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EXPERIMENTAL ARTICLES

Antioxidant and Antimicrobial Activity of Some Lichen Species[1]

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Abstract—The aim of the research is to explore the overall in vitro antioxidant activity, total phenol content, reduction power and antimicrobial activity of the methanol extracts of the lichens *Cetraria pinastri*, *Cladonia digitata*, *Cladonia fimbriata*, *Fulgensia fulgens*, *Ochrolechia parella* and *Parmelia crinita*. The methanol extract of the *Cetraria pinastri* showed a strong antioxidant activity, whereas the extracts of the species *Fulgenesi fulgens*, *Cladonia fimbriata* and *Parmelia crinita* showed the moderate one and the extract of the species *Ochrolechia parella* and *Cladonia digitata* the weak one. The methanol extract of the lichen *Cetraria pinastri* had the biggest total phenol content (32.9 mg/g of the dry extract). A certain correlation was established between the antioxidant activity and the total phenol content for the researched lichen extracts. The work also explores the antimicrobial activity of the methanol extracts of the mentioned species of lichens against six bacterial and eleven fungi species by the disc-diffusion method and by establishing the minimum inhibitory concentration (MIC). The methanol extracts of the lichens *Cetraria pinastri* and *Parmelia crinita* showed the strongest both antibacterial and antifungal activity against most of the tested microorganisms. These researches suggest that the lichens *Cetraria prunastri* can be used as new sources of the natural antioxidants and the substances with antimicrobial features.

Keywords: antimicrobial activity, antioxidant activity, methanol extract, *Cetraria pinastri, Cladonia digitata, Cladonia flmbriata, Fulgensia fulgens, Ochrolechia parella, Parmelia crinite*.

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The oxidative stress was established to be the main factor in causing many chronic and degenerative diseases including arteriosclerosis, diabetes, even a cancer [1–3]. Antioxidants, in either exogenous or endogenous way, either synthetic or natural, can be efficient in prevention of making free radicals, their release or razing [4–6] Therefore, the role of antioxidants has received increased attention during the past decade. In general, many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone, and propyl gallate are commercially available but are quite unsafe, and their toxicity is a source of concern [7]. Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant activity that may be used for human consumption. The importance of the antioxidant constituents from natural sources in keeping health of people and food production is a subject of interest of many researchers [8].

Lichens are symbiotic organisms consisting of one fungi partner and one alga or Cyanobacteria and have therapeutic effects on various diseases, and are often used in alternative medicine. Lately, certain attention has been paid to lichens as a source of natural antioxidants [9–13]. Lichens produce numerous secondary metabolites that attract big attention because of their antiviral, anti-micro bacterial, antitumor, anti- allergic, inhibitory activity of growth of plants and so on [14]. Because of growing resistance of microorganisms on existing antibiotics, their antimicrobial activity is also intensively explored [12, 15–17].

Therefore, the aim of this *in vitro* research is an assessment of the antioxidant and the antimicrobial activity of the methanol extract made of the six species of lichens that has not been explored yet: *Cetraria pinastri* (Scop.) Gray, *Cladonia digitata* (L.) Hoffm., *Cladonia flmbriata* (L.) Fr., *Fulgensia fulgens* (Sw.) Elenkin, *Ochrolechia parella* (L.)A. Massl., and *Parmelia crinite* Ach.

MATERIAL AND METHODS

Lichens

Samples of the lichens *Cetraria pinastri* (Scop.) Gray, *Cladonia digitata* (L.) Hoffm., *Cladonia flmbriata* (L.) Fr., *Fulgensia fulgens* (Sw.) Elenkin, *Ochrolechia parella* (L.) A. Massl., and *Parmelia crinite* Ach. were collected on Mounts Kopaonik and Durmitor during the summer of 2008. The demonstration samples are preserved in facilities of the Department of Biology of Kragujevac University's Faculty of Science. Determination of the investigated lichens was accomplished using standard methods [18, 19, 20, 21].

