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Full Length Research Paper

Antimicrobial activity of the lichen *Lecanora frustulosa* and *Parmeliopsis hyperopta* and their divaricatic acid and zeorin constituents

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Antibacterial and antifungal activity of the acetone, methanol and aqueous extracts of the lichen *Lecanora frustulosa* and *Parmeliopsis hyperopta* and their divaricatic acid and zeorin constituents has been screened *in vitro* against the following species of microorganisms: *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter cloaceae*, *Escerichia coli*, *Klebsiella pneumoniae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium oxysporum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurescens*, *Penicillium verrucosum* and *Trichoderma harsianum*. The antimicrobal activity was estimated by the disc-difusion method and determination of the minimal inhibitory concentration (MIC) by the Broth tube Dilution method. The bacteria were more sensitive related to the tested fungi. The smallest MIC values were 0.39 mg/mL against bacteria and 1.56 mg/mL against fungi. Acetone and methanol extracts of the investigated lichens showed relatively strong antimicrobial activity, whereas aqueous extracts were inactive. Divaricatic acid and zeorin also showed strong activity against bacteria and fungi. There was no antimicrobial activity against *E. coli* species. The lichen extracts maximum activity showed in the concentrations of 0.78 mg/mL and the lichen components demonstrated maximum activity in the concetrations of 0.39 mg/mL. Generally, the tested lichen extracts and lichen compounds demostrated a strong antimicrobial effect against the tested microorganisms. That suggest a possibility of their use in the treatment of various diseases caused by these and similar microorganisms.

Key words: Antimicrobial activity, lichen extracts, lichen components.

INTRODUCTION

Lichens are symbiotic organisms of fungal and algal, and/or cyanobacterial partner, they are considered edible or used for their medicinal properties by many cultures. They synthetise a variety of secondary metabolites "lichen substances", mostly from fungal metabolism (Brennan et al., 2009). Lichen substances include aliphatic, cycloaliphatic, aromatic and terpenic components. Up till now, about 350 biologically active components are known from lichens and approximately 200 have been characterized (Chand et al., 2009; Tay et al., 2004). They are extracellular products of relatively low molecular weight crystallized on the hyphal cell walls. Also, they are usually insoluble in water and can be extracted into organic solvents (Ötzürk et al., 1999). They make even more than 30% of the dry mass of thalus (Galun, 1988).

Various biological activities of some lichens and their components are known, such as: antiviral, anti-tumor, anti-inflammatory, analgesic, antipyretic, antiproliferative and antiprotozoal (Lawrey, 1986; Halama and Van Haluvin 2004; Huneck, 1999). Besides, many sorts are used for human nutrition, animal nutrition, for getting colours, perfumes, alcohol and in the medicine industry

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(Kirmizigül et al., 2003; Richardson, 1988; Romagni, 2002). Several lichens species have been used in folk medicine for treatment of stomach deseases, diabetes, whooping cough, pulmonary tuberculosis, cancer treatment and skin deseases (Baytop, 1999; Huneck, 1999; Richardson, 1991).

It has been proved that the lichen extracts and lichen components have a distinguished antimicrobal activity (Gulluce et al., 2006; Ranković et al., 2007a). Because of that, the purpose of this work is to investigate *in vitro* antimicrobal activity of the acetone, methanol and aqueous extract of the chosen lichens and their components in relation to a number of microorganisms, including agents of human, animal and plant diseases.

MATERIALS AND METHODS

Lichen samples

Samples of *Lecanora frustulosa* (Dicks.) Ach. and *Parmeliopsis hyperopta* (Ach.) Arnold., were collected from Kopaonik, Serbia, in May of 2009, and identified by Dr. B. Ranković, University of Kragujevac. The demonstration samples were preserved in facilities of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. Determination of the investigated lichens was accomplished using standard keys (Purvis et al., 1992; Wirth, 1995; Dobson, 2000).

