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Antimicrobial effect of south Ukrainian sea lavender *Limonium meyeri (Boiss.) O. Kuntze* and *Limonium* hypanicum Klok. with especial emphasizing against staphylococci and *Propionibacteria*

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Abstract
Antimicrobial activity of sea lavender species growing in the Black Sea area, *Limonium meyeri (Boiss.) O. Kuntze*. and *Limonium hypanicum Klok*., was revealed by agar microdiffusion screening assay. The sea lavender root extracts are much more active against various bacteria and *Candida* yeasts comparing with aerial parts extracts. Acetone root extracts and aethyl acetate fractions of 70% aqueous-acetone acetone 'extracts showed antimicrobial activity against methicillin resistant and sensitive clinical isolates of *S. aureus* and coagulase-negative staphylococci with MIC_{90} 250 μg/ml. For gram-negative bacteria MICs of these extracts ranged from 250 to 1,000 μg/ml. Bactericidal, activity of *Limonium hypanicum* root extract against *P. acnes* was noticed at dilutions 1:640-1:1,280. It was the same against the both antibiotic sensitive ATCC 6919 and multiple resistant clinical strains of *P. acnes*. Tannins or free gallic and ellagic acids may be responsible for the antibacterial activity of tested *Limonium* species roots.

Keywords: medicinal plants, antibacterial activity, methicillin resistant staphylococci, MLS-resistant staphylococci, gram-negative bacteria

Introduction
About 120 sea lavender (marsh rosemary) species are halophytic perennial plants of the genus *Limonium Mill*. (family *Plumbaginaceae Juss*.), widespread mostly in steppy zones of the Eurasian continent, in the Northern Africa, in Mediterranean and Pacific coast regions. *Limonium* plants are not enough investigated yet from the phytochemical and pharmacological point of view. No medicines produced of sea lavender are available in developed countries.
As soon as past few years the attention of some researchers was paid on these plants used earlier preferentially in the traditional medicine.
It was reported about antioxidant and free radical scavenging activity of the Okinawa medicinal plant *Limonium wrightii O. Kuntze* and *L. tetragonum* aqueous extracts [1, 6]. It shows cardio protective effect against myocardial ischemia-reperfusion injury in isolated rat hearts [12] and hepatoprotective effect in carbon tetrachloride induced mice liver toxicity *in vivo* as well [1]. Gallic acid was proved to be responsible for this kind of biological activity. The similar hepatoprotective properties were discovered in experiments with flavonoid-enriched extracts of *L. sinense (Girard) O. Kuntze* roots and leaves [2, 4]. Some publications concern to antibacterial, antifungal activity of *L. axillare* [8], antiviral activity of *L. sinense* [5, 7] and *L. tetragonum* [9]. A flavonol glycoside identified as myricetin 3-O-β-D-sorboside isolated from the leaves of *L. axillare* showed a moderate ant proliferative effect on Ehrlich ascites carcinoma cells [3]. *L. gmelinii* showed remarkable antioxidant, hepatoprotective, antimicrobial, ant mutagenic and antiviral activity in preclinical studies [13].
Steppy zone, Black Sea coast, lower flow of the Dnieper and South Bug, the Crimea are the growth area of Ukrainian sea lavender species, namely Meyer sea lavender *L. meyeri (Boiss.) O. Kuntze* (syn. *Statice meyeri Boiss.*) and south-bugean sea lavender *L. hypanicum Klok*. (syn. *L. gmelini (Willd.) O. Kuntze ssp. hypanicum (Klok.) Soo*). Sea lavender grows in steppe thickets on dry salt soils, on Black Sea and salty lakes coasts. These plants are widely used in the traditional medicine by population of south regions of Ukraine as a substitute of snakeroot (*Polygonum bistorta L.*), tormentil cinquefoil (*Potentilla erecta (L.) Hampe*) or great burnet (*Sanguisorba officinalis L.*) which are rarely met on this territories. Sea lavender roots due to hemostatic and antiinflammatory properties are traditional remedy for the treatment of gynecologic, acute gastrointestinal diseases (hyperacid gastritis, dysentery, enterocolitis),

hemorrhoids, internal hemorrhages, mouth and throat mucosa inflammatory processes. Local applications of sea lavender root decoctions and infusions are used as wound healing drug and to treat skin diseases (eczema). However, indigenous Ukrainian sea lavender species have been never investigated toward on their antimicrobial activity.