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Preparation of the methanol extract. Finely pulverized dried thally of the investigated lichens (100 g) were extracted with 1 L of methanol using a Soxchlet extractor for 7 hours at a temperature not exceeding the boiling point of the solvent. The extract was filtered using Whatman filter paper (No. 1) 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.

Antioxidant activity assay. Antioxidant activities of lyophilized lichen extracts were determined using the thiocyanate method [22]. Briefly, the sample (1 mg) in 1 mL solvent was mixed with 5 mL linoleic acid emulsion (0.02 M, pH 7.0) and 5 mL phosphate buffer (0.2 M, pH 7.0). Linoleic acid emulsion was prepared by mixing 0.5608 g of linoleic acid with 0.5608 g of Tween 20 as emulsifer, and 100 mL phosphate buffer, and the mixture was then homogenized. The reaction mixture was incubated at 37°C. Aliquots of 0.1 mL were taken at different intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding 4.7 ml ethanol (75%), 01 mL ammonium thiocyanate (30%), 0.1 mL sample solution, and 0.1 mL ferrous chloride (0.02 M, in 3.5% HCl), The mixture stood for 3 min and the peroxide value was then determined by reading the absorbance at 500 nm using a UV-visible spectrophotometer (Jenway G105, UK). A control was performed with linoleic acid but without the extract. Trolox and ascorbic acid solutions, prepared under conditions described above, were used as a positive control. The inhibition percent was calculated using the following equation:

1% = 1 - (absorbance of sample at 500 nm/absorbance of control at 500 nm) × 100.

1 = absorbence of control (blank) at 500 nm

All experiments were carried out in triplicate.

Determination of total phenolic content. Total phenolic content in the lichen extracts was determined with Folin-Ciocalteu reagent according to the method of [23] using gallic acid as a standard. In order to determine the total phenol content in the lichens by Folin-Ciocalteu assay, gallic acid and pyrocatechol are used as usual standards [3, 10–13, 24]. We have chosen the gallic acid, since it was structurally more similar to the lichen acids. An extract of 1 mL (contains 5 mg of dried extracts) was mixed with 1 mL of Folin-Ciocalteu reagent. The mixture was shaken vigorously and allowed to stand at room temperature for 5 min before the addition of 2 mL of 20% Na_2CO_3 was added and allowed to stand for 2 h with intermittent shaking. The absorbence was measured at 760 nm. The concentration of total phenolic compounds in the extract was determined as milligrams of gallic acid equivalent per gram of the dry extract. All experiments were carried out in triplicate.

Reducing power. The total reducing power of lichen extract was determined according to the method of [25]. Briefly, 1 mL of of the metanolic lichen extracts were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium fericyanide $[K_3Fe(CN)_6]$ (1%). The mixtura was then kept in a 50°C water bath for 20 minutes. The resulting solution was then cooled rapidly, spiked with 2.5 mL of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. The supernatant (5 mL) was then mixed with 5 mL of destilled water and 1 mL of 0.1% ferric chloride ($FeCl_3$). The absorbance of 700 nm was then detected after reaction for 10 minutes. The higher the absorbence represents the stronger the reducing power. The assay were carried out in triplicate and the results were expressed as mean values ± standard deviations. All experiments were carried out in triplicate.

Antimicrobial activity. ***Microbial Strains.*** The bacteria used in this study were *Enterococcus fecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 70063), *Micrococcus lysodeikticus* (ATCC 10259), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923). The fungi used as test organisms were: *Alternaria alternate* (ATCC 13963), *Aspergillus flavus* (ATCC 9170), *Aspergillus niger* (ATCC 6275), *Candida albicans* (ATCC 10259), *Cladosporium cladosporioides* (ATCC 13276), *Paecilomyces variotii* (ATCC 22319), *Acremonium chrysogenum* (DBFS 401), *Fusarium oxysporum* (DBFS 292), *Penicillium verrucosum* (DBFS 418) and *Trichoderma harsianum* (DBFS 379). These were obtained from the mycological collection maintained by the mycologicai laboratory within the Department of Biology of were University's Faculty of Science. Bacterial cultures were maintained on Miiller-Hinton agar substrates (Torlak, Belgrade). All cultures were stored at 4 C and sub-cultured every 15 days.

