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ANTIMICROBIAL ACTIVITIES OF DIFFERENT EXTRACTS OF *LECANORA ATRA LECANORA MURALIS, PARMELIA SAXATILIS*, *PARMELIA SULCATA* AND *PARMELIOPSIS AMBIGUA*

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Abstract

Antimicrobal activity of the acetone, methanol and aqueous extracts of the lichens *Lecanora atra*, *Lecanora muralis, Parmelia saxatilis*, *Parmelia sulcata* and *Parmeliopsis ambigua* was explored *In vitro* against to 6 species of bacteria and 10 species of fungi by the disc-difusion method and determination of the minimal inhibitory concentration (MIC) by the Broth tube Dilution method. The acqueous extracts of the tested lichens didn't show any antimicrobal activity on any of the test organisms, whereas the acetone and methanol ones showed an activity related to the tested species. The bacteria were very sensitive related to the tested fungi. The strongest antimicrobal activity was found in the acetone extract of the lichen *Parmelia sulcata* where the least measured MIC value was 0.78 mg/ml. Generally, among the bacteria the most sensitive was the species *Bacillus mycoides,* and among the fungi *Botrytis cinerea* and *Candida albicans*. The bacterium *Escerichia coli* was resistant to all the extracts of the explored lichens. Generally, all the explored lichens had a relatively strong antimicrobal activity, which can be very important in making the food bad and in curing numerous diseases caused by these and similar microorganisms.

Introduction

Lichens are symbiothic organisms built from fungi and a photosynethic partner, that can be an alga or a Cyanobacterium (Ahmadijan, 1993). They usually grow on rocks, non-fertile ground, as well as epiphytes on the trees and leaves (Taylor *et al.,* 1995). Lichens synthesise various bioactive components that sometimes make even more than 30% of the dry mass of talus (Galun & Shomer-Ilan, 1988). Although there are about 20,000 species of them around the world, and even they make 8% of the terrestrial ecosystems, their biological activity and biological components are not distinguished very much (Toma *et al.,* 2001). Various biological activities of some lichens and their components are known, such as: antiviral, anti-tumor, anti-inflammatory, analgetic, antipirethic, antiproliferative, antiprotosoal (Lawrey, 1986; Huneck, 1999; Davies *et al.,* 2002; Halama & Van Haluwin, 2004). Besides, many sorts are used for human nutrition, animal nutrition, for getting colours, perfumes, alcohol and in the medicine industry. (Richardson, 1988; Richardson, 1991; Romagni, 2002; Kirmizigül *et al*., 2003). Lichens have also, for hundreds of years, been used in many Europeans contry as a cure for stomach diseases, diabetes, cough, pulmonar tuberculosis, wounds curing, dermatological diseases (Richardson, 1991; Baytop, 1999; Huneck, 1999). The usage of some lichens for many years in the traditional medicine was later justified by numerous researches that confirmed their antimicrobal activity (Vartia, 1973; Choudhary *et al.,* 2005; Cansaran *et al.,* 2006; Gulluce *et al.,* 2006; Ranković *et al.,* 2008).

The aim of this study was to evaluate the antimicrobal activity of the acetone, methanol and aquatic extract of the lichens relative to the chosen test microorganisms, which are the causes of turning the food bad and cause the deseases of humans, animals and plants.

Materials and Methods

Lichen samples: Samples of the lichens of *Lecanora atra* (Hudson) Ach., *Lecanora muralis* (Schreber) Rabenh., *Parmelia saxatilis* (L.) Ach., *Parmelia sulcata* (Taylor) and *Parmeliopsis ambigua* (Wulf.) Nyl., were collected from Borač, Serbia, in August 2007, and identified by Dr. B. Ranković, University of Kragujevac. The demonstration samples are preserved in faculties of the Department of Biology and Ecology of Kragujevac, Faculty of Science. 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: *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 of Kragujevac University's Faculty of Science (DBFS). Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). Fungal cultures were maintained on potato dextrose agar and Sabourad dextrose agar (Torlak, Belgrade). All cultures were stored at 4°C and subcultured every 15 days.

