida albicans* as 32 mm. Methanol extracts of *Asperula* species were among the most active with the MIC values ranging from 7.6 to 14.8 mg/mL.

Keywords: *Asperula*; antimicrobial activity; Turkey.

Introduction

The use of medicinal plants still plays a vital role to cover the basic health needs in developing countries. Herbal remedies used in folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy which might help overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics. Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine (1, 2).

Turkey has an extraordinarily rich flora of nearly 10,000 natural plant species (3, 4) thanks to its geographic location and climate. There is also a wide knowledge of their medicinal properties.

The genus *Asperula* is represented in the Turkish flora by 39 species, 19 of which are endemic. Most of them grow in the south west and north east parts of Anatolia (5). Some species belonging to this genus contain quinonic compounds (anthraquinones, naphtho-quinones, naphthohydroquinones and their glycosides), iridoids, coumarins, triterpenes and flavonoids (6).

Despite the medicinal potential of plants in Turkey being considerable, knowledge of this area and studies on these plants is scarce (7). Some *Asperula* species are used in folk medicine as a diuretic and tonic and against diarrhea (6). To the best of our knowledge, no information is available on the antimicrobial nature of these plants.

This study aimed to determine the antimicrobial activity of the methanol and ether extracts of aerial parts of five endemic *Asperula* species, *Asperula antalyensis*, *A. brevifolia*, *A. pseudochlorantha*, *A. purpurea* subsp. *apiculata*, and *A. serotina* against various microorganisms.

Experimental

Plant materials

Asperula species were collected from

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Table 1. Collection time and location of *Asperula* species.

Taxa	Specimen location and habitat
A. brevifolia	Muğla: Marmaris, Datça to Knidos, limestone rocks 480 m, 21.05.2007 Minareci E. 451–23 (N: 36° 42' 53.39 E: 27° 34' 22.28)
A. pseudochlorantha	Antalya: Kemer, under *Pinus bruita* forest, 50–100 m 07.06.2007 Minareci E. 452–2, 453–2 (36° 35' 34.13 N, 30° 30' 31.03 E).
A. antalyensis	Antalya: Göynük, west slope of town, rocky area, 60 m, 11.06.2007 Minareci E. 452–49, 453-49 (36° 40› 30.60 N, 30° 32› 20.87 E).
A. serotina	Karaman: Ermenek towards Mut 62. km, 885 m, 13.07.2006, Minareci E. 454–89 (N: 36° 36' 0.39 E: 33° 03' 1.95)
A. purpurea subsp. ***apiculata***	Tekirdağ: Şarköy, 7 km from Uçmakdere to Yeniköy, 14.06.2007, Minareci E. 455–64 (N: 40° 50' 17.50 E: 27° 23' 07.70).

southwest and northeast parts of Anatolia. The collection time and location of these species are given in Table 1. Voucher specimens were deposited in the Herbarium of Botany, Department of Biology, Celal Bayar University. The aerial parts of these plants used in present study.

Microorganisms and growth conditions

Test microorganisms included the following bacteria: *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 39628, *Micrococcus luteus* ATCC 9341, *Bacillus cereus* CM 99, *Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *Salmonella typhimurium* CCM 5445, *Enterococcus faecalis* ATCC 29212, *Proteus vulgaris* ATCC 8427, *Pseudomonas fluorescens* ATCC 25289, *Serratia marcescens* CCM 583, *Klebsiella pneumoniae* UC 57 and for yeasts *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763. Cultures of these bacteria were grown in Mueller Hinton broth (Oxoid) at 37°C for 24 h and the studied yeasts were incubated in glucose yeast extract broth at 30°C for 48 h (2). Test microorganisms were obtained from the culture collection of Ege University, Faculty of Science, Basic and Industrial Microbiology Department.

Preparation of extracts

The aerial parts of the plants were dried at room temperature and then reduced to coarse powder. Twenty grams of the samples were extracted with methanol and ether separately at room temperature, under stirring for 7 days; the extraction solvents were then evaporated under vacuum to dryness. Sample solutions were prepared by dissolving the extracts of the aerial parts in dimethyl sulfoxide (DMSO) at 5 mg/mL (7).