Materials and Methods

Plant extracts preparation: The roots and aerial parts (leaves, stems and flowers) of *L. meyeri* and *L. hypanicum* were harvested on the Black Sea coast in Odessa region. The powdered air-dried crude drugs were extracted (crude drug/solvent 1:10) for 2 weeks at the room temperature and filtered to obtain 40% and 90% aqueous-ethanolic extracts.

Besides, 20.0 g portions of powdered roots were exhaustively extracted with pure acetone in Soxclet apparatus. Acetone extracts were concentrated by evaporation on the water bath and subsequently dried at room temperature.

Aqueous acetone extracts were prepared from 20.0 g of powdered roots in 200 ml 70% acetone using continuous extraction apparatus with back refrigerator on the water bath. Two fractions of this extract were collected after the separation with ethyl acetate and water. Resulted acetone extract and aqueous acetone extract fractions were evaporated. The residue was solubilized in ethanol/DMSO 1:1 solution before the use in microbiological experiments.

***Tested organisms*:** The organisms used in this study were *S. aureus* ATCC 6538P, *P. acnes* ATCC 6919, 51 strain of staphylococci (see Table 2) isolated from various clinical sources (presumably of skin and wound origin), including 14 methicillin-resistant *S. aureus* (MRSA), 18 methicillin-resistant coagulase-negative staphylococci (MR-CNS) strains, 2 MLS-resistant (Macrolide, Lincosamide, Streptogramin B) strains with constitutive and inducible phenotypes. MLS-resistant clinical strain *P. acnes* had inducible phenotype resistance. Besides of staphylococci and propionibacteria as common skin pathogens the wound isolates of *Enterococcus faecalis* and Gram-negative bacteria *E. coli*, *Citrobacter diversus*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterobacter gergoviae*, *Klebsiella pneumoniae*, *Providencia rettgeri*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* with various levels of antibiotic resistance were used as well. Identification was performed on the basis of morphology, culture properties and biochemical microtesting («STAPHYtest 16», «ENTEROtest 24», «NEFERMENTtest», «ANAEROtest 23», Lachema, Czech Republic).

P. acnes strains were cultured on Brain Heart Infusion Agar or Brain Heart Infusion Broth (HiMedia Laboratories Pvt. Ltd., India) in anaerobic conditions created by Gas generation pouch system (GasPak™ EZ, Bacton, Dickinson and Co., USA) for 5 days at 37 °C.

Agar micro diffusion screening assay: Bacterial strains (10^7 CFU/ml) were inoculated on nutrient agar plate. 20 μl of tested extracts were placed into the wells (diameter 4.0 mm). Solvents (90% aqueous ethanol, ethanol/DMSO 1:1) were dropped into a control wells. Acetone extracts solutions (in ethanol/DMSO 1:1) were used in the concentration of 10 mg/ml. Growth inhibition zones were observed after 24 h of incubation at 37 °C.

Minimum inhibitory concentrations: The minimum inhibitory concentrations (MICs) were measured by twofold dilution method. Aqueous-ethanolic extracts were tested starting from 1:8 dilution, to exclude antimicrobial action of the solvent. The final concentration of dried extracts in the medium was ranged from 2.000 to 62.5 μg/ml. Testing strains were inoculated by the replicator (25 strains on a plate) in the final dose of 10^4 CFU. After incubation at 37°C for 48 h the bacterial growth was examined by magnification ×10. Full inhibition of bacterial growth was registered. The concentration inhibitory for 90% strains tested (MIC_{90}) was established.

