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Full Length Research Paper

Evaluation of antimicrobial activity of *Ballota acetabulosa*

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The ethanol extracts obtained from *Ballota acetabulosa* (L.) Benth (Lamiaceae) were investigated for their antimicrobial activity against *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Stapylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 10538, *Proteus vulgaris* ATCC 6899, *Salmonella typhimurium* CCM 5445, *Psuedomonas aeruginosa* ATCC 27853, *Debaryomyces hansenii* DSM 70238, *Kluyveromyces fragilis* ATCC 8608 and *Rhodotorula rubra* DSM 70403 by disc diffusion method and micro dilution method. The extracts showed strong antibacterial activity against *E. coli*, with inhibition zones of 18.6 mm and with minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of 16 (32) µg/mL, respectively. *K. fragilis* is among the most susceptible in the yeast cultures, with inhibition zone of 18.2 mm and with MICs and minimum fungicidal concentrations (MFCs) of 16 (32) µg/mL, respectively. Also, the extracts exhibited moderate activity in the other test of micro-organisms. The results demonstrate that the ethanol extract of the aerial parts of *B. acetabulosa* has significant activity and suggest that it may be useful in the treatment of infections.

Key words: *Ballota acetabulosa*, ethanol extract, antimicrobial activity.

INTRODUCTION

The genus *Ballota* L. (Lamiaceae) consist of about 33 species growing mainly in the Mediterranean region. In Turkey, the genus *Ballota* is reprensented by eleven species, six subspecies, ten of which are endemic (Davis, 1982). Plants of this genus have been used traditionally for nausea, vomiting, nervous dyspepsia, specifically for vomiting of central origin ans also are used for antiemetic, sedative, antibacterial and mild astringent properties (Newall et al., 1996; Citoglu et al., 1998).

Ballota acetabulosa (L.) Benth is an herbaceous plant growing in rocks and rough ground in dry hills up to 900 m in Greece and Western Anatolia (Sahpaz et al., 2002). During our field excursions, it was determined that these plants have been used externally in the treatment of wounds and burns. Aerial parts of the plant are used internally to treat inflammation, to suppress cough and against gastrointestinal disorders. So, the aim of this works was to evaluate the antimicrobial activity of the plant as wild-growing in Turkey.

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MATERIALS AND METHODS

Plant materials

Aerial parts of the plant were collected from Gokceada, Canakkale, Turkey in September, 2009. Voucher specimens of the plant were deposited in the Biology Department at Canakkale Onsekiz Mart University, Canakkale, Turkey.

Preparation of extracts

The aerial of the plants were dried in an oven at 40°C (12 h) and powdered. Each dry powdered plant material (20 g) was extracted with 150 mL of 95% ethanol (Merck, Darmstadt, Germany) for 24 h by using Soxhlet equipment. The extract was filtered using Whatman filter paper no.1 and the filtrate solvent was evaporated under vacuum using a rotary evaporator at 55 °C (yield: 14.12% for ethanol). The resulting dried extract was stored in labeled sterile screw-capped bottles at -20°C. The extract (in the form of sticky

black substances) amounting to around 2 g was dissolved in 0.1 mL of DMSO (5 mg/g) (dimethyl sulfoxide) before testing.

Test micro-organisms

in vitro antimicrobial studies were carried out seven bacteria strains (*Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 7064, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 10538, *Proteus vulgaris* ATCC 6899, *Salmonella typhimurium* CCM 5445 and *Pseudomonas aeruginosa* ATCC 27853) and four yeast strains (*Candida albicans* ATCC 10239, *Debaryomyces hansenii* DSM 70238, *Kluyveromyces fragilis* ATCC 8608 and *Rhodotorula rubra* DSM 70403) obtained from the Microbiology Research Laboratory in Canakkale Onsekiz Mart University, Department of Biology, Turkey.

Disc diffusion method

The paper disc diffusion method was employed (Collins and Lyne, 1987). Sterile 6 mm disc filter paper disc (Schleicher and Schul, 2668, Dassel, Germany) were impregnated with 50 µL of the plant extracts. The bacterial cultures were inoculated on nutrient broth (Oxoid) and incubated for 24 h at 37 ± 0.1°C, while the yeast cultures were inoculated on malt extract broth (Oxoid) and incubated for 48 h at 28 ± 0.1°C. Adequate amounts of Mueller Hilton agar (Oxoid) were dispensed into sterile plates and allowed to solidify under aseptic conditions. The counts of bacterial cultures and yeast cultures were adjusted to yield *ca*. $1.0 \times 10^7 - 1.0 \times 10^8$ mL^{-1} and $1.0 \times 10^5 - 10^6$ mL^{-1}, respectively, using the standard McFarland counting method. The test micro-organisms (0.1 mL) were inoculated with a sterile swab on the surface of appropriate solid medium in plates. The agar plates inoculated with the test micro-organisms were incubated for 1 h before placing the extract impregnated paper disc on the plates. The bacterial plates were incubated at 37 ± 0.1°C for 24 h while yeast plates were incubated at 28 ± 0.1°C for 48 h. After incubation, all plates were observed for zones of growth inhibition and the parameters of these zones were measured in millimeters. All tests were performed under sterile conditions in duplicate and repeated three times. Penicillin (10 µg/disc), Tobramycin discs (10 µg/disc), Ampicillin (20 µg/disc), Nystatin discs (30 µg/disc), Clotrimazole (30 µg/disc) and Ketoconazole (20 µg/disc) were used as positive controls.

