

EVALUATION OF ANTIOXIDANT, ANTIMICROBIAL AND ANTICANCER PROPERTIES OF SELECTED LICHENS FROM SERBIA

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In the present investigation the methanol extracts of the lichens *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla* were tested for antioxidant, antimicrobial and anticancer potential. Antioxidant activity was evaluated by five separate methods: free radical scavenging, superoxide anion radical scavenging, reducing power, determination of total phenolic compounds and determination of total flavonoid content. Of the lichens tested, *Umbilicaria polyphylla* had largest free radical scavenging activity (90.08% inhibition). Moreover, the tested extracts had effective reducing power and superoxide anion radical scavenging. Total content of phenol and flavonoid in extracts were determined as pyrocatechol equivalent, and as rutin equivalent, respectively. The strong relationships between total phenolic and flavonoid contents and the antioxidant effect of tested extracts were observed. The antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method against six species of bacteria and ten species of fungi. The most active was extract of *Umbilicaria polyphylla* with minimum inhibitory concentration values ranging from 0.78 to 1.56 mg/mL. Cytotoxic activity was tested against colon cancer adenocarcinoma cell line HTC-116 using MTT method. All extracts were found to be strong cytotoxic activity toward cell line with IC₅₀ values ranging from 14.77 to 497 µg/mL. The present study shows that tested lichen extracts demonstrated a strong antioxidant, antimicrobial and cytotoxic effects. That suggest that lichens may be used as possible natural antioxidant, antimicrobial and anticancer agents and also, could be of significance for different applications in the food industry and to control various diseases.

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1. Introduction

Continuous and uncontrolled use of synthetic drugs has led to the need to find new preparations of natural origin in the control and prevention of various human, animal and plant diseases. It is known that long-term use of synthetic drugs often causes numerous side effects and sometimes resistance [1]. Unlike synthetic drugs, bioactive natural products have a beneficial effect on the whole organism and without causing unwanted effects. In search of new bioactive preparations of natural origin, lichens are the subject of many research teams.

Lichens are symbiotic organisms consisting of algae and fungi, and are important constituents of many ecosystems. They usually grow on rocks, non-fertile ground, and can also exist as epiphytes on the trees and leaves [2]. These organisms are used for human nutrition, animal nutrition, for getting colours, perfumes and alcohol. Lichens have also, for hundreds of years, been used in many contry as a cure for diseases of humans. For example, *Lobaria pulmonaria* and *Parmelia sulcata* have been used in the treatment of pulmonary and cranial diseases, respectively. Similarly, *Xanthoria parietina* was used to cure jaundice and *Letharia vulpina* in stomach diseases [3-5]. The usage of some lichens for many years in the traditional

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medicine was later justified by numerous researches that confirmed their various biological activity.

Lichens produce secondary metabolites the “lichen substances”, which comprise depsides, depsidones, dibenzofurans, xanthenes and terpene derivatives. These metabolites sometimes make up more than 30% of the dry mass of talus. Lichens and their metabolites have manifold biological activity: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory, antiherbivore, ecological roles and enzyme inhibitory [3,6,7]. The present study describes an evaluation of the antioxidant, antimicrobial and cytotoxic activities of the methanol extract of the lichens *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla*.

2. Experimental

2.1 Lichen samples

Lichen samples of *Lecanora muralis* (Schreber) Rabenh, *Parmelia saxatilis* (L.) Ach, *Parmeliopsis ambigua* (Wulf.) Nyl, *Umbilicaria crustulosa* (Ach.) Frey and *Umbilicaria polyphylla* (L.) Baumg, were collected from Kopaonik, Serbia, in September 2010. The samples were preserved in facilities of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. Identification of the investigated lichens was accomplished using standard methods.

2.2 Preparation of the lichen extracts

Extracts from finely ground dry thalli of the lichens (50 g) were obtained using methanol in a Soxhlet extractor. The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at -18°C until use in the tests. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for the experiments. All experiments were performed in triplicate.

