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EXPERIMENTAL ARTICLES

Evaluation of Antimicrobial Activity of the Lichens *Lasallia pustulata, Parmelia sulcata, Umbilicaria crustulosa*, and *Umbilicaria cylindrica*

B. Ranković[a,1], M. Mišić[a], S. Sukdolak[b]

[a] *Department of Biology, Faculty of Science, University of Kragujevac, Serbia*

[b] *Department of Chemistry, Faculty of Science, University of Kragujevac, Serbia*

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Abstract—The antimicrobial properties of acetone, methanol, and aqueous extracts of the lichens *Lasallia pustulata, Parmelia sulcata, Umbilicaria crustulosa*, and *Umbilicaria cylindrica* were studied comparatively in vitro. Antimicrobial activities of the extracts of different lichens were estimated by the disk diffusion test for Gram-positive bacteria, Gram-negative bacteria, and fungal organisms, as well as by determining the MIC (minimal inhibitory concentration). The obtained results showed that the acetone and methanol extracts of *Lasallia pustulata, Parmelia sulcata*, and *Umbilicaria crustulosa* manifest antibacterial activity against the majority of species of bacteria tested, in addition to selective antifungal activity. The MIC of lichen extracts was lowest (0.78 mg/ml) for the acetone extract of *Lasallia pustulata* against *Bacillus mycoides*. Aqueous extracts of all of the tested lichens were inactive. Extracts of the lichen Umbilicaria cylindrica manifested the weakest activity, inhibiting only three of the tested organisms.

Key words: antimicrobial activity, lichen extracts, *Lasallia pustulata, Parmelia sulcata, Umbilicaria crustulosa, Umbilicaria cylindrica*.

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Lichens are symbiotic organisms composed of fungi (mycobionts) and algae (photobionts), and about 20000 species of them have been recorded worldwide. The lichen organism has characteristics of both partners. Lichens are an important food for many animals, including man [1]. They are used in production of alcohols and paints, as well as in the perfume and pharmaceutical industries. In addition to this, lichens have been used in folk medicine for centuries: their biological activities were long ago recognized by native Americans, Haitians, Indians, Chinese, and Europeans, who use lichens in their traditional medicines to treat a variety of animals [2].

Many different bioactive secondary metabolites have been isolated from lichens [3], and some of them are used in pharmaceutical sciences. Several lichen extracts have been used for various remedies in folk medicine, and screening of lichens has revealed the frequent occurrence of metabolites with antibiotic, antimycobacterial, antiviral, antitumor, analgesic, and antipiretic properties [4–7].

Lichen-forming fungi produce antibiotic secondary metabolites that protect many animals from pathogenic microorganisms [6]. A number of investigators have studied the antibacterial and antifungal activity of lichens. The first study of the antibiotic properties of lichens was carried out by Burkholder in 1944 [8]. Vartia [4] reported antibacterial activity for several lichens, and other researchers have since then studied the antibacterial activity of several lichens against Gram-positive and Gram-negative bacteria, as well as the antifungal activity of lichen extract [9–12].

The development and spread of microbial resistance to available antibiotics has prompted investigators to study antimicrobial substances from other sources. Owing to pronounced antimicrobial activity of some of their secondary metabolites, lichens (together with algae, macrofungi, and higher plants) are attracting much attention among researchers as significant new sources of bioactive substances [7, 13].

Accordingly, the purpose of the present work was to conduct in vitro evaluation of the antimicrobial activity manifested by acetone, methanol, and aqueous extracts from four species of lichens (*Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa*, and *Umbilicaria cylindrica*) in relation to test microorganisms that included agents of human, animal, and plant diseases, producers of mycotoxins, and causers of food spoilage.

[1] Corresponding author; e-mail: rankovic@kg.ac.yu

MATERIALS AND METHODS

Lichen samples. Samples of the lichens *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa* and *Umbilicaria cylindrica* were collected on Mt. Kopaonik (Serbia) in August 2005. The samples were dried at room temperature for a period of 24 h. For identification of lichens, we used several standard keys [14, 15]. Documentation samples are deposited in the mycological herbarium of the Department of Biology, Faculty of Science, University of Kragujevac (MHDB): *Lasallia pustulata* (L.) Merat., 171; *Parmelia sulcata* Taylor, 134; *Umbilicaria crustulosa* (Ach.) Frey, 195; and *Umbilicaria cylindrica* (L.) Delise ex Duby, 241.

Preparation of lichen extracts. Three solvents (water, methyl alcohol, and acetone) were used to extract lichens. To obtain aqueous extracts, dried lichen thalli (50 g of material from each species separately) were ground to a particle size of <2.5 mm and flushed with distilled water. Extraction was performed in a Soxhlet extractor at 80°C for a period of 7 h. The obtained extract was filtered using Whatman No. 1 filter paper and then evaporated in a water bath at 80°C until dry material was obtained.