Results and Discussions

For the screening assay we have used microupdating of agar diffusion test. It is much more sensitive and allows reliably distinguish highly active plant specimens from that with weak or doubtful antimicrobial activity. Sea lavender 40% aqueous-ethanolic extracts lack noticeable antibacterial activity. More active are 90% aqueous-ethanolic extracts. Staphylococci among the all tested organisms appear have the best sensitivity. Sea lavender root extracts are much more active comparing with leaves stems and flowers extracts (the dates are presented in Table 1). Besides, *Limonium meyeri* and *Limonium hypanicum* root extracts showed fungistatic activity against *Candida sp*. (partial growth inhibition zones 6,1-9,4 mm).

Table 1: Comparative analysis of antimicrobial activity of *Limonium sp*. root and aerial parts 90% aqueous-ethanolic extracts

Strains	Growth inhibition zones (mm)					
	Limonium meyeri			*Limonium hypanicum*		
	roots	lives	stems and flowers	roots	leaves	stems and flowers
Staphyloococci						
S. aureus ATCC 6538-P	6.24±0.22*	4.25±0.19	4.62±0,40	6.09±0.26*	4.26±0.20	4.22±0.16
Clinical isolates:						
S. aureus MR	6.11±0.21*	4.92±0.26	4.51±0.25	5.67±0.12	4.26±0.09	4.29±0.07
S. epidermidis MR	6.42±0.36*	4.56±0.10	4.50±0.14	6.23±0.30*	5.05±0.17	4.89±0.14
S. epidermidis MS	7.48±0.41*	4.95±0.27	5.46±0.41	5.11±0.37	5.31±0.41	4.90±0.14
S. haemolyticus MR	5.31±0.13	4.78±0.22	4.79±0.28	5.13±0.12	4.71±0.27	4.29±0.07
S. hominis MS	5.53±0.16	4.67±0.33	5.00±0.58	5.04±0.17	4.74±0.09	4.88±0.14
S. cohnii ssp. cohnii MR	8.67±0.88*	4.41±0.11	4.60±0,13	5.29±0.24	4.71±0.23	5.26±0.28
S. epidermidis MLS	10.23±0.62*	10.73±0.8*	5.11±0.27	8.11±0,74*	7.12±0.6*	6.40±0.20*
S. aureus MLS	7.72±0.35*	7.44±1.03*	0	7.42±0.78*	0	0
Propionibacteria						
P. acnes ATCC 6919	13.47±0.76*	7.75±1.71*	8.61±0.29*	9.19±0.60*	9.69±0.39*	8.04±0.39*
P. acnes MLS	10.41±1.45*	9.18±0.54*	7.7±0.45*	11.24±1.4*	8.27±0.37*	5.67±0.59

Notes: 1. In the control studies growth inhibition by 90% ethanol: *S. aureus* – 4.3±0.27 mm, CNS – 4.4±0.09 mm.
2. The dates presented are the mean of 3 experiments for each strain.

3. MS – methicillin-sensitive strains, MR – methicillin-resistant strains.
4. MLS – macrolide, lincosamide, streptogramin B-resistant strains.
4. * – p<0.01 comparing with ethanol control.

Using agar twofold dilution technique MICs of 90% root extracts was determined. In the general, root extracts of the both *Limonium meyeri* and *Limonium hypanicum* inhibited *S. aureus* and coagulase-negative staphylococci growth at dilutions up to 1:32. MICs values were recalculated on the dry weight of extracted substances. The collection strain of *S. aureus* ATCC 6538-P was highly sensitive to sea lavender root extracts (MIC ≤ 62.5 μg/ml). Results of experiments on clinical isolates of staphylococci are presented in Table 2. Full growth inhibition of some MRSA and MR-CNS strains was detected in lower extracts dilutions comparing with methicillin-sensitive strains of the same species. Gram-negative bacteria were more resistant to active components of aqueous-ethanolic sea lavender root extracts than staphylococci.