2.3 Antioxidant activity

2.3.1 Scavenging DPPH radicals

The free radical scavenging activity of lichen extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH). The method used is a modified version of that described by Ibanez et al. and Dorman et al. [8,9]. Two milliliters of methanol solution of DPPH radical in the concentration of 0.05 mg/mL and 1 mL of plant extract (1 mg/mL) were placed in cuvettes. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a spectrophotometer (“Jenway” UK). Ascorbic acid, butylated hydroxyanisole (BHA) and α -tocopherol were each used as positive controls. The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = ((A_0 - A_1) / A_0) \times 100$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of reaction mixture or standards

2.3.2 Reducing power

The reducing power of extracts was determined according to the method of Oyaizu [10]. One milliliter of extract (1 mg/mL) was mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. Then, trichloroacetic acid (10%, 2.5 mL) was added and the mixture centrifuged. Finally the upper layer was removed and mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL; 0.1%). The absorbance of the solution was measured at 700 nm using the spectrophotometer (“Jenway” UK). Higher absorbance of the reaction mixture indicated that the reducing power is increased. Ascorbic acid, butylated hydroxyanisole (BHA) and α -tocopherol were each used as positive controls.

2.3.3 Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of lichen extract was detected according to the method of Nishimiki et al. [11]. Briefly, 0.1 mL of extracts (1 mg/mL) was mixed with 1 mL nitroblue tetrazolium (NBT) solution (156 μ M in 0.1 M phosphate buffer, pH 7.4) and 1 mL NADH solution (468 μ M in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 μ L of phenazine methosulphate (PMS) solution (60 μ M in 0.1 M phosphate buffer, pH 7.4). The mixture was incubated at room temperature for 5 min, and the absorbance was measured at 560 nm using the spectrophotometer ("Jenway" UK) against blank samples. Decreased absorbance indicated increased superoxide anion radical scavenging activity. Ascorbic acid, butylated hydroxyanisole (BHA) and α -tocopherol were used as positive controls. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging activity (\%)} = ((A_0 - A_1)/A_0) \times 100$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of reaction mixture or standards.

2.3.4 Determination of total phenolic compounds

Total soluble phenolic compounds in the lichen extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton [12], using pyrocatechol as a standard phenolic compound. Briefly, 1 mL of the lichen extract (1 mg/mL) in a volumetric flask was diluted with distilled water (46 mL). One milliliter of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min 3 mL of Na_2CO_3 (2%) was added and then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in spectrophotometer ("Jenway" UK). The total concentration of phenolic compounds in the extract determined as microgram of pyrocatechol equivalent (PE) per milligram of dry extracts by using an equation that was obtained from a standard pyrocatechol graph as follows:

$$\text{Absorbance} = 0.0021 \times \text{total phenols } [\mu\text{g PE/mg of dry extracts}] - 0.0092$$

$$(R^2 = 0.9934)$$

2.3.5 Total flavonoid content

The total flavonoid content was determined using the Dowd method [13]. Two milliliters of 2 % aluminium trichloride (AlCl_3) in methanol was mixed with the same volume of the extract solution (1 mg/mL). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in spectrophotometer ("Jenway" UK) against blank samples. The total flavonoid content was determined as microgram of rutin equivalent (RE) per milligram of dry extracts by using an equation that was obtained from a standard rutin graph as follows:

$$\text{Absorbance} = 0.0144 \times \text{total flavonoid } [\mu\text{g RE/mg of dry extracts}] + 0.0556$$

$$(R^2 = 0.9992)$$

2.4 Antimicrobial activity

2.4.1 Microorganisms and media

The following bacteria were used as test organisms in this study: *Bacillus mycoides* (IPH 197), *Staphylococcus aureus* (IPH 221), *Enterobacter cloacae* (IPH 241), *Escherichia coli* (IPH 246) and *Klebsiella pneumoniae* (IPH 251). All the bacteria used were isolates of the Institute for Protection of Health in Kragujevac (IPH) and the Faculty of Agriculture in Belgrade (FAB). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Botrytis cinerea* (DBFS 133), *Candida albicans* (IPH 1316), *Mucor mucedo* (ATCC 52568) and *Penicillium verrucosum* (DBFS 262). 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 (PD) agar and Sabourad dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4°C and subcultured every 15 days.

The sensitivity of microorganisms to extracts of the investigated species of lichens was tested by determining the minimal inhibitory concentration (MIC), as explained below.