Acetone extracts: powdered lichens (50 g) were extracted with 250 ml of acetone in a Soxhlet extractor for 7 h at a temperature not exceeding the boiling point of the solvent (56.2°C). The acetone extracts were filtered using Whatman No. 1 filter paper. The obtained extracts were evaporated on a rotary vacuum evaporator until a solid concentrate was produced.

Methanol extracts: powdered lichens (50 g) were extracted with 250 ml of methanol in a Soxhlet extractor for 7 h at a temperature not exceeding the boiling point of the solvent [16]. The methanol extracts were filtered using Whatman No. 1 filter paper and then concentrated in a vacuum at 40°C using a rotary evaporator.

The extracts were further dissolved in dimethyl sulfoxide (DMSO) for the disk diffusion test, and MIC values were determined. The final concentration of DMSO in the experiment did not exceed 2%.

Microorganisms and media. The test organisms used in this study were as follows: *Bacillus mycoides* (ATCC 6463), *Bacillus subtilis* (ATCC 6633), and *Staphylococcus aureus* (ATC 25923) (Gram-positive bacteria); and *Enterobacter cloaceae* (ATCC 29003), *Escherichia coli* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 29665) (Gram-negative bacteria). The following fungi were used in the study: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Botrytis cinerea* (DBFS 133), *Candida albicans* (IPH 1316), *Fusarium oxysporum* (DBFS 192), *Mucor mucedo* (ATCC 52568), *Paecilomyces variotii* (ATCC 22319), *Penicillium purpurescens* (DBFS 418), *Penicillium verrucosum* (DBFS 262), *Saccharomyces cerevisiae* (DBFS 234), and *Trichoderma harsianum* (DBFS 379). The fungi came from the mycological collection of the Mycological Laboratory, Department of Biology, Faculty of Science, University of Kragujevac (DBFS). Bacterial cultures were maintained on Mueller–Hinton media from Torlak (Belgrade, Serbia). Fungal cultures were maintained on potato dextrose agar and Sabourad dextrose agar from Torlak. All cultures were preserved at 4°C and subcultured every 15 days.

Antibacterial activity assay. The sensitivity of microorganisms to extracts of different species of lichens was ascertained by the standard disk diffusion method approved by the National Committee for Clinical Laboratory Standards or NCCLS [17, 18], in addition to which the MIC values of extracts were also determined.

Bacterial inocula were obtained from bacterial cultures incubated for 24 h at 37°C on Mueller–Hinton medium and diluted by approximately 10^8 CFU/ml in relation to the 0.5 McFarland standard. Suspensions of fungal spores were prepared from fresh mature (3- to 7-day-old) cultures reared at 30°C on Sabourad dextrose agar media. Spores were rinsed with sterile distilled water. Turbidity of spore suspensions was determined spectrophotometrically at 530 nm, after which they were further diluted to approximately 10^6 CFU/ml according to the procedure recommended by the aforementioned committee [19].

Disk diffusion was carried out as follows: suspensions of the tested microorganisms were poured onto solid Mueller–Hinton nutrient substrate (for bacteria) or Sabourad dextrose agar (for fungi). Filter paper disks (7 mm in diameter) were soaked with 15 μl of a lichen extract and placed on the inoculated substrates. After incubation at 37°C for a period of 24 h in the case of bacteria and at 27°C for a period of 2–14 days in the case of fungi, the diameter of the zone of inhibition of the tested microorganism by the given extract was measured. Disks soaked with 15 μl of DSMO in the same concentration as that of the lichen extract solution were used as a negative control. Streptomycin was used as a positive control of growth in the case of bacteria, while ketoconazole was used as a positive control of growth in the case of fungi. All experiments were performed in duplicate. Diameters of inhibition zones were measured in millimeters and expressed in mean values.

The MIC was determined by the broth tube dilution method by making a series of geometric dilutions in the range from 50 to 0.05 mg/ml of lichen extract. A series of 12 sterile test tubes with stoppers (1 to 12) was used for each extract against each microorganism tested in the experiment. The first test tube was given 2.0 ml of a solution of a certain lichen extract (50 mg/ml), which was then serially diluted with Mueller-Hinton broth (for bacteria) or Sabourad dextrose broth (for fungi) to an extract concentration of 0.05 mg/ml. The 12th tube served as a control and contained only 1 ml of the substrate. Each tube (1–12) was then given 20 μl of a bacterial or fungal culture. The tubes were subsequently incubated at 37°C for a period of 24 h in the case of bacteria or at 27°C for a period of 2–12 days in the case of fungi. The MIC was determined by establishing visible