Microorganisms and media

The bacteria used as test organisms in this study were as follows: *Bacillus mycoides* (IPH), *Bacillus subtilis* (IPH) and *Staphylococcus aureus* (IPH) (Gram-positive bacteria); and *Enterobacter cloaceae* (IPH), *Escherichia coli* (IPH) and *Klebsiella pneumoniae* (IPH), (Gram-negative bacteria). All of the bacteria used were isolates of the Institute for Protection of Health in Kragujevac (IPH) and the Faculty of Agriculture in Belgrade (FAB). Their identification was confirmed in the Microbiological Laboratory of Kragujevac University's Department of Biology. The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Botrytis cinerea* (DBFS 133), *Candida albicans* (IPH 1316), *Fusarium oxysporum* (DBFS 292), *Mucor mucedo* (ATCC 52568), *Paecilomyces variotii* (ATCC 22319), *Penicillium purpurescens* (DBFS 418), *Penicillium verrucosum* (DBFS 262) and *Trichoderma harsianum* (DBFS 379). They were from the mycological collection maintained by the Mycological Laboratory within the Department of Biology, Faculty of Science (DBFS), University of Kragujevac. Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). Fungal cultures were maintained on potato dextrose agar (PDA) and Sabouraud dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4°C and subcultured every 15 days.

Preparation of the lichen extracts and isolation of the component

Finely ground thalli of the investigated lichens (50 g) were extracted using acetone, methanol and water in a Soxchlet extractor. The crude extracts were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at -18°C until they were used in the tests. Lichen substances were isolated from the obtained extracts by the method of preparative chromatography (TLC – silica gel G in the system of the solvents toluene: dioxane: vinegar acid = 90 : 25 : 4). The mixtures were identified by the chemical and physical-chemical analysis [tt. $Rf_{(TLC)}$, IR, H^1-NMR i MS (mass spectrum)]. The extracts and components were dissolved in dimethyl sulphoxide (DMSO). Minimal inhibitory concentracion (MIC) was determined by preparing a series of dilutions in Müller-Hinton broth (for bacteria) or in SD broth (for fungi). The final concentration for the DMSO did not extend 2% in the experiment.

Antimicrobial assays

The sensitivity of microorganisms to acetone, methanol, aqueous extracts of the investigated species of lichens and their component was tested by measuring the zone of inhibition of a given concentration of extract by the disk diffusion method and by determining the minimal inhibitory concentration (MIC).

All of the tested microorganisms in this study were used and the bioactivity was based on disc-diffusion assay and broth tube dilution methods. Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37°C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/ml. Suspensions of fungal spores were prepared from fresh mature (three - to seven-day-old) cultures that grew at 30°C on a PDA substrate. Spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by the NCCLS (National Committee for Clinical Laboratory Standards M 38 - P, 1998).

A standard disk-diffusion method (Bauer et al., 1966 or NCCLS, M2-A5, 1993) was used to study antimicrobial activity. Müller-Hinton agar (for bacteria) or in SD agar (for fungi) was seeded with the appropriate inoculum. Paper disks (7 mm diameter) were laid on the inoculated substrate after being soaked with 15 µL of lichen extract (50 mg/ml). Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the disk. Streptomycin (for bacteria) and ketoconazole (for fungi) were used as controls. A DMSO solution was used as a negative control for the influence of the solvents. All experiments were performed in triplicate.

The MIC was determined by the broth tube dilution method (NCCLS, 1998). A series of dilutions with concentrations ranging from 50 to 0.195 mg/mL for extracts and 25 to 0.097 mg/mL for components was used in the experiment against every microorganism tested. The starting solutions of extracts and component were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of extracts and components were prepared in Müller-Hinton broth for bacterial cultures and SD broth for fungal cultures in test tubes. The MIC was determined by establishing visible growth of the microorganisms. The boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria and ketoconazole in the case of fungi. All experiments were performed in triplicate.

RESULTS

From the obtained lichen extracts, were isolated lichen components: divaricatic acid from the lichen *L. frustulosa* and zeorin from the lichen *P. hyperopta.* Structure data of these components were as follows: Divaricatic acid - mp:137-139°C (toluen). UV (CH_3OH)λ^{max}, nm, log ε,:215 (4.52), 269 (4.12), 309 (3.90). IR (KBr) $\nu_{max:}$ 3500 (OH acid, phenol), 3030 (ar. CH), 1660 (C=O), 1640, 1603, 1430 (ar.γ), 1465, 1360 (CH_3C), 1280, 1230, 1200, 1175,

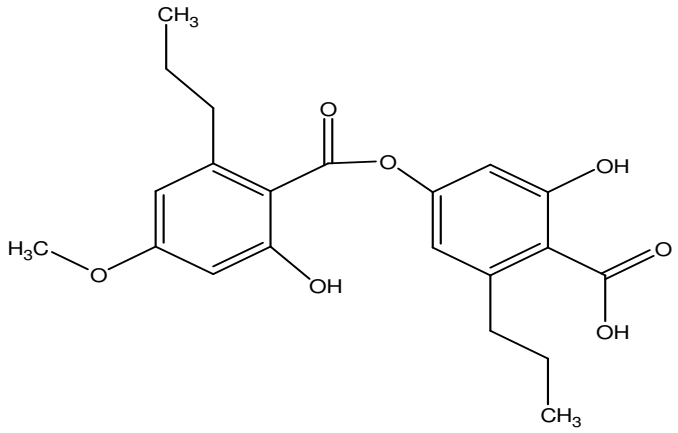

Figure 1. Structures of divaticatic acid.