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37°C on Müller-Hinton agar substrate and diluted 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 PD agar 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 NCCLS [14].

2.4.2 Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method using 96-well micro-titer plates [15]. A series of dilutions with concentrations ranging from 50 to 0.195 mg/mL for extracts was used in the experiment against every microorganism tested. The starting solutions of extracts 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. The minimal inhibitory concentration was determined with resazurin. 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 boundary dilution without any changing color of resazurin was defined as the minimal inhibitory concentration (MIC) for the tested microorganism at the given concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria, ketoconazole in the case of fungi. A DMSO solution was used as a negative control for the influence of the solvents.

2.5 Cytotoxic activity

2.5.1 Cell lines

The colon cancer adenocarcinoma cell line HCT-116 was obtained from the American Tissue Culture Collection (Manassas, VA, USA). These cells were propagated and maintained in DMEM (Dulbecco's Modified Eagle Medium), (Gibco, USA) and supplemented with 10% fetal bovine serum (PAA), antibiotics 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were grown in 75 cm² culture bottles supplied with 15 mL DMEM, and after a few passages cells were seeded in 96-well plates. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

2.5.2 Determination of cell survival (MTT test)

After 24 and 72 h of treatment the cell viability was determined by MTT assay. The proliferation test is based on the color reaction of mitochondrial dehydrogenase from living cells with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, USA). HCT-116 cells were seeded in a 96-well plate (10^4 cells per well). After 24 h incubation, cells were treated with 100 µL of each concentrations of lichen extracts (in concentration of 50-1000 µg/mL) for 24 and 72 h. Untreated cells served as a control. At the end of the treatment period, MTT (final concentration 5 mg/mL PBS) was added to each well, which was then incubated at 37 °C in 5% CO₂ for 2 h. The colored crystals of produced formazan were dissolved in DMSO. The absorbance was measured at 550 nm. Cell proliferation was calculated as a ratio of the absorbance of the treated group divided by the absorbance of control group, multiplied by 100 to give percentage proliferation [16].

2.5.3 Fluorescence microscopic analysis of cell death (AO/EB) double staining

For analysis of cell death, we used fluorescent assays Acridine orange/ethidium bromide (AO/EB) double staining. Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. We distinguished four types of cells according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. (1) Viable cells have uniform bright green nuclei with organized structure, also have orange cytoplasm. (2) Early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments. (3) Late apoptotic cells have orange to red nuclei with

condensed or fragmented chromatin. (4) Necrotic cells have a uniformly orange to red nuclei with organized structure [17].

HCT-116 cells were seeded in 6-well plates (3×10^4 cells per well). After 24 h incubation, cells were treated with 2 mL (250 $\mu\text{g/mL}$) of each of the lichen extracts for 24 and 72 h. Untreated cells served as a control. Incubation was performed at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity. After 24 and 72 h of treatment, 200 μL of dye mixture (100 μL of 100 mg/mL AO and 100 μL of 100 mg/mL EB in distilled water) were added to each well. The suspension was immediately (fast uptake) examined by fluorescence microscopy (NICON Eclipse Ti) at 400x magnification. A minimum of 300 cells was counted in every sample.

2.6 Statistical analyses

Statistical analyses were performed with the EXCEL and SPSS software packages. To determine the statistical significance of antioxidant activity, Student's t-test was used. Pearson's bivariate correlation test was carried out to calculate correlation coefficients (r) between the content of total phenolic and flavonoid and the DPPH radical scavenging activity, reducing power and superoxide anion radical scavenging. All values are expressed as mean \pm SD of three parallel measurements.

3. Results

3.1 Antioxidant activity

The scavenging of DPPH radicals of the studied lichen extracts is shown in Figure 1. Methanol extracts of the tested lichen showed a good scavenging activity on DPPH radical. There was a statistically significant difference between extracts and control ($P < 0.05$). The scavenging effects of all lichen extracts were 55.55–90.08%. Extracts from lichen *Umbilicaria polyphylla* showed the largest DPPH radical scavenging activity (90.08%), greater than standard antioxidants. The scavenging activity was also good for the lichens *Umbilicaria crustulosa* (79.85%) and *Parmeliopsis ambigua* (73.66%). The lichens *Lecanora muralis* and *Parmelia saxatilis* showed a slightly weaker activity DPPH radical scavenging activities (61.28% and 55.55%, respectively).