Microdilution method

Determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by Zgoda and Porter (2001) with some modifications. Dilution series of the extracts were prepared from 10 to 0.5 mg/mL in test tubes then transferred to the broth in 96–well micro titer plates. Final concentrations were 1000 to 50 µg/mL in the medium. Before inoculation of the test organisms, the bacteria strains and yeast strains were adjusted to 0.5 McFarland and diluted 1:1000 in Mueller Hinton broth (Oxoid) and malt extract broth (Oxoid), respectively. Plates were incubated at 35°C for 18 – 24 h for bacteria and at 30°C for 48 h for the yeast cultures. The entire test were performed in broth and repeated twice. Whereas the MIC values of the extracts were defined as the lowest concentration that showed no growth, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by plotting samples from clear wells onto Mueller Hinton agar and malt extract agar, respectively. MBC and MFC were defined as the lowest concentration yielding negative subcultures. Ampicillin and Streptomycin were used as standard antibacterial agents, whereas Nystatin was used as a standard antifungal agent. Their dilutions were prepared from 128 to 0.25 µg/mL concentrations in micro titer plates.

RESULTS AND DISCUSSIONS

The antimicrobial activities of *B. acetabulosa* extracts against the test micro-organisms examined in this study were qualitatively and quantitatively assessed by the presence of inhibition zones, MIC, MBC and MFC (Tables 1 and 2). The ethanolic extracts of *B. acetabulosa* were strong antimicrobial effects against the test micro-organisms, with inhibition zones at 11.6 to 18.6 mm. Notably, *E. coli* is more susceptible to the extract of *B. acetabulosa* as compared to all standard antibacterial antibiotics (inhibition zone is 18.6 mm). Similarly, the extracts showed higher antibacterial activity on *S. typhimurium* than those of all standard antibacterial antibiotics (inhibition zone is 14.2 mm). Antifungal effects of the extracts against *K. fragilis* are more susceptible to the extracts, as compared to all the standard antifungal antibiotics (inhibition zone is 18.2 mm), followed by *R. rubra* and *D. hansenii*.

The ethanolic extracts were further tested by micro dilution to determine the MICS and MBCs. The lowest MICs and MBCs of the extract were 16(32) µg/mL against *E. coli* and *K. fragilis*, followed by *R. rubra* and *D. hansenii*, with ranged from 32(32) and 64 (>.128) µg/mL, respectively. The extracts have a weak antimicrobial effect against the other test micro-organisms with MICs and MBCs or MFCs ranged from 1000 (1000) to 250 (500) µg/mL. These values are far below than the standard antibiotics. Ethanol was observed as the best solvent for extracting antimicrobial substances in previous study (Jonathan and Fasidi, 2003). The results in this study with ethanol are similar to those reported in the mentioned study. It is important to bear in mind that the concentration of extract used in the test may be correlated with a high activity of its chemical components.

Investigations of antimicrobial activity on the other *Ballota* species are limited. In previously studies, diterpenoids and flavonoids isolated from *Ballota inaequidens* are investigated for their activities against *B. subtilis, S. aureus, E. coli, P. aeruginosa, C. albicans* and *C. krusei* (Citoglu et al., 2004). In that study, the compounds tested have no important inhibitory activity against bacteria but showed good activities against *C. albicans* and *C. krusei*. In addition, it is reported that three diterpenoid obtained from the aerial parts of *Ballota saxatilis* subsp. *saxatilis* and their effects against gram-positive (*S. aureus*, *S. faecalis*) and gram-negative (*P. aeruginosa*, *E. coli*, *K. pneumonia*) micro-organisms and *C. albicans* in a previous study (Citoglu et al., 1998). Couladis et al. (2002) reported that essential oil of *Ballota pseudodictamnus* has been investigated for their anti-microbial activity against *S. aureus, E. coli, Enterobacter aerogenes, K. pnemoniae, P. aeroginosa, C. albicans, C. tropicalis* and *C. glabrata* using the dilution technique. Essential oil of the plant exhibited strong to moderate activity against all

