

ANTIMICROBIAL ACTIVITY OF THE LEAVES OF *Ballota acetabulosa* ON MICROORGANISMS ISOLATED FROM URINARY TRACT INFECTIONS

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Abstract

The methanolic extracts obtained from the leaves of *Ballota acetabulosa* (L.) Bentham (Lamiaceae) were investigated for their antimicrobial activities against the pathogens causing complicated urine tract infections (*Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans*) by disc diffusion method and microdilution method. Some antibacterial and antifungal antibiotics were used as a positive reference standard to determine the sensitivity of the test microorganisms. The extracts showed strong antimicrobial activity against *Escherichia coli* with inhibition zones of 18.6 mm, with MIC's and MBC's of 32(64) µg/mL. Also, the extracts exhibited moderate activity against the other test microorganisms. The results demonstrate that the methanolic extract of the leaves of *Ballota acetabulosa* has significant activity and suggest that it may be useful in the treatment of infections.

Key words : Urinary Tract Infection (UTI), Antimicrobial activity, *Ballota acetabulosa*

İdrar Yolu Enfeksiyonlarından İzole Edilen Mikroorganizmalar Üzerine *Ballota acetabulosa* Yapraklarının Antimikrobiyal Aktivitesi

Ballota acetabulosa (L.) Bentham (Lamiaceae) yapraklarından elde edilen metanol ekstralarının, komplike idrar yolu enfeksiyonları oluşturan (*Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* ve *Candida albicans*) patojenlere karşı antimikrobiyal aktiviteleri disk difüzyon ve mikrodilüsyon metotları kullanılarak araştırılmıştır. Test mikroorganizmalarının duyarlılığını belirlemede, bazı antibakteriyel ve antifungal referans antibiyotikler pozitif kontrol olarak kullanılmıştır. Ekstraktlar, *Escherichia coli* bakterisine karşı 18.6 mm inhibisyon zonu, 32(64) µg/mL MIC ve MBC değeri oluşturarak güçlü antimikrobiyal aktivite göstermiştir. Buna ilaveten, ekstraktlar diğer test mikroorganizmalarına karşı ise orta derecede bir aktivite oluşturmuşlardır. Bulgular, *Ballota acetabulosa* yapraklarının metanol ekstratları belirli bir aktiviteye sahip olduğunu, enfeksiyonların tedavisinde yararlı olabileceğini göstermiştir.

Anahtar kelimeler: İdrar yolu enfeksiyonları, Antimikrobiyal aktivite, *Ballota acetabulosa*

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INTRODUCTION

Plants have long been in use as medicine the world over. More recently, plant extracts have been developed and proposed for use as antimicrobials.

The *Lamiaceae* is one of the most diverse and widespread plant families in terms of ethnomedicine and its medicinal value is mainly based on the volatile oils concentration (1). It is well documented that some plants growing in Turkey belonging to this family possess antimicrobial properties (2-5). The genus *Ballota* L. (Lamiaceae) consists of about 33 species growing mainly in the Mediterranean region. In Turkey, the genus *Ballota* is represented by eleven species, six subspecies, ten of which are endemic (6). Plants of this genus have been used traditionally for nausea, vomiting, nervous dyspepsia, specifically for vomiting of central origin and also are used for antiemetic, sedative, antibacterial and mild astringent properties (7-8). *Ballota acetabulosa* (L.) Benth. is a herbaceous plant growing in rocks and rough ground in dry hills up to 900 m in Greece and Western Anatolia (9). 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 study was to evaluate the antimicrobial activity of the plant as wild-growing in Turkey.

EXPERIMENTAL

Plant Material

The plant material was collected from Gokceada, Canakkale, Turkey in September, 2009. Voucher specimens of the plant (voucher number BD183) were deposited in the Biology Department of Canakkale Onsekiz Mart University, Canakkale, Turkey.