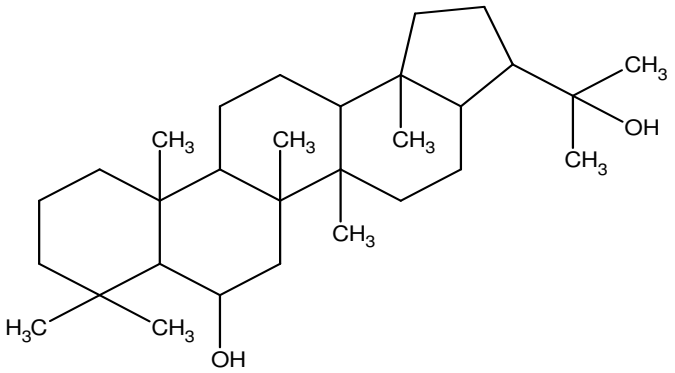

Figure 2. Structures of zeorin.

1160, 1140, 1044, 1020 (C-O), 980, 965, 875, 830, 760, 730, 710, 690 (ar.δcm $^{-1}$. ^{1}H NMR (TMS,)δ (acetone d_6): 0.92 (2xCH_3, t, J^3 = 7.8 Hz); 1.64 (2xCH_2, sex, J^3 = 7.8 Hz); 2.61 (2xCH_2, t, J^3 = 7.8 Hz); 3.81 (CH_3O, s); 11.48 (2xOH, br. s); 12.9 (COOH, s); 6.78, 6.50 (arom. ring A, 3H, 5H, s); 6.95, 7.19 (arom. ring B, 3H, 5H, s). MS m/z M^+ 388 (45 %), 370 (9%), 193 (100%), 179 (10%), 164 (13%), 138 (22%), 135 (19%), 107 (6%). TLC_{vol}(toluen: dioxane: acetic acid = 90: 25: 4) R_f = 0.39.

Zeorin - mp:237-240°C (ethanol). IR (KBr)$\nu_{max:}$ 3385 (OH), 2980 (CH, CH_2, CH_3), 1465, 1380 (CH_3C), 1260, 1250, 1210, 1160, 1140, 1044, 1020 (C-O), 980, 965, 875, 830, 760, 730, 710, 690 cm $^{-1}$. ^{1}H NMR (TMS,)δ($CDCl_3$): 0.76 (3H, CH_3 -28, s); 0.85 (3H, CH_3 -25, s); 0.99 (3H, CH_3 -27, s); 1.04 (3H, CH_3 -26, s); 1.09 (3H, s, CH_3 -23); 1.17 (6H, s, CH_3 -29, CH_3 -30); 4.28 (H. br.s, OH); 4.69 (H. br.s, OH). MS m/z M^+ 444 (45 %), 426 (9 %), 207 (100 %), 189 (88 %), 149 (73 %), 148 (24 %), 121 (19 %), 107 (32 %), 95 (79 %), 59 (93 %). TLC_{vol}(toluen: dioxane: acetic acid = 90: 25: 4) R_f = 0.52. Structural formulas of both components were shown in Figures 1 and 2.

The antimicrobal activity of the tested lichen extracts and lichen components against the tested microorganisms was shown in the tables, for lichen extracts (Table 1) and for lichen components (Table 2).

Disc-difusional method

The acetone and methanol extracts of the tested lichens showed relatively strong antimicrobial activity. The zones of inhibition for both extracts relative to the bacteria and fungi were 11 - 26 mm. The aqueous extracts of the tested lichens showed no antimicrobial activity against any of the test organisms.

The acetone and methanol extracts of the lichen *L. frustulosa* inhibited five out of six tested bacteria. The zones of inhibition relative to the bacteria were 12 - 24 mm. The largest zones of inhibition were recorded with the methanol extract, against *Bacillus mucoides* (24 mm). The antifungal activity of these extracts was selective, the acetone extract inhibited only *B. cinerea* and *P. variotii* species and methanol extract inhibited seven out of ten tested fungal species (Table 1). The zones of inhibition relative to the fungi were 13 - 17 mm.