Table 2: Growth inhibition of staphylococci clinical isolates (49 strains) with various level of methicillin resistance by *L. meyeri* and *L. hypanicum* root aqueous-ethanolic extracts

	No of	***L. meyeri (Boiss.) O. Kuntze***				***L. hypanicum Klok.***			
Clinical isolates	**strains**	**1:8***	**1:16**	**1:32**	**1:64**	**1:8**	**1:16**	**1:32**	**1:64**
	tested	2000**	1000	500	250	1250	625	312.5	125.25
S. aureus MS	8	8†/100††	8/100	7/87.5	1/1.25	8/100	8/100	4/50	1/12.5
S. aureus MR	14	14/100	14/100	9/64.3	1/7.1	14/100	13/92.9	7/50	1/7.1
S. epidermidis MS	4	4	4	4	0	4	4	3	0
S. epidermidis MR	4	4	4	3	1	4	4	3	0
S. haemolyticus MS	3	3	3	3	0	3	3	3	0
S. haemolyticus MR	9	9	9	9	0	9	8	7	0
S. hominis MS	1	1	1	1	1	1	1	1	0
S. hominis MR	3	3	3	3	0	3	3	2	0
S. cohnii ssp. cohnii MR	1	1	1	1	0	1	1	1	0
S. sciuri MR	1	1	1	1	0	1	0	0	0
S. saprophyticus MS	1	1	1	1	1	1	1	1	1
Total:									
S. aureus strains	22	22/100	22/100	17/77.3	2/9.1	22/100	20/90.9	11/50	2/9.1
CNS strains	27	27/100	27/100	26/96.3	3/11.1	27/100	25/92.6	21/77.8	1/3.7
MR strains	32	32/100	32/100	26/81.3	2/6.3	32/100	29/90.6	20/62.5	1/3.1
MS strains	17	17/100	17/100	17/100	3/17.6	17/100	16/94.1	12/70.6	2/11.8

Notes: 1. * – aqueous-ethanolic extracts dilutions.
2. ** – concentration of extracted substances (μg/ml).
3. † – No of strains inhibited.
4. †† – percentage of strains inhibited.

Therefore, we have concluded that sea lavender roots contain substances with antibiotic activity. Acetone and 70% aqueous-acetone root extracts were produced for the further microbiological researches. MICs of these extracts were determined by agar twofold dilution method and compared with activity of corresponding 90% aqueous-ethanolic extracts. Acetone extraction procedure looks to be more preferential. In general, acetone root extracts of *L. meyeri* and *L. hypanicum* almost do not differ on the level of their activity against gram-positive and gram-negative bacteria (Table 3).

Table 3: MICs of *L. meyeri* and *L. hypanicum* root acetone extracts

Strains	**MIC_{90} (μg/ml)**	
	Limonium meyeri	***Limonium hypanicum***
S. aureus ATCC 6538-P	250	250
MRSS	250	250
MRSA	250	250
CNS-MS	250	250
CNS-MR	250	250
Enterococcus faecalis	2,000	2,000
E. coli	500	500
Klebsiella pneumoniae	500	500
Citrobacter diversus	2,000	1,000
Enterobacter cloacae	500	500
Enterobacter aerogenes	1,000	250
Enterobacter gergoviae	500	250
Providencia rettgeri	500	500
Pseudomonas aeruginosa	250	250
Burkholderia cepacia	250	250

The same level of activity was discovered for the aethyl acetate fractions of 70% aqueous-acetone root extracts of the both *Limonium* species. The water fractions of these extracts showed week antimicrobial properties.

Table 4: MICs of *L. meyeri* and *L. hypanicum* root extracts.