Table 1. Antimicrobial activity of *B. acetabulosa.*

Microorganisms	Diameter of zone of inhibition (mm)*						
	EtOH Extract (µg/mL)	Standards					
		P	AMP	TOB	NYS	KETO	CLT
B. subtilis	11.6	14.0	12.0	24.0	Nt	Nt	Nt
B. cereus	12.4	13.0	16.0	18.0	Nt	Nt	Nt
E. coli	18.6	16.0	14.0	10.0	Nt	Nt	Nt
S. aureus	15.2	23.0	16.0	8.0	Nt	Nt	Nt
P. aeruginosa	12.8	8.0	10.0	12.0	Nt	Nt	Nt
P. vulgaris	12.4	10.0	16.0	13.0	Nt	Nt	Nt
S. typhimurium	14.2	13.0	13.0	10.0	Nt	Nt	Nt
D. hansenii	16.2	Nt	Nt	Nt	16.0	14.0	20.0
K. fragilis	18.2	Nt	Nt	Nt	18.0	16.0	18.0
R. rubra	17.4	Nt	Nt	Nt	18.0	22.0	16.0

* Zone of inhibition, including the diameter of the filter disc (6.0 mm), mean value of three independent experiments, Nt: not tested; P: Penicillin (10 µg/disc), TOB: Tobramycin discs (10 µg/disc), AMP: Ampicillin (20 µg/disc), NYS: Nystatin discs (30 µg/disc), KETO: Ketoconazole (20 µg/disc); CLO: Clotrimazole (30 µg/disc).

Table 2. Minimum inhibitory concentration (MIC) of *B. acetabulosa.*

Microorganisms	MIC (MBC or MFC)			
	EtOH extract (µg/mL)	Standards		
		ST	AMP	NYS
B. subtilis	1000(1000)	0.5(0.5)	0.5(2.0)	Nt
B. cereus	500(> 1000)	4.0(4.0)	8.0(8.0)	Nt
E. coli	16(32)	4.0(4.0)	64(128)	Nt
S. aureus	250(500)	2.0(4.0)	<0.25(0.35)	Nt
P. aeruginosa	500(> 1000)	1.0(1.0)	16(32)	Nt
P. vulgaris	500(> 1000)	8.0(8.0)	0.5(0.5)	Nt
S. typhimurium	1000(1000)	16(32)	1.0(4.0)	Nt
D. hansenii	64(> 128)	Nt	Nt	16(32)
K. fragilis	16(32)	Nt	Nt	16(16)
R. rubra	32(32)	Nt	Nt	16(16)

Nt: not tested,; ST: Streptomycin, AMP: Ampicillin, NYS: Nystatin.

tested bacteria (MIC values 0.45 -10.15 mg/mL), while it appeared inactive against the tested fungi. Another study reported that *B. acetabulosa* is used for the treatment of haemorrhoids as infusion in folk medicine (Mericli et al., 1988). The antimicrobial activities of some *Ballota* species growing in Turkey was reported by Citoglu et al. (2003). The antimicrobial activities of ethanol extracts of 16 *Ballota* species growing in Turkey were studied. The ethanolic extracts were tested *in vitro* against gram-negative strains (*E. coli*, *P. aeruginosa*) and the gram-positive strains (*S. aureus*, *B. subtilis*) and the yeast cultures (*C. albicans*, *C. Glabrata* and *C. krusei*) by the agar diffusion method. Among *Ballota* species studied, *B. acetabulosa* has a strong antibacterial activity against bacterial strains. In addition, the extracts have antifungal activity against *C. albicans*, *C. glabrata* and *C. krusei*, with inhibition zones varied from 12, 13 and 12 mm, respectively. Besides, ethanol extracts of some species were tested against four diffferent *Listeria* isolates (*Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. murrayi*) by the agar diffusion method. Among *Ballota* species, *B. acetabulosa* have a strong antilisterial effects against all *Listeria* species except for *L. innocua* (Yilmaz et al., 2005). Equally, in this study all the extracts of *B. acetabulosa* were presented antimicrobial activity to both bacteria and yeast cultures. The differences between our result and others may be due to several factors, for example the infra-specific variability in the production of secondary metabolites. In addition, there may be differences in the extraction protocols to recover the active metabolites and differences in the assay methods. Flavonoids and also phenylpropanoids have been re-

reported to exist in some *Ballota* species such as *B. acetabulosa*, *B. foetida*, *B. hirsuta* and *B. nigra* (Darbour et al., 1986; Ferreres et al., 1986; Mericli et al., 1988; Seidel et al., 1996; Bertrand et al., 2000). Flavonoids may be responsible for their antibacterial activity (Saeedi et al., 2008). The result indicated that *B. acetabulosa* possessed significant activity against both bacteria and yeast cultures. This activity may be indicative of the presence of metabolic toxins or the mentioned plant compounds. So, this plant extracts should be analyzed further, as it might provide a new compound effective against pathogens. This preliminary evaluation indicated that *B. acetabulosa* has significant activity against the test microorganisms. Fur-ther analyses are necessary to identify the main active constituents.

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