The acetone and methanol extracts of the lichen *P. hyperopta* showed strong antibacterial activity on all of the tested bacteria except *E. coli*, which was resistant. The zones of inhibition relative to the bacteria were 11 - 18 mm for the acetone and 11 - 21 mm for the methanol. Extracts of this lichen showed a strong antifungal activity on all of the tested fungi. The measured zones of inhibition were 12 - 26 mm. The maximum activity was found in the methanol extract against *P. variotii* (26 mm).

Minimal inhibitory concentration (MIC)

The MIC for the different extracts related to the tested bacteria and fungi were 0.78 - 12.5 mg/ml. The maximum antimicrobial activity was found in the extracts of the lichen *P. hyperopta*. The acetone and methanol extract of this lichen had approximately equal antibacterial activity, but antifungal activity was not equal. The methanol extract exerted stronger antifungal activity than acetone extract. The measured MIC values for the acetone and methanol extracts were 0.78 - 1.56 mg/mL against bacteria and 1.56 - 12.5 mg/mL against fungi.

The acetone and methanol extracts of the lichen *L. frustulosa* inhibited five out of six tested bacteria. The MIC values relative to the bacteria were 1.56 - 3.12 mg/mL for the acetone and 0.78 - 1.56 mg/mL for the methanol extract. The methanol extract of this lichen demonstrated stronger antifungal activity than acetone. The acetone extract inhibited only two fungal species in the concentrations of 12.5 mg/mL. The methanol extract inhibited seven fungal species. The MIC for the methanol extract related to the fungi was 3.12 - 12.5 mg/mL.

Divaricatic acid from lichen *L. frustulosa* and zeorin

Table 1. Antimicrobial activities of acetone, methanol and aqueous extracts of *L. frustulosa* and *P. hyperopta* against tested microorganisms based on disc-diffusion and broth tube dilution methods.

Test organisms	Lecanora frustulosa			Parmeliopsis hyperopta			Antibiotics	
	A[a]	B	C	A	B	C	S	K
	DD[b]-MIC[c]	DD-MIC	DD-MIC	DD-MIC	DD-MIC	DD-MIC	DD-MIC	DD-MIC
B. mycoides	22-1.56	24-0.78	- -	11-0.78	11-0.78	- -	28-7.81	- -
B. subtilis	13-3.12	15-1.56	- -	16-0.78	17-0.78	- -	26-7.81	- -
E. cloacae	12-1.56	17-0.78	- -	13-0.78	18-1.56	- -	25-1.95	- -
E. coli	- -	- -	- -	- -	- -	- -	15-31.25	- -
K. pneumoniae	19-1.56	23-0.78	- -	18-0.78	21-1.56	- -	40-1.95	- -
S. aureus	18-1.56	21-0.78	- -	15-0.78	15-0.78	- -	20-31.25	- -
A. flavus	- -	- -	- -	13-12.5	16-6.25	- -	- -	27-3.9
A. fumigatus	- -	15-12.5	- -	14-12.5	17-3.12	- -	- -	34-3.9
B. cinerea	14- 12.5	17-3.12	- -	21-6.25	24-3.12	- -	- -	39-1.95
C. albicans	- -	14-6.25	- -	20-12.5	22-3.12	- -	- -	40-1.95
F. oxysporum	- -	15-12.5	- -	12-12.5	16-3.12	- -	- -	35- 3.9
M. mucedo	- -	13-12.5	- -	12-12.5	15-3.12	- -	- -	17-31.25
Paecilomyces variotii	14-12.5	16-6.25	- -	12-12.5	26-1.56	- -	- -	40-1.95
P. purpurescens	- -	- -	- -	13-12.5	18-6.25	- -	- -	38- 3.9
P. verrucosum	- -	- -	- -	14-12.5	17-6.25	- -	- -	36- 3.9
T. harsianum	- -	16-3.12	- -	13-12.5	17-6.25	- -	- -	18-7.81

[a] A, acetone extract; B, methanol extract; C, aquaeos extract. [b] Diameter of inhibition zone (mm) including disc diameter of 7 mm. [c] Minimum inhibitory concentration (MIC); values are given as mg/ml for lichen extract and µg/ml for antibiotics. Values are the mean of three replicate. Antibiotics: K, ketaconazole, S, streptomycin.

Table 2. Minimum inhibitory concentration (MIC) of divaricatic acid and zeorin against the test organisms.