Strains	*P. acnes* ATCC 6919		*P. acnes* MLS	
	MIC	**MBC**	**MIC**	**MBC**
Aqueous-ethanolic extracts				
Limonium meyeri	1:640	1:320	1:640	1:320
	25	50	25	50
Limonium hypanicum	1:1,280	1:640	1:1,280	1:640
	7.81	15.625	7.81	15.625
Acetone extracts (fraction A*)				
Limonium meyeri	>1,000	>1,000	250	250
Limonium hypanicum	>1,000	>1,000	>1,000	>1,000
Acetone extracts (fraction B*)				
Limonium meyeri	500	1.000	500	1,000
Limonium hypanicum	500	1.000	250	250
Aethyl acetate fractions of 70% aqueous-acetone extracts				
Limonium meyeri	500	500	1,000	1,000
Limonium hypanicum	500	500	250	250

Notes: * Fraction of acetone extracts, soluble in cool acetone.
* Fraction of acetone extracts, non-soluble in cool acetone, soluble in hot acetone and in ethanol.

Aqueous-ethanolic extracts of see lavender roots exhibited remarkable antimicrobial activity against collection and clinical strains of *P. acnes* (Table 4). Bactericidal activity of *Limonium hypanicum* root extract against *P. acnes* was noticed at dilutions 1:640-1:1,280. It was the same against the both antibiotic sensitive ATCC 6919 and multiple resistant clinical strains of *P. acnes*. Activity of acetone and aqueous-acetone extracts was much weaker.

The chemical nature of *L. meyeri* and *L. hypanicum* antibacterial substances is specified exactly. It was confirmed earlier that antiviral properties of *Limonium sinense* roots are due to hydrolysable tannins. (-)-epigallocatechin 3-O-gallate and samarangenin B are compounds with potent inhibitory effect on HSV-1 replication that exceeds acyclovir activity [5, 7]. Tannins or free gallic and ellagic acids may be responsible for the antibacterial activity of tested *Limonium* species roots.

Limonium gmelini root extract contains 3-O-Я-D-glucopyranoside camphatherine, 3,5,7,3',4',6'-hexahydroxyflavan, 3,5,7,3',4',6'-hexahydroxyflavon, 3-O-б-L-arabinopyranoside myritsetin, epigallocatechin-(4Я→8)-(-)-3,5,7,3',4',6'-hexahydroxyflavan, (+)-gallocatechin-(4Я→8), б-galactopyranoside myritsetin that can be responsible for its antimicrobial properties [13].

Active compounds (m-coumaric acid, naringin and quercetin) of *Limonium awei* (De Not.) Brullo & Erben ethanolic extract showed strong antimicrobial activity against Gram-positive bacteria, such as *S. aureus* including MRSA strains (MIC and MBC values ranging from 7.81 to 62.50 mg/mL and from 500 to 2000 mg/mL respectively) [10].

A water extract of *L. wrightii* depressed production of reactive oxygen species from polymorph nuclear leukocytes stimulated by phorbor-12-myristate acetate [1].

It is the first publication concerning to antimicrobial activity of *L. meyeri (Boiss.) O. Kuntze* and *L. hypanicum Klok*. grow in the Black Sea area. A few years ago, the antibacterial and antifungal effects of indigenous Qatari medicinal plant *Limonium axillare* were demonstrated [8]. Its aerial parts crude butanol extract inhibited the growth of *E. coli*, *Ps. aeruginosa*, *Bacillus cereus*, *S. aureus*, *Candida albicans* and *Aspergilus flavus*. Further microbiological investigations are needed for the evaluation of protective effect of *L. meyeri* and *L. hypanicum* root extracts on experimental infections *in vivo*.

Conclusions

1. *Limonium meyeri (Boiss.) O. Kuntze*. and *Limonium hypanicum Klok*. have a potential as an antibacterial and antifangal plants against Gram positive bacteria including MRSA strain, *Candida sp*.
2. The present investigation demonstrates that *Limonium hypanicum* root extract possess strong anti - *P. acnes* activity, even to resistant strain and this herb is promising source of new antiacne drugs for dermatology and cosmetics.

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