The sensitivity of microorganisms to methanol 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 MIC.

Inoculums were obtained from bacterial cultures incubated for 24 h at 37°C and diluted according to the 0.5 McFarland standard to approximately 10^8 colony-forming units (cfu)/mL.

Suspensions of fungal spores were prepared from fresh mature (three to seven days old) cultures that grew at 30°C on a PDA substrate. Spores were rinsed with sterile distilled water, used to determine turbidity using spectrophotometry at 530 nm, and then diluted to approximately 10^6 cfu/mL according to the procedure recommended by the National Committee for Clinical Laboratory Standards [26].

Disk-diffusion assay. The dried plant extracts were dissolved in methanol to a final concentration of 30 mg/mL and sterilized by filtration through 0.45 μm Millipore filters. Antimicrobial tests were then carried out by the disk diffusion method [27] using 100 μL of suspension containing 10^8 cfu/mL of bacteria, 10^6 cfu/mL of fungi spread on Muller-Hinton agar

Table 1. Antioxidant activity, total phenolic content and reducing power of the lichens *Cetraria pinastri*, *Cladonia digitata*, *Cladonia fimbriata*, *Fulgensia fulgens*, *Ochrolechia parella* and *Parmelia crinita*

Lichen	Antioxidant activity[a]		Total phenolic content[a]	Reducing power[a]
	% Inhibition	Mean abs. at 500 nm (48 h)	(mg gallic acid/g dry extract)	(Abs. 700 nm)
Cetraria pinastri	48.79	0.635 ± 0.047	32.9 ± 1.148	0.188 ± 0.028
Cladonia digitata	9.75	1.119 ± 0.052	13.7 ± 1.236	0.085 ± 0.022
Cladonia fimbriata	20.40	0.987 ± 0.030	26.4 ± 1.180	0.114 ± 0.007
Fulgensia fulgens	21.61	0.972 ± 0.058	12.4 ±1.255	0.136 ± 0.024
Ochrolechia parella	8.5	1.134 ± 0.058	5.6 ± 1.112	0.077 ± 0.012
Parmelia crinita	16	1.040 ± 0.036	12.8 ± 1.302	0.172 ± 0.018
Trolax	99.60	0.005 ± 0.001	–	–
Ascorbic acid	35.01	0.805 ± 0.058	–	–
Control	–	1.240 ± 0.018	–	–

[a] The values are presented as mean ± SD.

(for bacteria) or SD agar (for fungi). The disks (6 mm in diameter) impregnated with 10 μL of 10 μL of the methanol solution of the dried plant extracts (300 μg/disk) were placed on the inoculated agar. Streptomycin (for bacteria) and ketoconazole (for fungi) were used as controls. Negative controls were prepared with the same solvents used to dissolve the plant extracts. One strain/isolate in each bacterial species tested. The inoculated plates were incubated at 37°C for 24 h for bacterial strains, and 72 h for fungi isolates. Plant-associated microorganisms were incubated at 27°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay was repeated thrice.

Minimal Inhibitory Concentration. The MIC was determined by the broth tube dilution method. A series of dilutions with concentrations ranging from from 30 to 0.12 mg/ml was used in the experiment for each extract against all microorganism tested. The initial concentration of 30 mg/ml was obtained by measuring a quantity of extract and disolved it in DMSO. Two-fold dilutions of extracts were prepared in Muller-Hinton for bacterial cultures and in SD broth for fungal cultures.

