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ANTILISTERIAL ACTIVITY OF *BALLOTA* SPECIES GROWING IN TURKEY

TÜRKİYE'DE YETİŞEN *BALLOTA* TÜRLERİNİN ANTİLİSTERİYAL AKTİVİTESİ

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ABSTRACT

The objective of this study was to determine the antibacterial effect of all Ballota species growing in Turkey on four strains of Listeria.

Ethanol extracts of sixteen Ballota species were tested against 4 different Listeria isolates (Listeria monocytogenes, L. ivanovii, L. innocua, L. murrayi) by the agar diffusion method.

All plants showed higher antilisterial activity against L. monocytogenes. The extracts of B. nigra subsp. anatolica, B. cristata, B. nigra subsp. foetida, B. rotundifolia, B. nigra subsp. uncinata, B. pseudodictamnus subsp. lycia and B. saxatilis subsp. saxatilis have highest antilisterial activity against L. monocytogenes. Among these species B. nigra subsp. anatolica, B. cristata and B. nigra subsp. foetida have also antilisterial activity against L.ivanovii, L. innocua and L. murrayi.

Keywords: *Ballota, Lamiaceae, Listeria, Antilisterial activity*

ÖZET

Bu çalışmanın amacı Türkiye'de yetişen Ballota türlerinin 4 Listeria suşu üzerine antibakteriyal etkilerini belirlemektir.

Onaltı Ballota türünün etanollü ekstreleri izole edilen 4 farklı Listeria (Listera monocytogenes, L. ivanovii, L. innocua, L. murrayi) suşuna karşı agar difüzyon metodu ile test edildi.

Bitkilerin tümü L. monocytogenes'e karşı güçlü antilisteriyal aktivite gösterdiler. L. monocytogenes'e karşı en güçlü antilisterial aktivide gösteren türler ise B. nigra subsp. anatolica, B. cristata, B. nigra subsp. foetida, B. rotundifolia, B. nigra subsp. uncinata, B. pseudodictamnus subsp. lycia

ve B. saxatilis subsp. saxatilis'tir. Bu türler arasında B. nigra subsp. anatolica, B. cristata ve B. nigra subsp. foetida aynı zamanda L.ivanovii, L. innocua ve L. murrayi'ye karşı da antilisteriyal aktivite göstermektedir.

***Anahtar Kelimeler**: Ballota, Lamiaceae, Listeria, Antilisteriyal aktivite*

INTRODUCTION

Listeria is a Gram (+) rod, aerobic, non-spore forming, motile by means of flagella, foodborne bacilli. Unusual among Gram (+) bacteria, it produces an endotoxin (1).

The genus *Listeria* has 6 species named *L. monocytogenes, L. innocua, L. seeligeri, L. ivanovii*, *L.grayi* and *L.murrayi*. These species are pathojenic for mice and other animals, while *L. monocytogenes* is commonly associated with human listeriosis (2).

L. monocytogenes is the most important *Listeria* species, causing a wide spectrum of clinical syndromes in humans, summarized as listeriosis. A wide variety of foods, including milk, cheese, beef, pork, chicken, seefoods, fruits, and vegetables have been identified as vehicles of *L. monocytogenes* in causing listeriosis (3). The manifestations of listeriosis include meningitis, septicemia, meningoencephalitis and intrauterine or cervical infection in pregnant women, which may result in spontaneous abortion or stillbirth *L. ivanovii* mainly causes abortion in sheep, but cases of listeriosis in cattle and humans have also been reported (4,5).

L. innocua present in food stuffs is the species that is closest to *L. monocytogenes*, but it is not pathogenic. Strains of nearly all *Listeria* species are consumed through food, drinks (including pasteurized milk) and water, often in large amounts, and pathogenic properties have been found in 'apathogenic' *Listeriae* as well (6).

L. monocytogenes is resistant to different environmental conditions, including acid pH, high NaCl concentration, and refrigeration temperatures. *L. monocytogenes* can grow in many foods when stored at refrigeration temperatures (7).