Antimicrobial assays

Agar well diffusion assay

In vitro antimicrobial studies were carried out by the agar well diffusion method against test microorganisms. Briefly, 50-µL inoculums (containing approximately 10^5 bacteria per milliliter and 10^4 yeast per milliliter) was added to 25 mL molten Mueller-Hinton agar (MHA) and Potato Dextrose agar (PDA) media cooled at 45°C. These media were then poured into 90-mm-diameter Petri dishes and maintained for 1 h at room temperature. Small wells (6 mm diameter) were cut in the agar plate using a cork borer; 100 µL of extract concentration with a negative control (DMSO, 100 µL) were loaded in the wells. The dishes were preincubated at 4°C for 2 h to allow uniform diffusion into the agar. After preincubation, for bacteria the plates were incubated aerobically at 37°C for 24 h and for yeasts at 30°C for 48 h (2). The antimicrobial activity was evaluated by measuring the inhibition zone diameter observed. In addition, commercial antibiotics, i.e. Penicillin G (10 IU), nalidixic acid (30 µg), novobiocin (30 µg), amphicillin (10 µg), vancomycin (30 µg), chloramphenicol (30 µg) and nystatin (10 µg) were used as positive control to determine the sensitivity of the strains (2). These studies were performed in triplicate.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined for five endemic *Asperula*

Table 2. Antimicrobial activity of the endemic *Asperula* species on some bacteria and yeasts mean ± SD (inhibitory zone diameter/ mm*).

Extracts	1	2	3	4	5	6	7	8	9	10	P	Na	No	C	A	V	N
Microorganisms																	
E. coli (EC)	-	-	-	18±2	-	-	14±1	-	-	20±2	-	26±2	-	26±0	-	-	-
E. aerogenes (EA)	14±1	-	-	23±2	18±1	12±2	15±2	-	14±1	22±0	-	26±3	17±1	12±1	-	-	-
P. vulgaris (PV)	10±1	-	-	20±1	15±1	-	10±2	-	-	10±0	10±1	12±0	26±2	15±1	10±0	22±2	-
B. cereus (BC)	12±2	11±2	15±1	-	-	-	-	10±1	14±2	16±1	10±0	28±2	25±0	28±2	-	15±1	-
B. subtilis (BS)	14±1	10±1	16±1	-	-	-	-	11±2	15±0	16±2	-	32±3	13±0	30±2	10±1	-	-
M. luteus (ML)	-	-	-	-	-	-	-	-	-	15±2	20±3	10±0	28±1	28±2	26±2	15±0	-
S. aureus (SA)	-	22±2	17±2	26±3	-	-	-	-	-	-	24±2	20±1	20±2	20±1	15±2	12±1	-
E. faecalis (EF)	12±1	11±1	14±2	20±1	-	-	-	-	15±1	17±1	24±3	30±2	28±1	30±0	16±1	16±1	-
S. typhimidium (ST)	13±1	10±1	11±1	-	12±0	12±1	11±1	10±2	12±1	22±3	-	-	40±2	40±2	-	28±2	-
P. fluorescens (PF)	-	-	-	15±2	-	-	-	-	-	15±0	-	30±2	20±2	12±1	-	-	-
S. marcescens (SM)	-	-	-	-	-	-	-	-	-	-	-	30±3	-	30±2	-	-	-
K. pneumoniae (KP)	-	-	-	-	-	-	-	-	-	-	-	20±1	22±1	-	-	-	-
C. albicans (CA)	32±3	29±2	17±2	30±3	-	-	14±1	-	-	-	-	-	-	-	-	-	22±2
S. cerevisiae (SC)	26±2	25±3	18±3	25±2	-	-	10±0	-	-	-	-	-	-	-	-	-	20±2

1: *A. antalyensis* methanol extract; **2**: *A. antalyensis* ether extract; **3**: *A. brevifolia* methanol extract; **4**: *A. brevifolia* ether extract
5: *A. pseudochlorantha* methanol extract; **6**: *A. pseudochlorantha* ether extract; **7**: *A. purpurea* methanol extract; **8**: *A. purpurea* ether extract
9: *A. serotina* methanol extract; **10**: *A. serotina* ether extract; **P**: Penicillin; **Na**: Nalidixic acid; **No**: Novobiocin; **C**: Chloramphenicol
A: Amphicillin; **V**: Vancomycin; **N**: Nystatin; -: No Activity, *: not including well diameter (6 mm)

species. The broth macrodilution method (8) was used to determine MIC of methanol extracts against selected test microorganisms, using Mueller-Hinton broth for bacteria and glucose yeast extract broth for yeasts. In these experiments, 0.5 mL of a microbial suspension containing 1×10^5 colony forming units (CFU)/ mL of bacteria and 1×10^4 CFU/mL of yeast was added to 4.5 mL of susceptibility test broth containing serial two-fold dilutions of the extract in glass test tubes according to NCCLS (9). All tubes were incubated at 37°C for 24 h for bacteria and at 28°C for 48 h for yeasts before being read. The MIC was considered the lowest concentration of the sample that prevented visible growth. All samples were examined in duplicate in three separate experiments.