Organisms	*B. m*	*B. s*	*E. cl*	*E. coli*	*K. p*	*S. a*	*A. fl*	*A. fu*	*B. c*	*C. al*	*Fu. o*	*M. m*	*Pe. ve*	*P. pur*	*Pen. v*	*T.h*
Lichen compounds																
Divaricatic acid	1.56[a]	1.56	0.78	-	0.78	1.56	12.5	12.5	6.25	6.25	6.25	6.25	6.25	12.5	12.5	6.25
Zeorin	0.39	0.39	0.39	-	0.39	0.78	6.25	6.25	3.12	3.12	3.12	6.25	3.12	6.25	6.25	3.12
S	7.81	7.81	1.95	31.25	1.95	31.25	-	-	-	-	-	-	-	-	-	-
K	-	-	-	-	-	-	3.9	3.9	1.95	1.95	3.9	31.25	1.95	3.9	3.9	7.8

[a]Minimum inhibitory concentration (MIC); values given as mg/ml for lichen extract and as µg/ml for antibiotics.
Antibiotics: K – ketaconazole, S – streptomycin, *B. m=Bacillus mycoides; B. s=Bacillus subtilis; E. cl=Enterobacter cloaceae; E. clil=E. coli; K. p=Klebsiella pneumoniae; S.a=Staphyloccocus aureus; A.fl=Aspergillus flavus; A. fu=Aspergillus fumigatus; B. c=Botrytis cinerea; C. al=Candida albicans; Fu. o=Fusarium oxysporum; M.m=Mucor mucedo; Pe. ve=Paecilomyces variotii; P. pur=Penicillium purpurescens; Pen. vr=Penicillium verrucosum; T. h=Trichoderma harsianum*

from lichen *P. hyperopta* demonstrated very strong antimicrobial activity, but bacteria demonstrating bigger sensitivity than the fungi. Zeorin showed stronger antibacterial activity than divaricatic acid. At a concentration of 0.39 mg/mL (maximum activity), zeorin inhibited four out of six tested bacteria. Divaricatic acid demonstrated activity in the concentrations of 0.78 mg/ml against two bacterial species and in the concentrations of 1.56 mg/ml against three bacterial species. The measured MIC values relative to the fungi were 3.12 - 6.25 mg/ml for zeorin and 6.25 - 12.5 mg/ml for divaricatic acid.

The antimicrobial activities were compared with the standard antibiotics, streptomycin (for bacteria) and ketoconazole (for fungi). The results showed that standard antibiotics had stronger activity than tested extracts and components as shown in Tables 1 and 2.

DISCUSSION

In this paper, for the first time, the antimicrobial activity of the extracts of the lichens *L. frustulosa* and *P. hyperopta* and their divaricatic acid and zeorin constituents has been presented. The tested lichen extracts and lichens acid show a relatively strong antimicrobial activity. The intensity of the antimicrobial effect depended on the sort of the extract, its concentration and the tested microorganism. Similar results were also noticed by other investigators (Ranković et al., 2007a). The aqueous extracts of the tested lichens did not show any antimicrobial activity. That is probably because the active components produced by lichens are either insoluble or poorly soluble in water (Kinoshita et al., 1994). The antibacterial effect is stronger realtive to the antifungal. These results could be expected considering the fact that numerous tests proved that bacteria are more sensitive to the antibiotic compared to fungi (Hugo and Russell, 1983). The reason of different sensitivity between the fungi and bacteria can be found in different transparency of the cell wall (Yang et al., 1999). The cell wall of the gram-positive bacteria consists of peptidoglucans (mureins) and teichoic acids, the cell wall of the gram-negative bacteria consists of lipo polysaccharides and lipopoliproteins (Hugenholtz, 2002; Mandelstam et al., 1982; Jean, 2001), whereas, the cell wall of fungi consists of polysaccharides such as hitchin and glucan (Griffin, 1994; Ruiz-Herrera, 1992).

Previous researches showed significant bioactive characteristics of taxanomic_similar lichens. Gulluce et al. (2006) found out that the methanol extract of the lichen *Parmelia saxatilis* had a strong antimicrobal influence. Similar results were reported by Candan et al. (2007) for different extracts extracted from the lichen *Parmelia sulcata* and its salazinic acid constituent. Ranković at al. (2007b) find an antimicrobal activity for the extracts of the lichens *Parmelia caperata* and *Parmelia pertusa*.

The obtained results showed that the tested lichen extracts and lichen components showed a significant antimicrobial activity relative to the tested bacteria and fungi, which could be of significance in human therapy, animal and plant diseases. Further studies should be done to search new compounds from lichens that exhibit strong antimicrobial activity.

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