Foodborne listeriosis presents as systemic disease in human, especially in the elderly, immunocompromised, pregnant as well as intestinal illness with fever (8-10). *Listeria* species have been reported as susceptible to antibiotics active against Gram (+) bacteria but more recently, reports of resistance in *Listeria* species have been published. Current therapy of choice for all forms of listeriosis is a combination ampicillin - gentamicin (11).

Herbal medicine has been improved in developing countries as an alternative solution to health problems and costs of pharmaceutical products. The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources, including plants. Plants used for traditional medicine contain a wide range of substances that are used to treat chronic as well as infectious diseases.

Ballota species have been used in Turkish folk medicine as antiulcer, antispasmodic, diuretic, choleretic, antihaemorrhoidal, and sedative agent (12-15). *Ballota* L. is represented by 16 taxa in Turkey (16) (Table 1). *Ballota nigra* is used externally, in the treatment of wounds and burns. It is orally taken to suppress cough upper respiratory inflammation (17-19). Vural et al. (13) reported that *Ballota nigra* subsp. *anatolica* and *Ballota larendana* have antidepressant activitiy. *Ballota larendana* has also anxiolytic activity. Another study reported that *Ballota acetabulosa* is used for the treatment of haemorrhoids as infusion in folk medicine (12). The antimicrobial (20) and antioxidant activities (21) of all *Ballota* species growing in Turkey was recently reported as well as the antifungal activities of some flavonoids isolated from *Ballota glandulosissima* (22). Water extract of *B. glandulosissima* has been reported to have antinociceptive (23), anti-inflammatory and hepatoprotective activities (24). Çitoğlu et al., (25) also reported that antifungal activities of some diterpenoids and flavonoids from *B. inaequidens*.

The main components of the *Ballota* species are flavonoids, labdane diterpenoids and phenylpropanoids (26). In our previous studies, three diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) and ten flavonoids (kumatakenin, pachypodol, 5-hydroxy-7,3',4'-trimethoxyflavone, velutin, corymbosin, 5-hydroxy- 3,7,4'-trimethoxyflavone, retusin, 5-hydroxy-7,4'-dimethoxyflavone, 5-hydroxy-3,6,7,4'- tetramethoxyflavone, ladanein) were isolated, chemically characterized and analysed by HPLC in different *Ballota* species (15,22,25-27).

This paper is a part of our on-going studies on this genus (15,20-27). The aim of this work is to assess the antilisterial activities of all Ballota species growing in Turkey. To our knowledge, no data is available with respect to antilisterial activities of these plants.

MATERIAL AND METHODS

Plant material

Sixteen taxa of *Ballota* genus were collected from different localities in Turkey. Taxonomic identies of the plants were confirmed by Ph.D.Bio. Fatma Tezcan. Designation of the individuals and their origin are given in Table 1.

Table1. The names and origins of the plants

B. acetabulosa	B1 İzmir: Yenifoça, 10 m, 18.6.1998, AEF 21602
B. pseudodictamnus subsp. *lycia*	C2 Muğla: Fethiye, 20 m, 12.6.1997, AEF 21603
B. cristata	C3 Isparta: Eğridir, 910 m, 17.7.1997 AEF 19899
B. inaequidens	C3 Antalya: Alanya, 200 m, 20.7.1997, AEF 19901
B. saxatilis subsp. *saxatilis*	C4 İçel: Anamur, 1530m, 20.7.1997, AEF 19904
B. saxatilis subsp. *brachyodonta*	C4 İçel: Silifke, 1400 m, 3.7.1998, AEF 21505
B. glandulosissima	C3 Antalya: Kumluca, 500 m, 19.7.1997, AEF 19900
B. larendana	A4 Ankara: Kızılcahamam, 830 m, 28.6.1998, AEF 21604
B. latibracteolata	C3 Antalya: Gazipaşa, 425 m, 20.7.1997, AEF 19902
B. rotundifolia	A8 Erzurum: Tortum lake, 1200 m, 1.9.1998, AEF 21606
B. macrodonta	B5 Kayseri: Yahyalı, 1150 m, 2.8.1997, AEF 19907
B. nigra subsp. *nigra*	A5 Sinop: Boyabat, 370 m, 9.10.1998, AEF 21607
B. nigra subsp. *foetida*	C2 Muğla: Döğüşbelen, 600 m, 12.7.1999, AEF 21608
B. nigra subsp. *uncinata*	B1 İzmir: Gökçealan, 250 m, 19.6.1998, AEF 21607
B. nigra subsp. *anatolica*	B4 Ankara: Gölbaşı, 800 m, 28.6.1998, AEF 21601
B. antalyense	C3 Antalya: Turunçova, 150 m, 19.7.1997, not published