Statistical Analysis

The mean values were statistically analyzed with the MINITAB Release 13.20 program by the general one-way (unstacked) analysis of variance (ANOVA) to find out the most effective extracts and the most sensitive test organisms. Similarity (%) of microorganisms in relation to their susceptibility to the plant extracts was analyzed by the multivariate cluster analysis according to the data obtained from well diffusion assay.

Results and Discussion

Antimicrobial activity of five endemic *Asperula* species has been evaluated in vitro against 12 bacterial species and two yeasts that are known to cause dermic and mucosal infections besides other infections in humans.

All *Asperula* species studied in this work showed antimicrobial activity against at least one of the test microorganisms with inhibition zones ranging from 10 to 32 mm (Table 2). This result showed that the studied plants are potentially a rich source of antimicrobial agents. However, the plants differ significantly in their activity against test microorganisms. According to one-way ANOVA results, antimicrobial activity has also shown differences among the taxa (P=0.0062, F=3.76, R=0.1). The most active species were *Asperula brevifolia* and *A. serotina* which showed broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, whereas the least active species were *A. pseudochlorantha* and *A. purpurea* subsp. *apiculata*. *Asperula antalyensis* and *A. brevifolia*

Table 3. MIC values of plant methanol extracts against selected microorganisms.

	MIC (mg/mL)				
	E. coli	*E. aerogenes*	*B. subtilis*	*S. aureus*	*C. albicans*
A. antalyensis	ND	8.2	8.6	ND	7.6
A. brevifolia	12.8	ND	7.8	9.2	10.2
A. pseudochlorantha	ND	7.6	ND	ND	ND
A. purpurea	9.8	9.6	ND	ND	ND
A. serotina	ND	10.0	9.0	14.8	ND

MIC: minimum inhibitory concentration; ND: not determined

demonstrated high antiyeast activity against *Candida albicans* and *Saccharomyces cerevisiae* (Figure 1).

Maximum inhibition was shown by methanol extract of *A. antalyensis* against *Candida albicans* as 32 mm. High inhibition zone diameters, i.e. 30 and 29 mm against *C. albicans* were obtained by ether extracts of *A. brevifolia* and *A. antalyensis,* respectively. Maximum antibacterial effect was shown by ether extract of *A. brevifolia* against *Staphylococcus aureus* as 26 mm (Table 2).

The ether extract of *A. antalyensis* and ether and methanol extracts of *A. brevifolia* were found to be more active against *S. aureus* than control antibiotics. Also, the various extracts of *Asperula* species studied in this work were determined to have effectiveness similar to control antibiotics against *Enterobacter aerogenes*, *Proteus vulgaris*, *Bacillus cereus*, *B. subtilis* and *Enterococcus faecalis* (Table 2).

Susceptibility of test strains, in decreasing order was as follows: *C. albicans* > *E. aerogenes*> *S. typhimidium* > *S. cerevisiae* > *E. faecalis* > *B. subtilis* > *B. cereus* > *S. aureus* > *P. vulgaris* > *E. coli* > *P. fluorescens* > *M. luteus* > *S. marcescens*> *K. pneumoniae* (Figure 1). Figure 2 summarizes the similarity of microorganisms in relation to their susceptibility to the plant extracts.

Significant antimicrobial effects expressed as MIC of crude methanol extracts against *C. albicans*, *E. coli*, *E. aerogenes*, *B. subtilis* and *S. aureus*, are shown in Table 3. Methanol extracts of *Asperula* species were among the most active with the MIC values ranging from