The MIC was determined by establishing visible growth of the microorganisms. The last dilution to show no visible growth was defined as the MIC for the tested microorganism at the given lichen extract concentration. Streptomycin and ketoconazole were used as positive controls. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Antioxidant activity. The antioxidant activity of the methanol extracts of the lichens *Cetraria pinastri, Cladonia digitaty, Cladonia flmbriata, Fulgensia fulgens, Ochrolechia parella* and *Parmelia crinite* was researched by peroxidation of the linoleic acid. The results of the researched activity were shown in the Table 1 as a percent (%) of the inhibition. Trolox and ascorbic acid were used as positive controls. The oxidation of the linoleic acid was inhibited by all the tested lichen extracts. The methanol extract of the researched species of the lichens showed a various degree of the antioxidant activity. The methanol extract of the lichen *Cetraria pinastri* showed a relatively strong antioxidant activity with 48.79% of the inhibition of the linoleic acid, whereas somewhat weaker activity was shown in the species *Fulgensia fulgens* (21.61%) and *Cladonia fimbriata* (20.40%) with the very weak activity in *Ochrolechia parella* (8.5%). The total content of the phenol was given as the equivalent of the gallic acid and was also shown in the Table 1. The extract of the lichen *Cetraria pinastri* had the highest total phenol content, followed by the *Cladonia flmbriata, Cladonia digitata, Parmelia crinite* and *Fulgensia fulgens* with similar content, whereas the lichen *Ochrolechia parella* had an extremely low total content of the phenol. The results of the experiment of the reduction power showed that the biggest reduction power had been in the methanol extract of the lichen *Cetraria pinastri*. It was established that there was a certain connection between the values of the antioxidant activity and the total phenol content of the methanol extracts of the researched species of lichens. Since depsides, depsidones and dibenzofurans are characteristic phenol components that lichens synthesize [11, 28] that goes in favour of this research, where an important correlation was noticed between the total phenol content and the antioxidant activity and reduction power. A correlation like this in the researched extracts of other lichen species is not always present. Given that the antioxidant activity can be a feature of non-phenol components as well, certain phenols can have a different antioxidant activity [11].

Antimicrobial activity. The antimicrobial activity of the methanol extracts of the lichens *Cetraria pinastri,*

Table 2. Antibacterial activities of methanolic extracts of *Cetraria pinastri*, *Cladonia digitata*, *Cladonia fimbriata*, *Fulgensia fulgens*, *Ochrolechia parella* and *Parmelia crinita* against the organisms tested based on disk-diffusion and Broth tube dilution assay

Organisms	*Ent. faecalis*		*E. coli*		*K. pneumoniae*		*M. lysodeikticus*		*P. aeruginosa*		*S. aureus*	
Lichen	DD[a]	MIC[b]	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
C. pinastri	27[c]	0.23[d]	17	1.87	22	0.94	28	0.46	25	0.94	27	0.94
Cl. digitata	8	15	–	–	–	–	10	30	12	15	–	–
Cl. fimbriata	14	7.5	–	–	15	7.5	17	7.5	18	3.75	10	7.5
F. fulgens	17	3.75	10	7.5	14	3.75	20	1.87	22	1.87	17	3.75
O. parella	–	–	–	–	–	–	10	7.5	8	15	–	–
P. crinita	26	0.94	15	3.75	20	0.94	28	0.94	22	0.94	20	1.87
Antibiotic (S)[c]	24	7.5	20	30	22	1.87	27	1.87	31	1.87	15	30

[a] DD = Diameter of disc diffusion (mm); Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 300 μg/disc of metanol extract and 30 μg/ml for antibiotics. Values are the mean of three replication.

[b] Minimal inhibitory concentration (MIC); values given as mg/ml for lichen extract and as μg/ml for antibiotics.

[c] Antibiotic (S) = Streptomycin μg/ml.