Extraction of plant materials

Air dried and powdered aerial parts of *Ballota* species (20 g of each) were extracted with ethanol (75 % aqueous, 150 ml of each) for 24 hours by using a Soxhlet apparatus (28).

Microbiology

Preparation of inoculum

In this study 4 different isolates of *Listeria* (*L. monocytogenes, L. ivanovii, L.innocua, L. murrayi*) were used. These isolates were obtained from meat or meat products, by using Oxford Agar (Merck, Germany) (1).

The plates incubated at 37 °C for 48 h suspect *Listeria* colonies, which are small (1mm diameter), black and surrounded by black halos after 24 h. After 48 h colonies are 2-3 mm in diameter, black with black halo were selected for confirmation. They were streaked onto Tryptone Soya Yeast Extract Agar (Biokar Diagnostic, France) plates and incubated 37 °C, 24 h. All isolates were tested by using standard methods (1,29).

Results were obtained as positive for Gram stain, catalase positive, urea negative, and produce an acid slant and butt in TSI without production of H_2S, hydrolysis of sodium hippurate, esculin and mannitol, motile at room temperature, ß hemolysis; All species were given +/+ reactions in MR-VP broth. *L. monocytogenes, L. ivanovii*, produced hemolysis in sheep blood agar

and were also positive in the CAMP test. *L. innocua* and *L. murrayi* were given negative reaction in the CAMP test (6). This results are given in Table 2.

Gram (+), catalase positive, motile cultures were tested API *Listeria* (bioMerieux, France) system.

From the plates, single colonies were inoculated on 10 ml Brain Heart Infusion Broth (Oxoid),and incubated at 30°C for 24 h. The turbidity of the suspensions was adjusted to the McFarland I standard.

Table 2. Characteristics differentiating the species of the genus *Listeria*

Characteristics	***L. monocytogenes***	***L. innocua***	***L. ivanovii***	**L. murrayi**
Gram stain	+	+	+	+
Beta-Hemolysis	+	-	+	-
Mannitol	-	-	-	+
Hippurate	+	+	+	-
Voges proskauer	+	+	+	+
Esculin	+	+	+	+
***CAMP-test* S.aureus**	+	-	+	-

Antilisterial activity assay

The Bauer- Kirby disc diffusion procedure according to the NCCLS (National Committee for Clinical Laboratory Standards) regulations was used for determination of antilisterial activity of the extracts (30).

The disc diffusion method performed on Mueller-Hinton Agar (MHA) (Difco) supplemented with 5% defibrinated sheep blood and before *Listeria* isolates had been suspended in Brain Heart Infusion Broth (Oxoid) to the density of a 1.0 Mc Farland standards and this inoculum were inoculated onto the entire surface of a dried Mueller- Hinton Agar (MHA) (Oxoid) plate by using a cotton swab. The plates were held at room temperature for 10 min to allow absorption of free surface liquid.

All extracts were dissolved in 75 % aqueous ethanol to obtain 133 mg/ml extract concentration.

The ethanolic extracts were impregnated on sterile paper discs of 6 mm diameter, 0.02 ml capacity (Schleicher and Shüll No. 2668, Germany) and then a filter paper discs impregnated with a solution were placed on the surface of each inoculated plate. Discs were impregnated with pure

ethanol as negative control. Plates were incubated at 37°C for 48 h After incubation , the diameter (mm) of the zon arround each disc was measured.

The solvent control did not show any antimicrobial activity. Standard antibiotic disc ofloxacin (10µg/ disc Oxoid) was used for positive control. All tests were performed under sterile conditions in duplicate and repeated three times.