Table 1. Antimicrobial activities of different extracts of *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa* and *Umbilicaria cylindrica* using disk diffusion method

Organisms	Lichen species													
	L. pustulata			*P. sulcata*			*U. crustulosa*			*U. cylindrica*			Antibiotics	
	A*	B	C	A	B	C	A	B	C	A	B	C	S	K
B. mycoides	19**	18	–	18	13	–	13	14	–	–	–	–	28	–
B. subtilis	20	20	–	25	13	–	17	16	–	–	–	–	26	–
E. cloaceae	19	16	–	19	16	–	14	18	–	–	–	–	25	–
E. coli	–	–	–	24	20	–	–	–	–	–	–	–	15	–
K. pneumoniae	22	19	–	18	24	–	15	15	–	–	–	–	40	–
S. aureus	13	12	–	–	–	–	13	13	–	–	8	–	20	–
A. flavus	10	15	–	20	18	–	10	16	–	–	–	–	–	27
A. fumigatus	12	19	–	14	18	–	14	18	–	–	–	–	–	34
B. cinerea	–	–	–	–	–	–	–	–	–	–	–	–	–	39
C. albicans	15	16	–	14	12	–	–	–	–	12	10	–	–	40
F. oxysporum	–	–	–	12	11	–	8	11	–	–	–	–	–	35
M. mucedo	17	19	–	16	14	–	12	13	–	–	–	–	–	17
P. variotii	18	22	–	–	–	–	–	25	–	–	–	–	–	40
P. purpurescens	12	16	–	12	12	–	–	20	–	–	–	–	–	38
P. verrucosum	10	12	–	13	12	–	19	21	–	–	–	–	–	36
S. cerevisiae	10	10	–	18	16	–	–	–	–	–	–	–	–	30
T. harsianum	16	18	–	14	11	–	18	23	–	–	–	–	–	18

Notes: * A – acetone extract; B – methanol extract; C – aquaeos extract.

** Diameter of inhibition zone (mm) including disc diameter of 7 mm. Values are the mean of three replicates. Antibiotics: K – ketaconazole, S – streptomycin.

growth of microorganisms in the tubes. The boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration of a certain lichen extract. The antibiotic streptomycin was used as a positive control for inhibition of bacterial growth, while ketoconazole was used as a positive control for inhibition of fungal growth. The starting concentration in studying the antimicrobial activity of lichen extracts was 1 mg/ml. All experiments were performed in duplicate.

RESULTS AND DISCUSSION

The antimicrobial activity of acetone, methanol, and aqueous extracts of the lichens *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa*, and *Umbilicaria cylindrica* against the tested microorganisms was estimated on the basis of the presence or absence of inhibitory zones, their diameters, and values of the MIC. The results are presented in Tables 1 and 2.

The acetone and methanol extracts of *Lasallia pustulata* manifested significant activity against Gram-positive and Gram-negative bacteria with the exception of *Escherichia coli*, which was resistant. The largest zone of inhibition (22 mm in diameter) was obtained for the acetone extract against the species *Klebsiella pneumoniae*. The MIC ranged from 0.78 to 6.25 mg/ml of extract in relation to the tested bacteria. Extracts of this lichen acted selectively on the tested fungi. Antifungal activity was manifested against nine of the 11 fungal species. The acetone and methanol extracts were most active in relation to the species *Paecilomyces variotii*, in whose case the zones of inhibition were large (18 and 22 mm in diameter, respectively). The MIC fluctuated in the range from 3.12 to 25 mg/ml of extract. The aqueous extract manifested neither antibacterial nor antifungal activity. *Lasallia pustulata* contains the lichen substances gyrophoric acid, arabitol, mannitol, and umbilicarin [20].

The acetone and methanol extracts of *Parmelia sulcata* manifested significant inhibitory activity against all of the tested bacteria with the exception of *Staphylococcus aureus*, which was resistant. The acetone extract had the largest zones of inhibition, which were especially large in the cases of *Bacillus subtilis* (25 mm) and *Escherichia coli* (24 mm in diameter). The MIC ranged from 1.56 mg/ml against *Escherichia coli* to 6.25 mg/ml against *Klebsiella pneumoniae*…. The antifungal action of these extracts was selective: growth of nine out of 11 species of fungi was inhibited. The MIC ranged from

Table 2. Minimum inhibitory concentration (MIC) of *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa* and *Umbilicaria cylindrica* extracts against the test organisms