Preparation of the lichen extracts: Finely pulsverised thalli of the investigated lichens (50 g) were extracted using acetone, methanol and water in a Soxchlet extractor. The 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. The extracts were dissolved in dimethyl sulphoxide (DMSO) for the disk diffusion test. Minimal inhibitory concentracion (MIC) was determined by preparing a series of dilutions in Müller-Hinton broth (for bacteria) or in SD broth (for fungi) in the range 50 to 0.195 mg/mL. The final concentration for the DMSO didn't extend 2% in the experiment.

Antimicrobial assays: The sensitivity of microorganisms to acetone, methanol and aqueous extracts of the investigated species of lichens 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) (Iftikhar *et al.,* 2010; Jabeen *et al.,* 2011).

Bacterial inocula 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 (3- to 7-day-old) cultures that grew at 30°C on a PDA substrate. Spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometriaclly at 530 nm, and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by the Anon., (1998).

A standard disk-diffusion method (Anon., 1993) was used to study antimicrobial activity. Müller-Hinton agar (for bacteria) or in SD agar (for fungy) 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 minimal inhibitory concentration (MIC) was determined by the broth tube dilution method. A series of dilutions with concentrations ranging from 50 to 0.0037 mg/mL was used in the experiment for each extract against every microorganism tested. The starting solutions of extracts with a concentration of 50 mg/mL were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of extracts were prepared in Müller-Hinton broth for bacterial cultures and SD broth for fungal cultures in test tubes. The minimal inhibitory concentration was determined by establishing visible growth of the microorganisms. The boundary dilution without any visible growth was defined as the minimal inhibitory concentration (MIC) for the tested microorganism at the given lichen extract concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria, ketoconazole in the case of fungi. All experiments were performed in triplicate.

Results

The antimicrobal activity of the tested lichen extracts against the tested microorganisms is shown in the tables for the disc-difusional method (Table 1) and the minimal inhibitory concentration (Table 2).

Table 1. Antimicrobial activities of different extracts of *Lecanora atra*, *Lecanora muralis, Parmelia saxatilis*, *Parmelia sulcata* and *Parmeliopsis ambigua* by using agar disc diffusion method.

Organisms	Lichen species																
	L. atra			*L. muralis*			*P. saxatilis*			*P. sulcata*			*P.ambigua*			Antibiot.	
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	S	K
Bacillus mycoides	22[b]	25	-	23	26	-	22	24	-	28	26	-	16	11	-	28	-
Bacillus subtilis	14	14	-	13	15	-	18	20	-	20	16	-	16	15	-	26	-
Enterobacter cloacae	13	13	-	14	16	-	16	16	-	16	13	-	15	17	-	25	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	-
Klebsiella pneumoniae	16	14	-	18	24	-	17	19	-	18	14	-	21	19	-	40	-
Staphiloccocus aureus	16	13	-	17	18	-	18	20	-	26	25	-	14	14	-	20	-
Aspergillus flavus	16	18	-	-	-	-	13	17	-	23	23	-	14	16	-	-	27
Aspergillus fumigatus	12	15	-	-	13	-	18	24	-	18	19	-	14	16	-	-	34
Botrytis cinerea	20	16	-	12	15	-	22	28	-	30	27	-	20	23	-	-	39
Candida albicans	20	21	-	-	12	-	20	22	-	24	23	-	19	23	-	-	40
Fusarium oxysporum	12	18	-	-	14	-	13	17	-	14	16	-	12	17	-	-	35
Mucor mucedo	12	16	-	-	14	-	17	19	-	13	16	-	12	16	-	-	17
Paecilomyces variotii	10	20	-	13	16	-	20	22	-	22	25	-	13	24	-	-	40
Penicillium purpurescens	17	21	-	-	-	-	15	18	-	17	20	-	12	15	-	-	38
Penicillium verrucosum	15	19	-	-	-	-	15	22	-	17	19	-	15	15	-	-	36
Trichoderma harsianum	11	19	-	-	15	-	20	24	-	18	20	-	15	17	-	-	18