RESULTS

The antilisterial activities of the extracts of *Ballota* species were determined by using disc diffusion method against *Listeria monocytogenes, L. ivanovii, L. innocua and L. murrayi.* The results are shown in Table 3.

Table 3. The inhibition zones diameters (mm) of free and ethanolic extracts of the plants.

Sample name	*L.monocytogenes*	*L. ivanovii*	*L. innocua*	*L. murrayi*
B. acetabulosa	15	6	0	10
B. antalyense	9	0	0	0
B. cristata	18	8	10	15
B. glandulosissima	14	0	0	0
B. inaequidens	15	15	0	10
B. larendana	15	10	9	15
B. latibracteolata	12	11	13	10
B. macrodonta	10	5	0	10
B. nigra subsp. *anatolica*	20	15	15	16
B. nigra subsp. *foetida*	18	15	10	15
B. nigra subsp. *nigra*	11	10	0	10
B. nigra subsp. *uncinata*	16	20	0	10
B. pseudodictamnus subsp. *lycia*	17	9	15	10
B. rotundifolia	18	20	0	10
B. saxatilis subsp. *brachyodonta*	17	0	10	0
B. saxatilis subsp. *saxatilis*	17	17	10	11
Ofloxacin	22	20	20	20

As shown in Table 3 B. cristata, *B. larendana, B. latibracteolata, B. nigra* subsp. *anatolica*, *B. nigra* subsp. *foetida*, *B. pseudodictamnus* subsp. *lycia*, *B. saxatilis* subsp. *saxatilis* were indicated wide inhibition zone against *Listeria monocytogenes, L. ivanovii, L. innocua, L. murrayi.* As can clearly be seen in Table 3 *B. nigra* subsp. *anatolica* exhibited the best antilisterial activity. *B. nigra* subsp. *uncinata*, *B. rotundifolia* showed the best activity with 20 mm inhibition zone diameter against *L. ivanovii* which was show similar activity with ofloxacin.

DISCUSSION

In our previous study on ethanolic extracts of all *Ballota* species growing in Turkey, we observed their effects against Gram (+) (*S. aureus, B. subtilis*) and Gram (-) (*P. aeruginosa, E. coli*) bacteria and yeast (*C. albicans, C. galabrata, C. krusei*). According to this research, *B. inaequidens* have to greatest antimicrobial efficacy, followed by *B. saxatilis* subsp. *saxatilis* and *B. rotundifolia*. It is note worty to mentioned that the antifungal efficacy of all extracts had more than their antibacterial efficacy (20).

We also previously reported three diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) obtained from the aerial parts of *Ballota saxatilis* subsp. *saxatilis* and their effects against gram-(+) (*S. aureus, S. faecalis*) and gram-(-) (*P. aeruginosa*, *E. coli, K. pneumoniae*) microorganisms and *Candida albicans*. All compounds were found effective against *C. albicans* in low concentrations and active against bacteria (15).

In addition, previously we isolated some flavonoids and diterpenoids (pachypodol, 5-hydroxy-7,3',4'-trimethoxyflavone, 5-hydroxy-3,7,4'-trimethoxyflavone, retusin, 5-hydroxy-7,4'-dimethoxyflavone, 5-hydroxy-3,6,7,4'- tetramethoxyflavone, hispanolone, ballonigrine) from *Ballota inaequidens* and tested against *B.subtilis, S. aureus*, *E. coli, P. aeruginosa, C. albicans and C. krusei*. All the compounds tested had inhibitory activity against bacteria and showed good activities against *C. albicans* and *C. crusei* (25).

One of the undisputed functions of flavonoids is to play a role in protecting plants against microbial invasion. Thus, the antimicrobial activity was established to be mainly due to flavonoids (31,32).

In this study, the antilisterial activity of *Ballota* species evaluated by agar disc diffusion method against *L. monocytogenes, L. ivanovii, L. innocua, L. murrayi.*

When compared with the standard antibiotic, *B. nigra* subsp. anatolica was found to have good activities against *L. monocytogenes, L. ivanovii, L. innocua* and *L. murrayi*.

In conclusion, the present study shows that the extracts examined have variable antilisterial activities.

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