Preparation of extracts

The leaves of the plant were dried in an oven at 40 °C for 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 using a Soxhlet extractor (10). The extract was filtered with Whatman filter paper no.1, and the filtrate was evaporated under vacuum in a rotary evaporator at 55 °C. The extract yield obtained was 12.4%. The dry extract, which was sticky and black, was stored in labeled sterile screw-capped bottles at -20°C pending use. Prior to testing, 1 g was dissolved in 0.2 L of dimethyl sulfoxide (DMSO) (5 mg/mL).

Microorganisms

Urinary tract pathogens (*Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans*) were isolated from the urine of patients diagnosed with urinary infections in Medical Faculty of Canakkale Onsekiz Mart University, Canakkale, Turkey and from Trakya University, Edirne, Turkey. VITEK 2 (bioMérieux, France) system was used for identification.

Disc diffusion method

The paper disc diffusion method was employed (11). Sterile 6 mm disc filter paper disc (Schleicher & Schul, No. 2668, Dassel, Germany) were impregnated with 50 µL of the plant extract. 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±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 and yeast cultures were adjusted to yield 10⁷-10⁸ CFU (colony forming unite mL⁻¹) and

10^5 - 10^6 CFU mL⁻¹, respectively, using the standard McFarland counting method. The test microorganisms (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 microorganisms 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 diameter of these zones was measured in millimetres. All tests were performed under sterile conditions in duplicate and repeated three times. Penicillin (10 µg/disc) (Oxoid), tobramycin (10 µg/disc) (Oxoid), Ampicillin/Sulbactam 1:1 (20 µg/disc) (Oxoid), nystatin (30 µg/disc) (Hi-Media), clotrimazole (30 µg/disc) (Abtek Biologicals) and ketoconazole (20 µg/disc) (Liofilchem) discs 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 (12). A dilution series of the extract, ranging from 10 to 0.5 mg/mL, were prepared and then transferred to the broth in 96-well microtitre plates. The final concentrations were in the range 1000 to 50 µg/mL in the medium. Before inoculation of the test organisms, the bacterial and yeast strains were adjusted to 0.5 McFarland and diluted 1:1000 in Mueller Hinton Broth (Oxoid) and Malt Extract Broth (Oxoid), respectively. The plates were incubated at 35 °C for 18-24 h for bacteria and 30 °C for 48 h for the yeast cultures. All the tests were performed in broth and repeated twice. While 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 plating samples from clear wells onto Mueller Hinton Agar and Malt Extract Agar, respectively. MBC and MFC were defined as the lowest concentration yielding negative subculture. Reference antibacterial agents of ampicillin (Faco), streptomycin (Sigma) as well as reference antifungal agent of nystatin (Sigma) were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin, pH: 8.0; 0.1 mol mL⁻¹), DMSO (nystatin), or in water (streptomycin). The stock solutions of the agents were prepared in medium according to the Clinical and Laboratory Standards Institute (CLSI) (13).

RESULTS AND DISCUSSION

The antimicrobial activities of *B. acetabulosa* extracts against the pathogens causing complicated urinary tract infections examined in this study were qualitatively and quantitatively assessed by the presence of inhibition zones, MIC, MBC and MFC (Table 1 and Table 2).

The methanolic extracts obtained from the leaves of *B. acetabulosa* were strong antimicrobial activities against the pathogens with inhibition zones at 12.8 – 18.6 mm. *Escherichia coli* is susceptible to the extracts of the plant as compared to all standard antibacterial agents such as Penicillin, Ampicillin/Sulbactam and Tobramycin (inhibition zone is 18.6 mm). *Enterococcus faecalis* and *Proteus mirabilis* are more susceptible to the standard antibacterial antibiotics Penicillin and Ampicillin. The extracts have shown the weaker activity against *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Candida albicans* than those of the standard agents.

The methanolic extracts were further tested by microdilution to determine the MICs and MBCs or MFCs. The lowest MICs and MBCs or MFCs of the extracts were 32(64) µg/mL against *Escherichia coli*, followed *Enterococcus faecalis* and *Canida albicans* (MIC values are 64(128) µg/mL). The extracts have weak antimicrobial activity against the other microorganisms with MICs and MBCs ranged from 512(1024) to 1024(1024) µg/mL. These values are far below than the standard antibiotic agents.

