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Antimicrobial Activity of Endemic *Ballota nigra* subsp. *anatolica* in Turkey

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The ethanol extracts obtained from *Ballota nigra* subsp. *anatolica* P.H. Davis (Lamiaceae) were investigated for their antimicrobial activities. The antimicrobial activity of the extract was tested against *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, *Candida albicans* ATCC 10239, *Debaryomyces hansenii* DSM 70238, *Kluyveromyces fragilis* ATCC 8608 and *Rhodotorula rubra* DSM 70403 by disk diffusion method and microdilution method. Some antibacterial and antifungal antibiotics were used as a positive reference standard to determine the sensitivity of the strains. The extracts showed strong antibacterial activity against *Escherichia coli*, with inhibition zones of 13.0 mm and with minimum inhibitory concentrations (MIC's) and minimum bactericidal concentrations (MBC's) of 250 (500) µg/mL, respectively. The extracts also exhibited moderate activity against the other test microorganisms. The results demonstrate that the ethanol extract of *B. nigra* subsp. *anatolica* has significant activity and suggest that it may be useful in the treatment of infections.

Key Words: *Ballota nigra* subsp. *anatolica*, Ethanol extract, Antimicrobial activity.

INTRODUCTION

Medicinal plants have been known for their healing or disease-curing qualities for centuries. *Ballota* species have been used in Turkish folk medicine as antiulcer, antispasmodic, diuretic, choleretic, antihaemorrhoidal and sedative agent[1-3]. *Ballota nigra* is used externally, in the treatment of wounds and burns. Aerial parts of some subspecies of *Ballota nigra* are used internally to treat inflammation, to suppress cough and against gastrointestinal disorders[4,5]. It is reported that *Ballota nigra* subsp. *anatolica* and *B. larendana* have antidepressant activity[1].

Ballota nigra subsp. *anatolica* P.H. Davis (Lamiaceae) is endemic to Turkey[6]. A bibliographical survey showed that there were no reports on the antimicrobial activity of this plant. So, the aim of this work is to evaluate the antimicrobial activity of the plant as wild-growing in Turkey.

EXPERIMENTAL

The plant material was collected from Icel, Turkey in July and August, 2008. Voucher specimens of the plants were deposited in the Biology Department at Canakkale Onsekiz Mart University.

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: 13.48 % 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 *ca.* 2 g was dissolved in 0.1 mL of DMSO (5 mg/g) before testing.

Microorganisms: *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[7]. Sterile 6 mm disc filter paper disc (Schleicher & Schul, No. 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 ºC. 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.* 1.0×10^7–1.0×10^8 mL^{-1} and 1.0×10^5–10^6 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 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 with some modifications[8]. 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 microtiter 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 microtiter plates.

RESULTS AND DISCUSSION

The antimicrobial activities of *B. nigra* subsp. *anatolica* extracts against the test microorganisms 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. nigra* subsp. *anatolica* were moderate antimicrobial effects against the test microorganisms, with inhibition zones at 9.0 to 13.0 mm. Notably, *Escherichia coli* is more susceptible to the extract of *B. nigra* subsp. anatolica (inhibition zones is 13.0 mm) as compared to standard antibacterial antibiotics tobramycin. Similarly, the extracts showed higher antibacterial activity on *Pseudomonas aeruginosa* than those of the standard antibacterial antibiotics penicillin and ampicillin (inhibition zones is 11.0 mm). Besides, the extracts have more antibacterial effect than those of tobramycin. *Proteus vulgaris* is equal to the standard antibacterial penicillin. Antifungal effects of the extracts against all tested fungal strains are far below than the standard antifungal antibiotics.

The ethanolic extracts were further tested by microdilution to determine the MICs and MBCs. The lowest MICs and MBCs (or MFCs) of the extract were 250 (500) µg/mL against *Escherichia coli*, followed by *Bacillus subtilis, Staphylococcus aureus, Candida albicans* and *Rhodotorula rubra*, with MIC values at 500 (> 1000) µg/mL, respectively. Against the other microorganisms, the extracts have a weak antimicrobial effect with MIC values at 1000(1000) µg/mL. These values are far below than the standard antibiotics.

Ethanol was observed as the best solvent for extracting antimicrobial substances in a previous study[9]. The results obtained in this study with ethanol are similar to those reported in the mentioned study. It is important to note that the concentration of extract used in the test may be correlated with a high activity of its chemical components.

TABLE-1
ANTIMICROBIAL ACTIVITY OF *B. nigra* subsp. *anatolica*

Microorganisms	Diameter of zone of inhibition (mm)*						
	EtOH extract (µg/mL)	Standards					
		P	AMP	TOB	NYS	KETO	CLT
Bacillus subtilis	11.0	14.0	12.0	24.0	Nt	Nt	Nt
Bacillus cereus	8.0	13.0	16.0	18.0	Nt	Nt	Nt
Escherichia coli	13.0	16.0	14.0	10.0	Nt	Nt	Nt
Stapylococcus aureus	9.0	23.0	16.0	8.0	Nt	Nt	Nt
Pseudomonas aeruginosa	11.0	8.0	10.0	12.0	Nt	Nt	Nt
Proteus vulgaris	10.0	10.0	16.0	13.0	Nt	Nt	Nt
Salmonella typhimurium	9.0	13.0	13.0	10.0	Nt	Nt	Nt
Candida albicans	10.0	Nt	Nt	Nt	20.0	21.0	15.0
Debaryomyces hansenii	9.0	Nt	Nt	Nt	16.0	14.0	20.0
Kluyveromyces fragilis	9.0	Nt	Nt	Nt	18.0	16.0	18.0
Rhodotorula rubra	11.0	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. nigra* subsp. *anatolica*

