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EXPLORING ANTIMICROBIAL ACTIVITY OF HOREHOUND, *Marrubium peregrinum* L. EXTRACTS

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ABSTRACT. Antimicrobial activity of methanol, acetone and ethyl acetate extracts from whole herb of *Marrubium peregrinum* L. (Lamiaceae) were investigated in this study. Horehound, a perennial plant, was collected from the region of Suva Planina Mt. in eastern Serbia. Testing was preformed by microdilution method and minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) have been determined. Testing was conducted against 22 microorganisms, of which 15 strains of bacteria and 7 species of fungi. The strongest antimicrobial activity was detected on G+ bacteria while the activities on other species were moderate. The activity of tested extracts varied depending on the species and type and concentration of the extract. The comparative analyses showed that the most active was methanol extract (MIC from 0.3125 mg/ml to 40 mg/ml) followed by ethyl acetate (MIC from 0.0781 mg/ml to 40 mg/ml) and acetone extract (MIC from 0.1563 mg/ml to 40 mg/ml). The most sensitive bacteria were *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and especially ethyl acetate extract on *Bacillus subtilis* (MIC 0.0781 mg/ml). The best antifungal effect demonstrated methanol extract on *Aspergillus niger* ATCC 16404 (MIC 0.625 mg/ml). The antimicrobial properties of *Marrubium peregrinum* L. are insufficiently tested. Overall, extracts showed potential for further investigation and use.

Key words: antibacterial; antifungal; *Marrubium peregrinum* extracts.

INTRODUCTION

The genus *Marrubium* L. (Lamiaceae) has approximately the 30 species indigenous in Europe, the Mediterranean area and Asia (CANTINO *et al.*, 1992). In Serbian flora, four *Marrubium* species had been recognized. One of them, horehound, *Marrubium peregrinum* L. is a species of herbaceous perennial plant, with a stem up to 100 cm high, branched in the upper part, yellowish to white-felted. Rhizomes of this species are ligneous, leaves oblong, flowers 7-10 mm long, grouped in many small axillary clusters. It inhabits open dryish habitats in Europe and Middle Asia and belongs to the Pontic-Mediterranean floristic element (DIKLIĆ, 1974).

Some species of genus *Marrubium* are widely used in traditional and modern medicine (STANKOVIĆ *et al.,* 2011). Among them only *M. vulgare* (white horehound) has been extensively investigated. MEYRE-SILVA and CECHINEL-FILHO (2010) provide a review of the chemical and pharmacological aspects of the genus *Marrubium*, with emphasis on *M. vulgare*.

The chemical composition essential oils and extracts of *M. peregrinum* has been a lot studied (SALEI *et al.,* 1967, NAGY *et al.,* 1996, TELEK *et al.,* 1997, NAGY AND SVAJDLENKA, 1998, LAZARI *et al.,* 1999, SAHPAZ *et al*., 2002, JANICSÁK *et al.,* 2006, HENNEBELLE *et al.,* 2007, JOVIN *et al.,* 2008, ALKHATIB *et al*., 2010).

Some chemical substances isolated from *Marrubium peregrinum* exhibit antihypertensive (EL BARDAI *et al.,* 2004), antispasmodic (RIGANO *et al.,* 2009) and antiviral effect (HAID *et al.,* 2012, CALLAND *et al.,* 2012)

The essential oils and extracts of *Marrubium peregrinum* express strong antioxidant activity and capability to reduce lipid peroxidation (KAURINOVIĆ and POPOVIĆ, 2012). Results of antioxidant activity show that extracts of *M. peregrinum* are efficient in protection of tissues and cells from oxidative stress (KAURINOVIĆ *et al.,* 2011, STANKOVIĆ *et al.,* 2011). These results are in correlation with previous investigation (KAURINOVIĆ *et al.,* 2010).

For the diferent extracts of *M. perreginum*, except anti-*Aspergillus* activity (RADOJEVIĆ *et al.,* 2011), are no available data on antimicrobial activity. It is therefore, the aim of this paper is to investigate, antibacterial and antifungal activities *in vitro*, diferent extracts the whole herb of *Marrubium peregrinum* L.

MATERIALS AND METHODS

Chemicals

Acetone, methanol and ethyl acetate were purchased from "Zorka pharma" Šabac, Serbia. Mueller–Hinton broth was purchased from Liofilchem, Italy, while Sabouraud dextrose broth was obtained from Torlak, Belgrade. Doxycycline antibiotic was purchased from Galenika A.D., Belgrade, and fluconazole antifungal, was from Pfizer Inc., USA. All other solvents and chemicals were of analytical grade.