The results of the reducing power assay of lichen extracts are summarized in Figure 2. High absorbance indicates high reducing power. Measured values of absorbance varied from 0.066 to 0.089. Among the tested lichen species, *Parmeliopsis ambigua* gave the highest reducing power. The reducing power in the methanol lichen extracts decreased in the following order: *Parmeliopsis ambigua* > *Lecanora muralis* > *Parmelia saxatilis* > *Umbilicaria polyphylla* > *Umbilicaria crustulosa*.

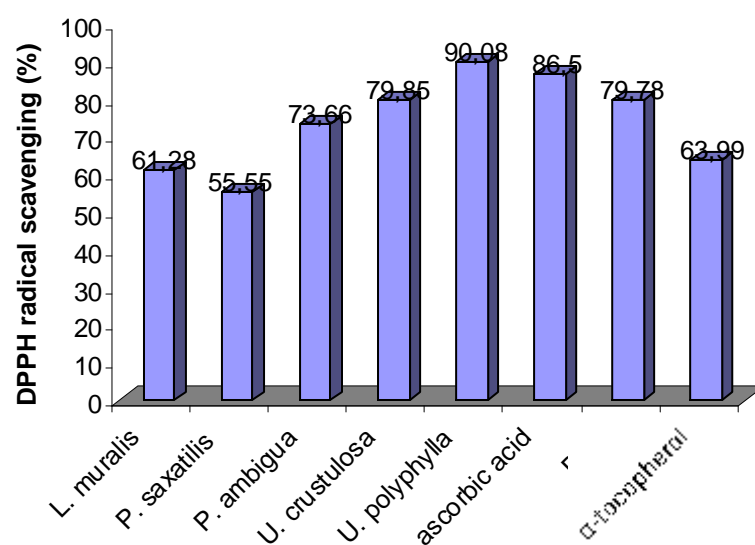


Fig. 1. DPPH radical scavenging of methanol extracts of the lichens *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla*

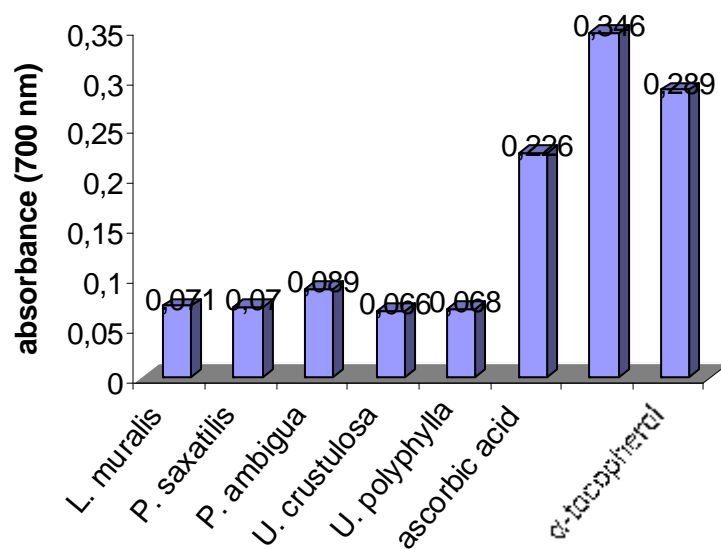


Fig. 2. Reducing power of methanol extracts of the lichens *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla*

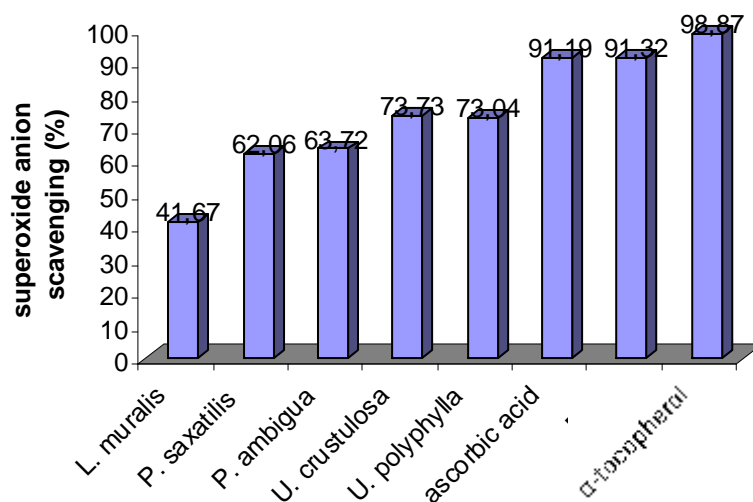


Fig. 3. Superoxide anion scavenging of methanol extracts of the lichens *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla*