Cladonia digitata., Cladonia flmbriata, Fulgensia fulgens, Ochrolechia parella and Parmelia crinite was analyzed against seventeen tested microorganisms by the disc-diffusion method (by measuring the diameter of the inhibited zones) and MIC values. The results are shown in the Tables 2 and 3 and show that the methanol extract of the lichen *Cetraria pinastri* has the strongest antimicrobial activity. It inhibited the growth of all the tested species of bacteria and fungi. With zones of inhibition comparable to those produced by the streptomycin standard. Values of the MIC ranged from 0.23 for the bacteria *Enterococcus fecalis* to 1.87 mg/mL for *Escherichia coli*. In relation to the fungi tested, values of the MIC ranged from 0.94 mg/mL for *Candida albicans* to 15 mg/mL for *Penicillium verrucosum*.

The methanol extract of the lichen *Parmelia crinite* also inhibited the growth of all the tested bacteria with the zones of inhibition from 28 mm for *Micrococcus lysodeikticus* to 15 mm for *Escherichia coli* and relatively low MIC values from 0.94 to 3.75 mg/mL of the extract for the same microorganisms. Related to the fungi, this extract had a selective activity, inhibiting the growth in 9 out of 11 tested species with the diameter of the inhibited zones ranging from 11 to 18 mm. The MIC values were from 3.75 to 30 mg/mL of the extract related to the sensitive microorganisms.

The methanol extract of the lichen *Fulgensia fulgens* had a moderate antimicrobial activity, inhibited the growth of all tested species of bacteria and six out of eleven tested species of fungi.

The methanol extract of the lichen *Cladonia flmbriata* manifested relatively strong antibacterial activity, inhibiting five out of six tested species of bacteria. *E. coli* showed the greatest resistance to the investigated extract. The extract of the lichen showed selective antifungal activity, inhibiting only 5 out of 11 tested species. The values of the MIC ranged from 3.75 to 7.5 mg/mL of the extract related to the bacteria tested and from 3.75 to 15 mg/mL of extract in relation to the fungi.

The methanol extract of the lichen *Cladonia digitata* inhibiting three out of six tested species of bacteria, and 5 out of 11 tested species. The values of the MIC ranged from 3.75 to 15 mg/mL of extract in related to the bacteria tested and from 3.75 to 30 mg/mL of extract in relation to the fungi.

The methanol extract of the lichen *Ochrolechia parella* showed the weakest antibacterial activity, inhibiting three out six tested species of bacteria, and it had even a weaker influence on fungi, inhibiting only one out of 11 tested species with small values of the diameter of the inhibition of growth of the sensitive microorganisms (8–12 mm) and the high MIC values up to 30 mg/mL of the extract.

DMSO as a negative control in the used concentration of the solvent of the dry lichen extracts didn't have the inhibiting effect on the tested organisms. Streptomycin, used as a positive control, inhibited growth of all the tested bacteria, and ketoconazole of fungi.

In order to study the antimicrobial activities of lichen compounds, methanol extracts were used. The water extracts showed the antimicrobial activity in our research of the antimicrobial activity of different lichen extracts in few trials, although more than two hundered species of lichens were included in the research [17, 29]. Similar results were obtained by other researches as well. The most probable antimicrobial components of lichens are depsides, depsidones, dibenzofurans and other, not soluble in water, but soluble in methanol, in acetone... etc. Some of them we had isolated from certain species of lichens in our previous study [30]. The lichen *Cetraria pinastri* has the usnic acid and pinastric acid; the *Cladonia digitata* contains the thamnolic acid and rhodocladonic acid; the *Cladonia flmbriata* contains the antranorin

Table 3. Antifungal activities of methanolic extracts of *Cetraria pinastri*, *Cladonia digitata*, *Cladonia fimbriata*, *Fulgensia fulgens*, *Ochrolechia parella* and *Parmelia crinita* against the organisms tested based on disk-diffusion and broth tube dilution assay