Plant material

Marrubium peregrinum was collected in August 2009**,** from the region of Prokuplje, in southeast Serbia. The voucher specimen of *M. peregrinum* was confirmed and deposited in Herbarium at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac (No. 95/09). The collected plant material was air-dried in darkness at room temperature (20°C). Dried plant parts were cut up and stored in tight-seal dark containers until needed.

Preparation of plant extracts

Prepared plant material (10 g) was transferred to dark-coloured flasks, mixed with 200 ml of solvent and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40°C using rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C.

In vitro antimicrobial assay

Test microorganisms

Antimicrobial activity of acetone, ethyl acetate and methanol extract was tested against 22 microorganisms including fifteen strains of bacteria (standard strains: *Escherichia*

coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Bacillus pumilus* NCTC 8241 and clinical strains: *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Proteus mirabilis, Sarcina lutea, Salmonella enterica, Bacillus subtilis and Bacillus cereus*) and seven species of fungi (*Aspergillus niger* ATCC 16404, *Penicillium italicum* PMFKG-F29, *Trichothecium roseum* PMFKG-F32, *Botrytis cinerea* PMFKG-F33; *Candida albicans* (clinical isolate); *Rhodotorula sp.* PMFKG-F27 and *Saccharomyces boulardii* PMFKG-P34). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

Bacterial suspensions and yeast suspension were prepared by the direct colony method. The colonies were taken directly from the plate and were suspended in 5 mL of sterile 0.85% saline. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard (0.5 mL 1.17% w/v $BaCl_2 \times 2H_2O$ + 99.5 mL 1% w/v H_2SO_4) (ANDREWS, 2005). When adjusted to the turbidity of the 0.5 McFarland's standard, bacteria suspension contains about 10^8 colony forming unites (CFU)/mL and suspension of yeast contains 10^6 CFU/mL. 1: 100 dilutions of initial suspension were additionally prepared into sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spore from slopes with growing aspergilli. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) using microdilution method with resazurin (SARKER *et al.,* 2007). The 96-well plates were prepared by dispensing 100 μL of nutrient broth, Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi and yeasts, into each well. A 100 μL from the stock solution of tested compound (concentration of 80 mg/mL) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a multichannel pipette. The obtained concentration range was from 40 to 0.039 mg/ml. A 10 μL of diluted bacterial, yeast suspension and suspension of spores was added to each well to give a final concentration of 5 x 10^5 CFU/mL for bacteria and 5 x 10^3 CFU/mL for fungi and yeast. Finally, 10 μL resazurin solution was added to each well inoculated with bacteria and yeast. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37°C for 24 h for bacteria, 28°C for 48 h for the yeast and 28°C for 72 h for fungi. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink. For fungi, MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelia growth.

Doxycycline and fluconazole were used as a positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Also, in the experiment, the concentration of DMSO was additionally decreased because of the twofold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Minimum bactericidal and fungicidal concentration was determined by plating 10 μL of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration.

Statistical analysis

All statistical analyses were performed using SPSS 17 for Windows (SPSS Inc., Chicago, IL, USA). Mean differences were established by Student's t-test. Data were analyzed using one-way analysis of variance (ANOVA). In all cases, $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The results of *in vitro* testing antibacterial and antifungal activities of the three extracts are shown in Table 1. and Table 2. For comparison, MIC and MMC values of doxycycline and fluconazole are also listed in Table 1. and Table 2. The solvent (10% DMSO) did not inhibit the growth of the tested microorganisms.

Antimicrobial activity of tested extracts was evaluated by determining MICs and MMCs in relation to the 22 species of microorganisms. MICs and MMCs values were in range from 0.0781 mg/ml to 40 mg/ml. The intensity of antimicrobial action varied depending on the groups of microorganisms (Gram+, Gram–, bacteria, filamentous fungi, yeasts) and on the type of the extracts. The tested extracts showed different levels of antimicrobial activity in relation to the tested species.

In general, the tested extracts demonstrated selective antimicrobial activity, while showing more potent inhibitory effects on the growth of G+ bacteria than to other tested microorganisms. Statistically significant difference in activity between the extracts of *M. peregrinum* was not observed.

The tested extracts showed high antibacterial activity against G+ bacteria (except for *Enter. faecalis*, clinical isolates and standard strains). MICs values for *Bacillus* sp were in range from 0.0781 mg/mL to 1.25 mg/mL and MMCs values were from 0.625 mg/ml to 2.5 mg/ml. Extracts showed significant effect on food spoilage isolates *Sarcina lutea* and *Staphylococcus aureus* (see Table 1.). Based on this information, plant extracts from this plant could be used as natural sources of preservatives substances with high importance in food industry.

The tested extracts showed very low activity on the growth of clinical isolates and standard strains of G-bacteria (MIC and MMC ranged from 1.25 mg/ml to 40 mg/ml). The exception was the methanol and acetone extracts of the species *P. aeruginosa* ATCC 27853, where MIC value was 1.25 mg/ml and *Proteus mirabilis* (MIC 1.25/2.5 mg/ml).