[a] A – acetone extract; B – methanol extract; C – aquaeos extract

[b] Diameter of inhibition zone (mm) including disc diameter of 7 mm. Values are the mean of three replicate

Antibiotics: K – ketaconazole, S – streptomycin

Table 2. Minimum inhibitory concentration (MIC) of *Lecanora atra*, *Lecanora muralis, Parmelia saxatilis*, *Parmelia sulcata* and *Parmeliopsis ambigua* extracts against the test organisms.

Organisms	Lichen species																
	L. atra			*L. muralis*			*P. saxatilis*			*P. sulcata*			*P.ambigua*			Antibiot.	
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	S	K
B. mycoides	1.56[b]	1.56	-	3.12	1.56	-	3.12	1.56	-	0.78	1.56	-	1.56	1.56	-	7.81	0
B. subtilis	1.56	3.12	-	6.25	3.12	-	3.12	3.12	-	0.78	1.56	-	1.56	1.56	-	7.81	0
E. cloaceae	3.12	3.12	-	3.12	1.56	-	3.12	1.56	-	0.78	1.56	-	1.56	3.12	-	1.95	0
E. coli	-	-	-	-	-	-	0	0	-	0	0	-	0	0	-	31.25	0
K. pneumoniae	3.12	3.12	-	3.12	1.56	-	3.12	1.56	-	0.78	1.56	-	1.56	3.12	-	1.95	0
S. aureus	3.12	3.12	-	3.12	1.56	-	3.12	3.12	-	0.78	1.56	-	1.56	1.56	-	31.25	0
A. flavus	25	6.25	-	-	-	-	25	12.5	-	6.25	3.12	-	25	12.5	-	0	3.9
A. fumigatus	25	3.12	-	-	25	-	25	6.25	-	6.25	3.12	-	25	6.25	-	0	3.9
B. cinerea	25	3.12	-	25	12.5	-	3.12	1.56	-	1.56	1.56	-	12.5	6.25	-	0	1.95
C. albicans	12.5	1.56	-	-	25	-	3.12	1.56	-	1.56	1.56	-	25	6.25	-	0	1.95
F. oxysporum	25	6.25	-	-	25	-	25	12.5	-	6.25	3.12	-	25	6.25	-	0	3.9
M. mucedo	25	6.25	-	-	25	-	12.5	6.25	-	6.25	3.12	-	25	6.25	-	0	31.25
P. variotii	25	3.12	-	25	12.5	-	6.25	3.12	-	3.12	1.56	-	25	3.12	-	0	1.95
P. purpurescens	25	3.12	-	-	-	-	25	12.5	-	12.5	6.25	-	25	12.5	-	0	3.9
P. verrucosum	25	3.12	-	-	-	-	25	6.25	-	12.5	6.25	-	25	12.5	-	0	3.9
T. harsianum	25	6.25	-	-	-	-	12.5	6.25	-	6.25	3.12	-	25	12.5	-	0	7.81

[a] A – acetone extract; B – methanol extract; C – aquaeos extract
[b] Minimum inhibitory concentration (MIC); values given as mg/ml for lichen extract and as μg/ml for antibiotics
Antibiotics: K – ketaconazole, S – streptomycin

Disc-difusional method: The acetone and methanol extracts of the tested lichens showed a strong antimicrobal activity. The extracts of the lichen *Lecanora atra* inhibited 5 out of 6 tested bacteria. The greatest sensitivity to the tested species was shown by the species *Bacillus mycoides* in which the largest zones of inhibition were measured (22 cm for the acetonic and 25mm for the methanol extract). The extracts of the lichen *Lecanora atra* also showed an antifungal activity relative to the all tested fungi. The zones of inhibition for the acetone and the methanol ectracts were within the range 11-21 mm.