Results of superoxide anion scavenging activities of tested extracts are shown in Figure 3. All extracts revealed a relatively good superoxide anion scavenging activity. The superoxide anion scavenging activity for different lichens was within the range 41.67– 73.73%. There was a statistically significant difference between extracts and control ($P < 0.05$). Maximum scavenging activity (73.73%) was in the methanol extracts of the lichen *Umbilicaria crustulosa*. Methanol extract of lichen *Lecanora muralis* demonstrated weakest superoxide anion scavenging activity (41.67%).

Total phenolic and flavonoid constituents of tested extracts are given in Table 1.

Table 1. Total phenolics and flavonoid content of methanol extracts of *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla*

Lichen species	Phenolics content ($\mu\text{g PE/mg}$ of extract)	Flavonoid content ($\mu\text{g RE/mg}$ of extract)
<i>L. muralis</i>	50.93 ± 1.191	39.91 ± 1.066
<i>P. saxatilis</i>	53.08 ± 1.269	20.77 ± 1.113
<i>P. ambigua</i>	52.67 ± 1.211	33.92 ± 1.195
<i>U. crustulosa</i>	55.03 ± 1.096	41.52 ± 1.168
<i>U. polyphylla</i>	90.09 ± 1.176	47.24 ± 1.213

The amount of total phenolic compounds was determined as the pyrocatechol equivalent using an equation obtained from a standard pyrocatechol graph ($y = 0.0021 \times \text{total phenols } [\mu\text{g PE/mg of dry extracts}] - 0.0092$, $R^2 = 0.9934$). Highest phenolic compounds was identified in methanol extract of *Umbilicaria polyphylla* at a $90.09 \mu\text{g PE/mg}$ while methanol extracts of *Lecanora muralis* showed the lowest content at $50.93 \mu\text{g PE/mg}$.

The amount of total flavonoid compounds was determined as the rutin equivalent using an equation obtained from a standard rutin graph ($y = 0.0144 \times \text{total flavonoid } [\mu\text{g RE/mg of dry extracts}] + 0.0556$, $R^2 = 0.9992$). The total flavonoid content for methanol extracts of *Lecanora*

muralis, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla* were 39.91, 20.77, 33.92, 41.52 and 47.24 µg RE/mg, respectively.

The lichens with the highest radical scavenging activity also had the greatest quantity of phenolic and flavonoid contents. The correlation coefficient between phenolic and flavonoid compounds of the tested extracts and free radical scavenging activity were $r=0.757$ and $r=0.804$, respectively.

Various antioxidant activities (DPPH radical scavenging, superoxide anion radical scavenging and reducing power) were compared to the activities of standard antioxidants ascorbic acid, butylated hydroxyanisole (BHA) and α -tocopherol. The results showed that standard antioxidants had stronger activity than tested extracts in terms of superoxide anion scavenging and reducing power, but *Umbilicaria polyphylla* had greater DPPH radical scavenging than all three standard antioxidants

3.2 Antimicrobial activity

The antimicrobial activity of the tested lichen extracts against the tested microorganisms was shown in the Table 2.