Organisms	*A. ch*		*A. al*		*A. fl*		*A. ng*		*C. al*		*C. cl*		*F. o*		*M. m*		*P. v*		*P. ver*		*T. h*	
Lichen	DD[a]	MIC[b]	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
C. pinastri	18	3.75	22	1.87	20	7.5	18	3.75	25	1.87	27	0.94	18	7.5	20	7.5	12	15	16	15	24	3.75
Cl. digitata	–	–	8	15	–	–	10	30	–	–	12	15	–	–	–	–	–	–	–	–	–	–
Cl. fimbriata	–	–	12	7.5	–	–	13	15	18	7.5	15	7.5	–	–	–	–	18	7.5	–	–	–	–
F. fulgens	–	–	14	3.75	7	30	–	–	16	7.5	19	7.5	12	30	–	–	12	15	–	–	–	–
O. parella	–	–	–	–	–	–	–	–	–	–	12	30	–	–	–	–	–	–	–	–	–	–
P. crinita	11	7.5	15	3.75	12	7.5	–	–	14	3.75	18	3.75	12	15	12	30	14	30	–	–	17	15
Antibiotic (K)[c]	17	30	28	3.75	27	3.75	34	3.75	40	1.87	44	1.87	35	3.75	17	30	40	1.87	36	3.75	18	7.5

[a] DD = Diameter of disc diffusion (mm); Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 300 μg/disc of metanol extract and 30 μg/ml for antibiotics. Values are the mean of three replication.

[b] Minimal inhibitory concentracion (MIC); values given as mg/ml for lichen extract and as μg/ml for antibiotics.

[c] Antibiotic (K) = Ketoconazole μg/ml.

A. ch – Acremonium chrysogenum; *A. al – Alternaria alternata*; *A. fl – Aspergillus flavus*; *A. ng – Aspergillus niger*; *C. al – Candida albicans*; *C. cl – Cladosporium cladosporioides*; *F. o – Fusarium oxysporum*; *M. m – Mucor mucedo*; *P. v – Paecilomyces variotii*; *P. ver – Penicillium verrucosum*; *Ò. h – Trichoderma harsianum*.

and fumaprotocetraric acid; the *Fulgensia fulgens* has the parietin (sometimes as physcion); the *Ochrolechia parille* contains the variolaric acid; the *Parmelia crinite* contains atranorin and stictic acid [28].

The results of the antimicrobial activity of the methanol extracts showed a relatively strong inhibitory activity, depending on the sort of lichens, related to the tested bacteria and fungi. In many authors' researches of the antimicrobial activity of the extracts of lichens there are either similarities or differences comparing with our results, which depends on species of lichen, extracting solvent used, and the concentration of the lichen extract. [15] got similar results, researching the antifungal activity of the lichen extracts related to fitopatogenous fungi. Gulluce et al. [12], Rankovic et al. [17] established that the methanol extracts had a bigger antibacterial than antifungal activity and they have a stronger influence on Gram-positive than on Gram-negative bacteria. The reason for different sensitivity between Gram-positive and Gram-negative bacteria could be attributed to the morphological differences between the organisms, before all in differences of porousness of the cell wall [31]. Canden et al. [16] established an antimicrobial activity of different extracts of the lichen *Xanthoparmelia pokornyi* against the bacteria and yeasts but not against filamentous fungi. In contrast, in Shahi *et al.,* [32] based on the present results, it can be noticed that the methanol extract of the species *Cetraria pinastri* showed the strongest antimicrobial activity related to the tested species of lichens. Our results show that the tested extracts showed a strong but different antimicrobial activity. That suggests separating of active components from the extracts of the researched species of lichens and their antimicrobial research as well as their possible use in preservation of food and the pharmaceutical industry. Since taluses of some lichens, including the three researched species from this work (*Cetraria pinastri, Fulgensia fulgens*, and *Cladonia fimbriata*), have a bigger percent of the phenol components which were proven to have numerous biological effects including the antioxidant activity, they can be of a big importance in the food industry, given the fact that they keep the oxidative processes, that way improving the quality and its nutritional value, so they can be used as additives. The antioxidant constituents from the taluses of the researched lichens can be used in preventing and treatment of some diseases caused by the oxidative stress.

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