The lichen *Lecanora muralis* showed a relatively strong antibacterial activity. The largest zone of inhibition (26mm) was measured in the methanol extract relative to the species *Bacillus mycoides*. The extracts of this lichen showed a weak antifungal activity. The acetone extract inhibited two and the methanol one seven out of ten tested fungi. The zone of inhibition for the acetone and methanol extracts range 12-16 mm.

The acetone and methanol extracts of the lichen *Parmelia saxatilis* showed a very strong inhibitory influence to the tested bacteria. Bigger zones of inhibition were noticed in inluencing of the methanol extract, especially relative to the species *Bacillus mycoides* (24 mm). The zones of inhibition in both extracts relative to the tested fungi ranged 13-28mm.

The extracts of the lichen *Parmelia sulcata* showed the strongest antibacterial activity. The acetone extract showed a stronger antibacterial effect compared to the methanol one. The zones of inhibition relative to the bacteria were large. They were within the range of 16-28mm for the acetone and 13 – 26mm for the methanol one. The lichen *Parmelia sulcata* had a strong antifungal activity. The measured zones of inhibition related to the fungi were also large.

The acetone and methanol extracts of the lichen *Parmeliopsis ambigua* showed a relatively strong antimicrobial activity. The zones of inhibition of both the extracts realtive to the bacteriae and fungi were relatively large (11–24 mm).

Minimal inhibitory concentration (MIC): The MIC for the different extract related to the tested bacteria and fungi were within the range of 0.78-25 mg/ml. The biggest antibacterial activity was in the extracts of the lichen *Pamela sulcata*, particularly the acetone extract, which inhibited the tested bacteria in a very low concentration (0.78 mg/mL). The lichen *Parmelia sulcata* had a very strong antifungal activity as well. The measured MIC values related to the tested fungi were relatively low (1.56-12.5 mg/mL). The extracts of the lichens *Lecanora atra, Parmelia saxatilis* and *Parmeliopsis ambigua* showed relatively equal antimicrobial activity, although it should be stressed that the methanol extracts had shown a stronger inhibitory influence than the acetone ones. The lichen *Lecanora muralis* showed a relatively strong antibacterial effect but the antifungal effect was weak. The MIC for the acetone and methanol extracts of the lichen *Lecanora muralis* were within the range 1.56-3.12 mg/mL related to the bacteria and 12.5-25 mg/mL related to the fungi.

Discussion

The tested lichen extracts show a relatively strong antimicrobial activity. The intensity of the antimicrobial effect of the tested extracts depended on the sort of the extract, its concentration and the tested microorganism. The aquatic extracts of the tested lichens didn't show any antimicrobial activity. That is probably because the active

components produced by lichens can't be diluted or can be little diluted (Kinoshita *et al*., 1994). The antibacterial effect is stronger realtive to the antifungal one. These results could be expected considering the fact that numerous tests proved that bacteria are more sensitive to the antibiotisc compared with fungi (Hugo & 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 cells consists of lipopolysaccharides, and lipopoliproteins (Mandelstam *et al.,* 1982; Jean van Heijenoort; 2001; Hugenholtz, 2002) whereas the cell wall of fungi consists of polysaccharides such as hitchin and glucan (Ruiz-Herrera, 1992; Griffin, 1994).

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

In this work, for the first time study was carried out on the antimicrobal activity of the lichens *Lecanora atra, Lecanora muralis* and *Parmeliopsis ambigua*. The obtained results showed that the tested lichen extracts showed a significant antimicrobial influence relative to the tested bacteria and fungi. That can be very useful in prevention of turning food bad and therapies of many diseases caused by these and similar microorganisms.

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