Table 2. Minimum inhibitory concentration (MIC) of methanol extracts of *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla*

Lichen species	<i>L. muralis</i>	<i>P. saxatilis</i>	<i>P. ambigua</i>	<i>U. crustulosa</i>	<i>U. polyphylla</i>	S	-	K
<i>B. mycoides</i>	1.56 ^a	1.56	1.56	6.25	0.78	7.81	-	-
<i>E. cloacae</i>	1.56	1.56	3.12	6.25	0.78	1.95	-	-
<i>E. coli</i>	-	-	-	-	1.56	31.25	-	-
<i>K. pneumoniae</i>	1.56	3.12	3.12	6.25	0.78	1.95	-	-
<i>S. aureus</i>	3.12	1.56	1.56	6.25	1.56	31.25	-	-
<i>A. flavus</i>	25	12.5	12.5	12.5	1.56	-	-	3.9
<i>B. cinerea</i>	1.56	6.25	6.25	-	1.56	-	-	1.95
<i>C. albicans</i>	1.56	6.25	6.25	-	1.56	-	-	1.95
<i>M. mucedo</i>	6.25	6.25	6.25	12.5	1.56	-	-	3.9
<i>P. verrucosum</i>	6.25	12.5	12.5	12.5	1.56	-	-	3.9

^aMinimum inhibitory concentration (MIC); values given as mg/mL for lichen extract and as µg/mL for antibiotics. Values are the mean of three replicate

Antibiotics: K – ketoconazole, S – streptomycin

The maximum antimicrobial activity was found in the methanol extracts of the lichen *Umbilicaria polyphylla*, which, in relatively low concentrations inhibited the tested bacteria and fungi. The MIC for bacteria ranged from 0.78 mg/mL against *Bacillus mycoides*, *Enterobacter cloacae* and *Klebsiella pneumoniae* to 1.56 mg/mL against *Escherichia coli* and *Staphylococcus aureus*. This lichen inhibited all of the tested fungi in concentration of 1.56 mg/mL

Extracts of the lichen *Lecanora muralis* manifested relatively strong antimicrobial activity. The MIC was 1.56 to 3.12 mg/mL against the tested bacteria, excepting *Escherichia coli*, which was resistant, while MIC for fungi ranged from 1.56 mg/mL against *Botrytis cinerea* and *Candida albicans* to 25 mg/mL against *Aspergillus flavus*

The lichen *Parmelia saxatilis* manifested a good antimicrobial effect. The MIC was 1.56 to 3.12 mg/mL for the bacteria and 6.25 to 25 mg/mL for the fungi. The *Escherichia coli* was resistant.

Extracts of the lichen *Parmeliopsis ambigua* manifested relatively strong antimicrobial activity. The MIC was 1.56 to 3.12 mg/mL for tested bacteria, excepting *Escherichia coli*, which was resistant, while MIC for fungi ranged from 6.25 to 12.5 mg/mL.

The lichen *Umbilicaria crustulosa* showed a slightly weaker antimicrobial activity. The MIC was 6.25 mg/mL against four species of bacteria and 12.5 mg/mL against three species of fungi. The microorganisms *Escherichia coli*, *Botrytis cinerea* and *Candida albicans* were resistant.

The antimicrobial activity was compared with the standard antibiotics, streptomycin (for bacteria) and ketoconazole (for fungi). The results showed that standard antibiotics had stronger activity than tested extracts as shown in Table 2. In a negative control, DMSO had no inhibitory effect on the tested organisms.

3.3 Cytotoxic activity

The cytotoxic activity of the studied lichen extracts on the tested cell line HCT-116 line was shown in the Table 3. Our results indicate that there was inhibition of cell growth in a dose-dependent and time-dependent manner. The tested extracts showed better activity at higher concentration. The longer time exposure induced higher cell sensitivity.

Table 3. Growth inhibitory effects of methanol extracts of *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla* on HCT-116 cell line

Lichen species	24 h	72 h
	IC ₅₀ (µg/mL)	
<i>L. muralis</i>	331.11 ± 1.62	286.81 ± 1.77
<i>P. saxatilis</i>	96.86 ± 0.18	14.77 ± 0.96
<i>P. ambigua</i>	299.58 ± 2.58	280.43 ± 0.66
<i>U. crustulosa</i>	401.44 ± 0.35	497.66 ± 0.31
<i>U. polyphylla</i>	194.41 ± 1.89	153.96 ± 1.97

The tested lichen extracts manifested a strong cytotoxic activity against target cell *in vitro*. The inhibition concentration at 50 % inhibition (IC₅₀) was the parameter used to compare the cytotoxic activity. A lower IC₅₀ meant better cytotoxic activity.