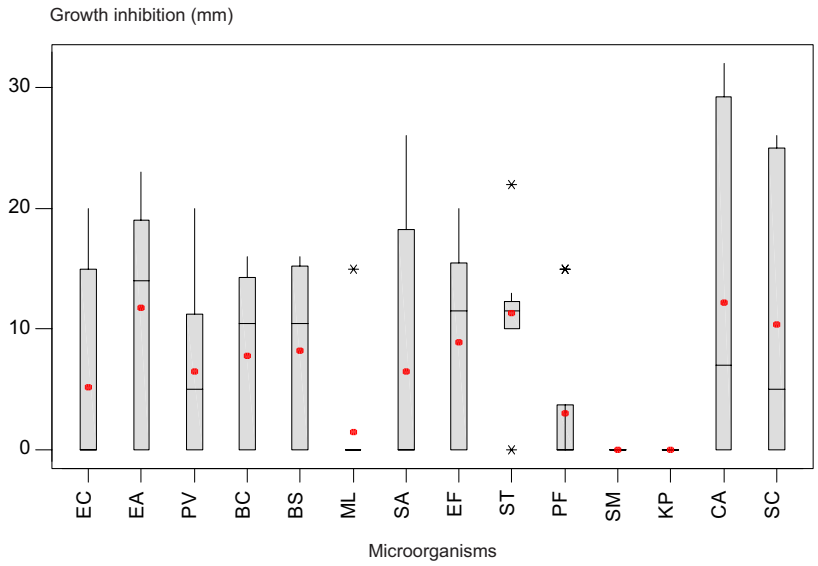

Figure 1. Mean values of microorganisms in relation to their susceptibility to the plant extracts. *Means are indicated by solid circles. EC: *Escherichia coli*, EA: *Enterobacter aerogenes*, PV: *Proteus vulgaris.* BC: *Bacillus cereus*, BS: *Bacillus subtilis*, ML: *Micrococcus luteus*. SA: *Staphylococcus aureus*, EF: *Enterococcus faecalis*, ST: *Salmonella typhimidium.* PF: *Pseudomonas fluorescens*, SM: *Serratia marcescens*, KP: *Klebsiella pneumoniae.* CA: *Candida albicans*, SC: *Saccharomyces cerevisiae.*

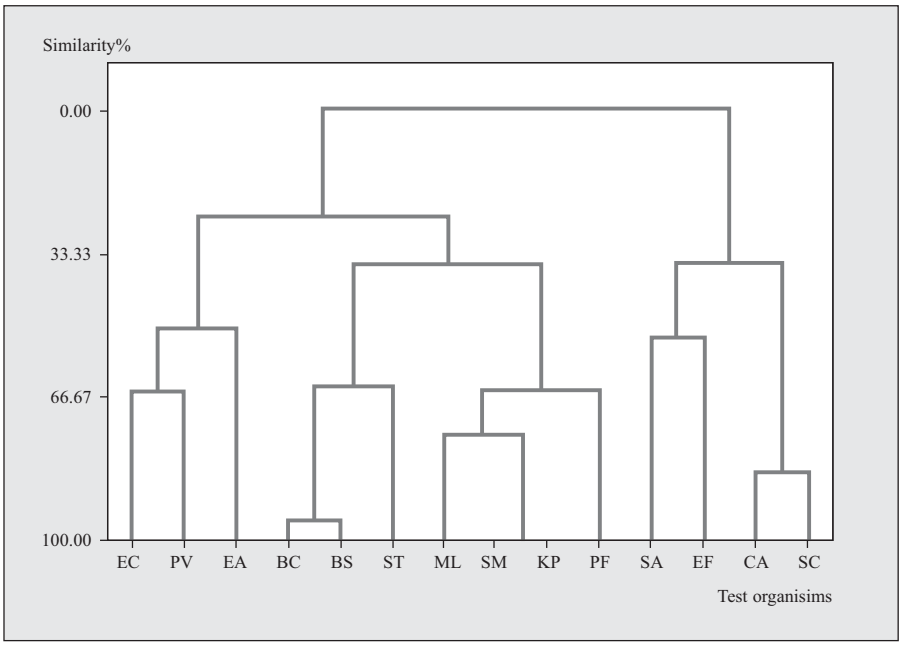

Figure 2. Similarity (%) of microorganisms in relation to their susceptibility to the plant extracts. EC: *Escherichia coli*, EA: *Enterobacter aerogenes*, PV: *Proteus vulgaris.* BC: *Bacillus cereus*, BS: *Bacillus subtilis*, ML: *Micrococcus luteus*. SA: *Staphylococcus aureus*, EF: *Enterococcus faecalis*, ST: *Salmonella typhimidium.* PF: *Pseudomonas fluorescens*, SM: *Serratia marcescens*, KP: *Klebsiella pneumoniae* CA: *Candida albicans*, SC: *Saccharomyces cerevisiae*

7.6 to 14.8 mg/mL. Among the plants tested, methanol extracts of *A. antalyensis* showed very strong activity against *C. albicans* with the best MIC (7.6 mg/mL). According to the literature data, no information is available on the antimicrobial nature of these species. However, a study reported that methanol extract of *A. nitida* subsp. *subcapitellata* showed weak inhibitory effect against *B. subtilis*, *B cereus* and *S. aureus* (4, 4 and 4.5 mm inhibition zone diameter, respectively) (10). In our study, methanol extracts of *A. serotina* and *A. brevifolia* showed 15 and 16 mm inhibition zone against *B.* subtilis and 14 and 15 mm inhibition zone against *B. cereus,* respectively (Table 2). Observed dissimilar results may be attributed to differences in techniques and extracts because different methods were used and the variable susceptibility of different microorganisms to chemical substances relates to different resistance levels between the strains.

The results of the current investigation clearly indicate that the antibacterial and antifungal activity vary with the endemic species of *Asperula*. Further, the active phytocompounds of these plants against some bacteria and yeasts should be characterized and their toxicity should be evaluated in vivo.

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