The tested extracts showed low antifungal activity. Methanol extract showed a significant effect on species *Aspergillus niger* ATCC 16404, where the MIC and MMC 0.625 mg/mL. MICs and MMCs for yeasts was 40 mg/ml. Slightly better effect of ethyl acetate extract can be seen in *Rhodotorula* sp. where the value 10 mg/ml.

Very little data is currently available about antimicrobial activity of different extracts the whole plant *M. peregrinum.* Chemical substance, ladanein, isolated from *M. peregrinum* show high antiviral effect aganist hepatitis C virus (HAID *et al.,* 2012, CALLAND *et al.,* 2012).

With the exception of the assay of anti-*Aspergillus* activities (RADOJEVIĆ *et al*., 2011), this is the first study on the antimicrobial activity of the metanol, acetone and ethyl acetate extracts of *M. peregrinum*. The results of our research indicated a good antibacterial potential of *M. peregrinum* against G+ bacteria, especially food spoilage isolates, based on which it could be considered as a source of potential antibacterial substances.

CONCLUSION

Antimicrobial activity extracts of *M. peregrinum* was tested by microdilution method and both minimal inhibitory and microbicidal concentration were determined. These tested extracts demonstrated the significant antibacterial activity against pathogenic bacteria *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus, Bacillus subtilis* ATCC 6633*, Bacillus subtilis, Bacillus cereus, Bacillus pumilus* NCTC 8241 and *Sarcina lutea*. On the other hand, these extracts demonstrated moderate and low antifungal activity.

The results of our study suggest the great value of the species *M. peregrinum* for use in food industry and phytotherapy. Therefore, the aerial sterile stems of this plant can be a potential source of antibacterial substances.

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Table 1. Antibacterial activities of acetone, ethyl acetate and methanol extracts of *Marrubium peregrinum* L. (Lamiaceae) against tested microorganisms based on microdilution method.

	Acetone extract		**Ethyl acetate extract**		**Methanol extract**		**Doxycycline**	
Species	**MIC***	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**
Escherichia coli ATCC 25922	5	10	10	10	5	5	15.625	31.25
Escherichia coli	20	20	20	20	20	20	7.81	15.625
Pseud. aeruginosa ATCC 27853	1.25	10	2.5	10	1.25	10	62.5	125
Pseud. aeruginosa	2.5	10	5	10	2.5	5	250	> 250
Salmonella enterica	20	20	20	20	20	20	15.625	31.25
Proteus mirabilis	2.5	5	10	10	1.25	2.5	250	> 250
Enter. faecalis ATCC 29212	10	40	10	20	10	40	7.81	62.5
Enter. faecalis	10	10	10	10	10	10	7.81	62.5
Staphylococcus aureus ATCC 25923	0.3125	1.25	0.3125	1.25	1.25	5	0.224	3.75
Staphylococcus aureus	1.25	5	2.5	5	0.625	1.25	0.448	7.81
Sarcina lutea	0.1563	0.625	0.3125	0.625	0.3125	1.25	< 0.448	3.75
Bacillus subtilis ATCC 6633	0.3125	0.625	0.625	1.25	0.625	0.625	1.953	31.25
Bacillus subtilis	0.3125	0.625	0.0781	0.625	0.3125	1.25	0.112	1.953
Bacillus cereus	1.25	2.5	1.25	1.25	0.3125	0.625	0.977	7.81
Bacillus pumilus NCTC 8241	1.25	1.25	0.3125	1.25	0.3125	0.625	0.112	7.81

*Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values are given as mg/ml for plant extracts and μg/ml for antibiotic. Antibiotic: doxycycline

Table 2. Antifungal activities of acetone, ethyl acetate and methanol extracts of *Marrubium peregrinum* L. (Lamiaceae) against tested microorganisms based on microdilution method.

	Acetone extract		Ethyl acetate extract		Methanol extract		Fluconazole	
Species	**MIC***	**MMC**	**MIC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**
Candida albicans	40	40	40	40	40	40	62.5	1000
Rhodotorula sp.	40	40	10	10	40	40	62.5	1000
Saccharomyces boulardii	40	40	40	40	40	40	31.25	1000
Aspergillus niger ATCC 16404	2.5	10	2.5	10	0.625	0.625	62.5	62.5
Penicillium italicum	10	20	2.5	20	5	10	1000	1000
Trichothecium roseum	20	20	10	20	2.5	10	500	500
Botrytis cinerea	20	20	10	20	20	20	31.25	500

*Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values are given as mg/ml for plant extracts and μg/ml for antibiotic. Antibiotic: fluconazole.