The *Parmelia saxatilis* was exhibited the best cytotoxic activity. The IC₅₀ against HCT-116 cell line was 96.86 µg/mL after 24 h and 14.77 µg/mL after 72 h.

The extract of *Umbilicaria polyphylla* also showed a good cytotoxic activity against HCT-116 cell line. The IC₅₀ value was 194.41 µg/mL after 24 h and 153.96 µg/mL after 72 h.

Extract of *Parmeliopsis ambigua* manifested intermediate activity against tested cell line. The IC₅₀ value after 24-hours and 72-hours exposure was 299.58 µg/mL and 280.43 µg/mL, respectively.

The *Lecanora muralis* showed a slightly weaker cytotoxic activity. The IC₅₀ value was 331.11 and 286.81 µg/mL after 24 and 72 h, respectively.

The *Umbilicaria crustulosa* manifested weakest cytotoxic activity. The IC₅₀ value was 401.44 µg/mL after 24 h and 497.66 µg/mL after 72 h.

3.4 The results obtained with AO/EB double staining

The ability of the lichens extracts to induce apoptosis was initially screened by using acridine orange/ethidium bromide staining. Control cells showed bright green nucleus with uniform intensity and had not taken up ethidium bromide, where the apoptotic cells appeared orange in color (Figure 4 and Figure 5). All the cells treated with lichen extracts showed obvious nuclear condensation after 24 h treatment. The results are represented in Table 4 and Table 5.

Florescence microscopic images clearly showed nuclear disintegration of cells which were treated by methanolic extracts of all lichenes species compared with that of the untreated control cells when stained with acridine orange and ethidium bromide. Compared with spontaneous apoptosis observed in control cells (early apoptotic 3.2%, 0% late apoptotic and 0% necrotic cells) HCT-116 was treated by 250 µg/mL methanolic extract of all lichenes showed high and early apoptotic for 24 h. The methanolic extract of *Parmelia saxatilis* which exhibited the highest antiproliferative potential with IC₅₀ of 96.85 µg/mL (Table 3) showed increased percentages of early apoptotic (90.15%), increased percentage of late apoptotic (0.98%) cells after 24 h. Acridine

orange/ethidium bromide staining of HCT 116 cells to detect apoptosis induced by 250 µg/mL dose of tested lichens extracts. Compared with the spontaneous apoptosis observed in control cells (early apoptotic 28.8% after 72 h), HCT 116 treated with 250 µg/mL dose of any lichen extract showed increased percentages of late apoptotic cells (Table 5). All cells treated with lichen extracts showed obvious nuclear condensation after 72 h treatment. After 72 h, there was a high percentage of cells exhibiting late apoptosis (29.25%) or necrosis (70.75%) which had been treated extract of *Parmelia saxatilis* (IC₅₀=14.7 µg/mL).

Table 4. Effect of lichens extract on apoptosis HCT 116 cells were stained with AO/EB and analyzed under a fluorescence microscope after 24 h

24 h	Viable cells (%)	Early apoptotic Cells (%)	Late apoptotic cells (%)	Necrosis cells (%)
Control	96.8	3.2		
<i>U. crustulosa</i>	53.51	44.69	1.8	
<i>P. ambigua</i>	23.74	58.99	12.94	4.3
<i>U. polyphylla</i>	50.49	42.24	2.6	4.6
<i>L. muralis</i>	34.07	62.92	1.48	1.48
<i>P. saxatilis</i>		90.15	9.8	

Table 5. Effect of lichens extract on apoptosis HCT 116 cells were stained with AO/EB and analyzed under a fluorescence microscope after 72 h

72 h	Viable cells (%)	Early apoptotic Cells (%)	Late apoptotic cells (%)	Necrosis cells (%)
Control	71.12	28.88		
<i>U. crustulosa</i>	32.78	67.21		
<i>P. ambigua</i>		33.03	46.69	20.26
<i>U. polyphylla</i>	13.23	85.81	0.96	
<i>L. muralis</i>	4.7	91.18	4.06	
<i>P. saxatilis</i>			29.25	70.75