Organisms	Lichen species													
	L. pustulata			*P. sulcata*			*U. crustulosa*			*U. cylindrica*			Antibiotics	
	A*	B	C	A	B	C	A	B	C	A	B	C	S	K
B. mycoides	0.78**	1.56	–	3.12	3.12	–	3.12	6.25	–	–	–	–	7.81	–
B. subtilis	1.56	3.12	–	3.12	1.56	–	6.25	6.25	–	–	–	–	7.81	–
E. cloaceae	1.56	3.12	–	3.12	1.56	–	3.12	6.25	–	–	–	–	1.95	–
E. coli	–	–	–	1.56	1.56	–	–	–	–	–	–	–	31.25	–
K. pneumoniae	1.56	3.12	–	6.25	6.25	–	12.5	6.25	–	–	–	–	1.95	–
S. aureus	6.25	6.25	–	–	–	–	6.25	6.25	–	–	50	–	31.25	–
A. flavus	25	6.25	–	50	50	–	25	12.5	–	–	–	–	–	3.9
A. fumigatus	12.5	6.25	–	25	25	–	12.5	6.25	–	–	–	–	–	3.9
B. cinerea	–	–	–	–	–	–	–	–	–	–	–	–	–	1.95
C. albicans	6.25	6.25	–	0.78	1.56	–	–	–	–	12.5	50	–	–	1.95
F. oxysporum	–	–	–	25	25	–	25	6.25	–	–	–	–	–	3.9
M. mucedo	12.5	6.25	–	0.78	3.12	–	12.5	12.5	–	–	–	–	–	31.25
P. variotii	6.25	3.12	–	–	–	–	–	6.25	–	–	–	–	–	1.95
P. purpurescens	25	6.25	–	25	25	–	–	6.25	–	–	–	–	–	3.9
P. verrucosum	12.5	6.25	–	25	25	–	12.5	12.5	–	–	–	–	–	3.9
S. cerevisiae	12.5	12.5	–	0.78	0.78	–	–	–	–	–	–	–	–	1.95
T. harsianum	12.5	6.25	–	12.5	25	–	12.5	6.25	–	–	–	–	–	7.81

Notes: * A – acetone extract; B – methanol extract; C – aquaeos extract.

** Minimum inhibitory concentration (MIC); values given as mg/ml for lichen extract and as μg/ml for antibiiotics. Antibiotics: K – ketaconazole, S – streptomycin.

0.78 mg/ml against *Candida albicans*, *Mucor mucedo*, and *Saccharomyces cerevisiae* to 50 mg/ml against *Aspergillus flavus*. The aqueous extract of this lichen was inactive in relation to the tested microorganisms. The lichen *Parmelia sulcata* contains atranorin and salazinic acid [20].

Among extracts of the lichen *Umbilicaria crustulosa*, the acetone and methanol extracts inhibited all of the tested bacteria except *Escherichia coli*. The MIC ranged from 3.12 to 12.5 mg/ml for the acetone extract and was 6.25 mg/ml for the methanol extract. The methanol extract exerted inhibitory action on eight species of fungi, while the acetone extract inhibited six species. The MIC against different species of fungi ranged from 12.5 to 25 mg/ml for the ethanol extract and from 6.25 to 12.5 mg/ml for the acetone extract. The aqueous extract did not inhibit any of the tested organisms. No lichen substances have been isolated to date from the lichen *Umbilicaria crustulosa* [15].

Among extracts of the lichen *Umbilicaria cylindrica*, weak antibacterial activity was manifested only by the methanol extract against *Staphylococcus aureus* (the MIC was extremely high: 50 mg/ml). Antifungal activity was recorded for the acetone and methanol extracts against *Candida albicans* (the MIC was 12.5 mg/ml for the acetone extract and extremely high: 50 mg/ml for the methanol extract). The acetone and methanol extracts of this lichen were inactive against the other fungi, while the aqueous extract was inactive against all of the tested bacteria and fungi. The species *Umbilicaria cylindrica* contains the lichen substances arabitol and mannitol [20].

As a negative control, DMSO in the employed concentrations of lichen extract solvents had no inhibiting effect on the tested organisms.

Used as a positive control, streptomycin inhibited the growth of all of the tested bacteria, while ketoconazole inhibited growth of the fungi.

Indicating differences of antimicrobial activity between lichen extracts as a function of the species of lichen and depending on the extracting solvent used, the results obtained in the present work corroborate the findings of previous studies [11, 12, 21]. The aqueous extracts did not inhibit any of the tested organisms. The probable reason for this is that the majority of active substances present in the thalli of lichens are either insoluble or poorly soluble in water [22].

Different crude extracts of the studied lichens moderately and in some cases significantly inhibited the tested microorganisms, the majority of which are pathogens of man, animals, or plants. The obtained results suggest what kinds of extracts from the investigated species of lichens possess potentially isolable components with antibacterial and antifungal properties. Naturally, if the active ingredients of different extracts of these lichens prove to be isolable, it can then be asserted that the lichens in question represent an interesting new source of bioactive substances useful as an alternative to synthetic antimicrobial medicines in treating bacterial and fungal infections and diseases.

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