Based on the results, it is possible to conclude that ethanol extract has stronger and broader spectrum of antibacterial activity as compared to the others. This information confirmed the evidence in previous study reported that ethanol is a better solvent for extraction of antimicrobial substances from medicinal plants than water and methanol (14).

Table 1. Summary of antimicrobial activity of *B. acetabulosa* and some standard antibiotics

Microorganisms	Inhibition zones (mm) ^a						
	Plant Extracts (250 µg/disc)	Standard antibiotics					
		P 10	SAM 20	TOB 10	NYS 30	KETO 20	CLT 30
<i>Enterococcus faecalis</i>	16.2	14.0	16.0	18.0	Nt	Nt	Nt
<i>Escherichia coli</i>	18.6	16.0	14.0	10.0	Nt	Nt	Nt
<i>Klebsiella pneumoniae</i>	13.2	18.0	14.0	15.0	Nt	Nt	Nt
<i>Pseudomonas aeruginosa</i>	9.6	8.0	10.0	12.0	Nt	Nt	Nt
<i>Proteus mirabilis</i>	13.4	13.0	16.0	14.0	Nt	Nt	Nt
<i>Candida albicans</i>	15.8	Nt	Nt	Nt	18.0	22.0	16.0

^a includes diameter of disk (6 mm); mean value of three independent experiments; Nt = not tested; P = penicillin (10 µg/disc); TOB = tobramycin discs (10 µg/disc); SAM = Ampicillin/Sulbactam (20 µg/disc); NYS = nystatin discs (30 µg/disc); KETO = ketoconazole (20 µg/disc); CLT = clotrimazole (30 µg/disc)

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

Microorganisms	MIC (MBC or MFC) (µg/mL)			
	Extract	Standards		
		ST	AMP	NYS
<i>Enterococcus faecalis</i>	64 (128)	2.0 (4.0)	1.0 (4.0)	Nt
<i>Escherichia coli</i>	32 (64)	4.0 (4.0)	32 (64)	Nt
<i>Klebsiella pneumoniae</i>	512 (1024)	8.0 (16.0)	8.0 (8.0)	Nt
<i>Pseudomonas aeruginosa</i>	1024 (1024)	1.0 (1.0)	16 (32)	Nt
<i>Proteus mirabilis</i>	512 (1024)	4.0 (8.0)	0.5 (1.0)	Nt
<i>Candida albicans</i>	64 (128)	Nt	Nt	8.0 (16)

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

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 *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Candida krusei* (15). 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. pneumoniae*) microorganisms and *C. albicans* in a previous study (8). In previous study, essential oil of *Ballota pseudodictamnus* has been investigated for their antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida tropicalis* and *Candida glabrata* using the dilution technique. Essential oil of the plant exhibited strong to moderate activity against all tested bacteria (MIC values 0.45-10.15 mg/mL), while it appeared inactive against the tested

fungi (16). Another study reported that *Ballota acetabulosa* is used for the treatment of haemorrhoids as infusion in folk medicine (17). 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 (*Escherichia coli*, *Pseudomonas aeruginosa*) and the Gram-positive strains (*Staphylococcus aureus*, *Bacillus subtilis*) and the yeast cultures (*Candida albicans*, *Candida glabrata*, *Candida krusei*) by the agar diffusion method. Among *Ballota* species studied, *Ballota 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 (18). Besides, ethanol extracts of some species were tested against four different *Listeria* isolates (*Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. murrayi*) by the agar diffusion method. Among *Ballota* species, *Ballota acetabulosa* have a strong antilisterial effects against all *Listeria* species except for *L. innocua* (19). Equally, in this study all the extracts of *Ballota 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 phenylpranoids have been reported to exist in some *Ballota* species such as *Ballota acetabulosa*, *B. foetida*, *B. hirsuta* and *B. nigra* (17, 20-24). Flavonoids may be responsible for their antibacterial activity (25). The result indicated that *Ballota 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.

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