Microorganisms	MIC (MBC or MFC)			
	EtOH extract (µg/mL)	Standards		
		ST	AMP	NYS
Bacillus subtilis	500(>1000)	0.5(0.5)	0.5(2.0)	Nt
Bacillus cereus	1000(1000)	4.0(4.0)	8.0(8.0)	Nt
Escherichia coli	250(500)	4.0(4.0)	64(128)	Nt
Stapylococcus aureus	500(>1000)	2.0(4.0)	<0.25(0.35)	Nt
Pseudomonas aeruginosa	1000(1000)	1.0(1.0)	16(32)	Nt
Proteus vulgaris	1000(1000)	8.0(8.0)	0.5(0.5)	Nt
Salmonella typhimurium	1000(1000)	16(32)	1.0(4.0)	Nt
Candida albicans	500(>1000)	Nt	Nt	8.0(16)
Debaryomyces hansenii	1000(1000)	Nt	Nt	16(32)
Kluyveromyces fragilis	1000(1000)	Nt	Nt	16(16)
Rhodotorula rubra	500(>1000)	Nt	Nt	16(16)

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

There are no reports on the antimicrobial activity studies of *Ballota nigra* subsp. *anatolica*. Also, investigations of antimicrobial activity on the other *Ballota* species are limited. In previously studies, the antimicrobial activity of some endemic *Ballota* species growing in Turkey was reported[10] as well as the antifungal activities of some flavonoids isolated from *Ballota glandulosissima*[11]. In another study, diterpenoids and flavonoids isolated from *Ballota inaequidens* are investigated for

their activities against *Bacillus subtilis, Stapylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and *Candida krusei*[12]. 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*) microorganisms and *C. albicans* in a previous study[3]. In addition, Couladis *et al.*[13] reported that essential oil of *Ballota pseudodictamnus* has been investigated for their antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes, Klebsiella pnemoniae, Pseudomonas aeroginosa, 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. Equally, in this study all the extracts of *Ballota nigra* subsp. *anatolica* were presented antimicrobial activity to both bacteria and yeast cultures. The differences between present results and other results 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. hirsute* and *B. nigra*[14-20]. Flavonoids may be responsible for their antibacterial activity[21]. The result indicated that *Ballota nigra* subsp. *anatolica* 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 *Ballota nigra* subsp. *anatolica* has significant activity against the test microorganisms. Further analyses are necessary to identify the main active constituents.

REFERENCES

1. K. Vural, N. Ezer, K. Erol and F.P. Sahin, *J. Fac. Pharm.*, **13**, 29 (1996).
2. T. Baytop, Therapy with Medicinal Plants in Turkey-Past and Present (in Turkish), Nobel Tip Basimevi: Istanbul, edn. 2, p. 193 (1999).
3. G. Citoglu, M. Tanker, B. Sever, J. Englert, R. Anton and N. Altanlar, *Planta Med.*, **64**, 484 (1998).
4. E. Yesilada, G. Honda, E. Sezik, M. Tabata, T. Fujita, T. Tanaka, Y. Takeda and Y. Takaishi, *J. Ethnopharmacol.*, **46**, 133 (1995).
5. E. Tuzlaci and E. Tolon, *Fitoterapia*, **71**, 673 (2000).
6. P.H. Davis. Flora of Turkey and the East Aegean Islands, Edinburgh University Press: Edinburgh, Vol. 7, pp. 156-160 (1982).
7. C.M. Collins and P.M. Lyne, Microbiological Methods. Butterworths and Co. Ltd., London, p. 316 (1987).

8. J.R. Zgoda and J.R. Porter, *Pharm. Biol.*, **39**, 221 (2001).
9. S.G. Jonathan and I.O. Fasidi, *Afr. J. Biomed. Res.*, **6**, 85 (2003).
10. G.S. Citoglu, B.S. Yilmaz and N. Altanlar, *J. Fac. Pharm.*, **32**, 93 (2003).
11. G.S. Citoglu, B. Sever, S. Antus, E. Baitz-Gacs and N. Altanlar, *Pharm. Biol.*, **41**, 483 (2003).
12. G.S. Citoglu, B. Sever, S. Antus, E. Baitz-Gac and N. Altanlar, *Pharm. Biol.*, **42**, 659 (2003).
13. M. Couladis, I.B. Chinou, O. Tzakou and A. Loukis, *Phytother. Res.*, **16**, 723 (2002).
14. N. Darbour, F. Baltassa and J. Raynaud, *Pharmazie*, **41**, 605 (1986).
15. F. Ferreres, F.A. Tomas-Barberana and F. Tomas-Lorente, *J. Nat. Prod.*, **49**, 554 (1986).
16. A.H. Meriçli, F. Meriçli and E. Tuzlaci, *Acta Pharm. Turc.*, **30**, 143 (1988).
17. V. Seidel, F. Bailleul and F. Tillequin, *Planta Med.*, **62**, 186 (1996).
18. N. Didry, V. Seidel, L. Dubreuil, F. Tillequin and F. Bailleul, *J. Ethnopharmacol.*, **67**, 197 (1999).
19. M.C. Bertrand, F. Tillequin and F. Bailleul, *Biochem. Syst. Ecol.*, **28**, 1031 (2000).
20. S. Sahpaz, A.L. Skaltsounis and F. Bailleul, *Biochem. Syst. Ecol.*, **30**, 601 (2002).
21. M. Saeedi, K. Morteza-Semnani, M.R. Mahdavi and F. Rahimi, *Indian J. Pharm. Sci.*, **70**, 403 (2008).

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