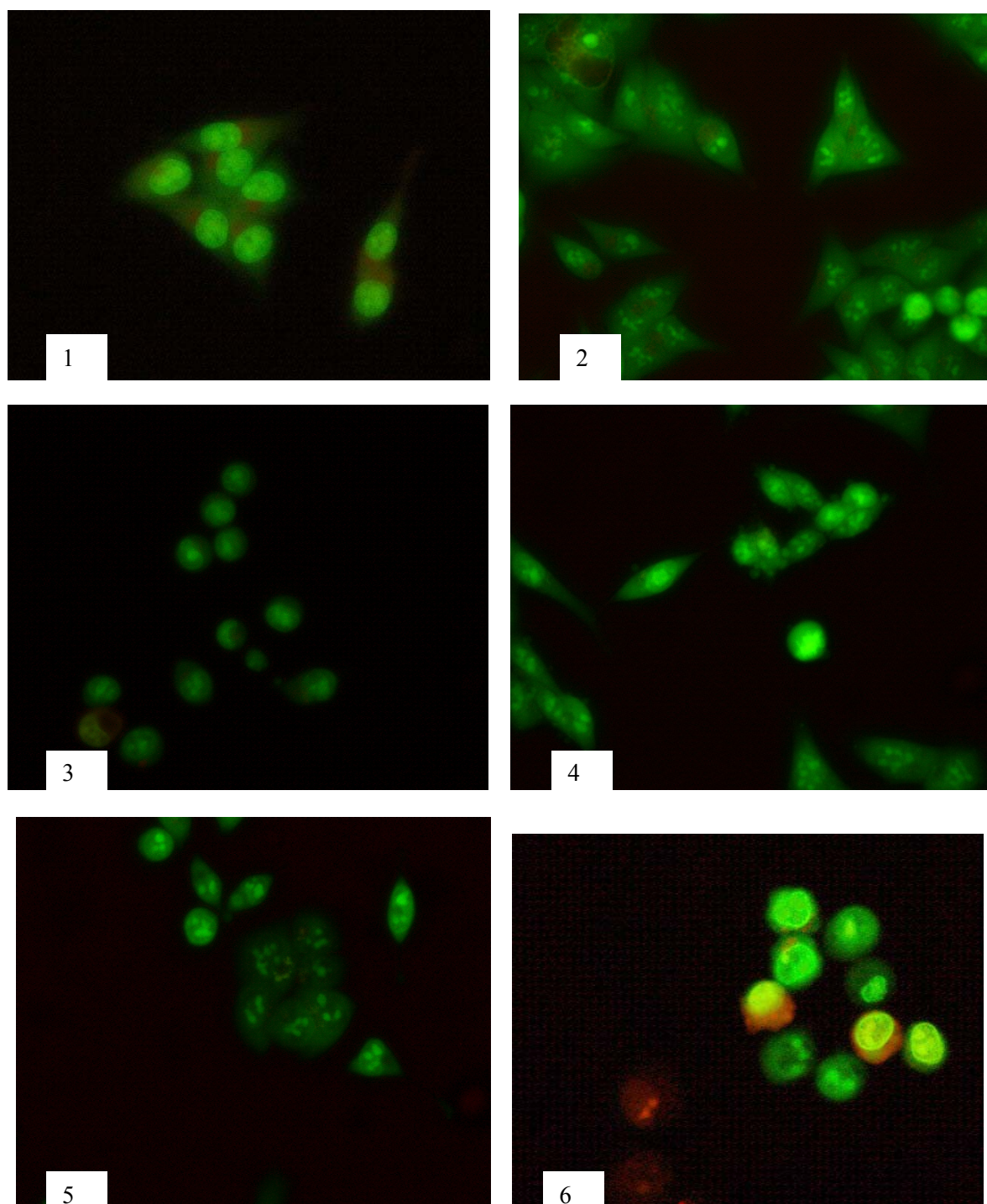


Fig. 4. AO/EB staining of HCT-116 cells to detect apoptosis and necrosis induced by methanolic extracts of *U. crustulosa* (2), *P. ambigua* (3), *U. polyphylla* (4), *L. muralis* (5), *P. saxatilis* (6) in concentration of 250 $\mu\text{g/mL}$, after 24 hours of exposure. Untreated cells were observed as a control cells (1). Magnification on fluorescent microscope was 400 \times .

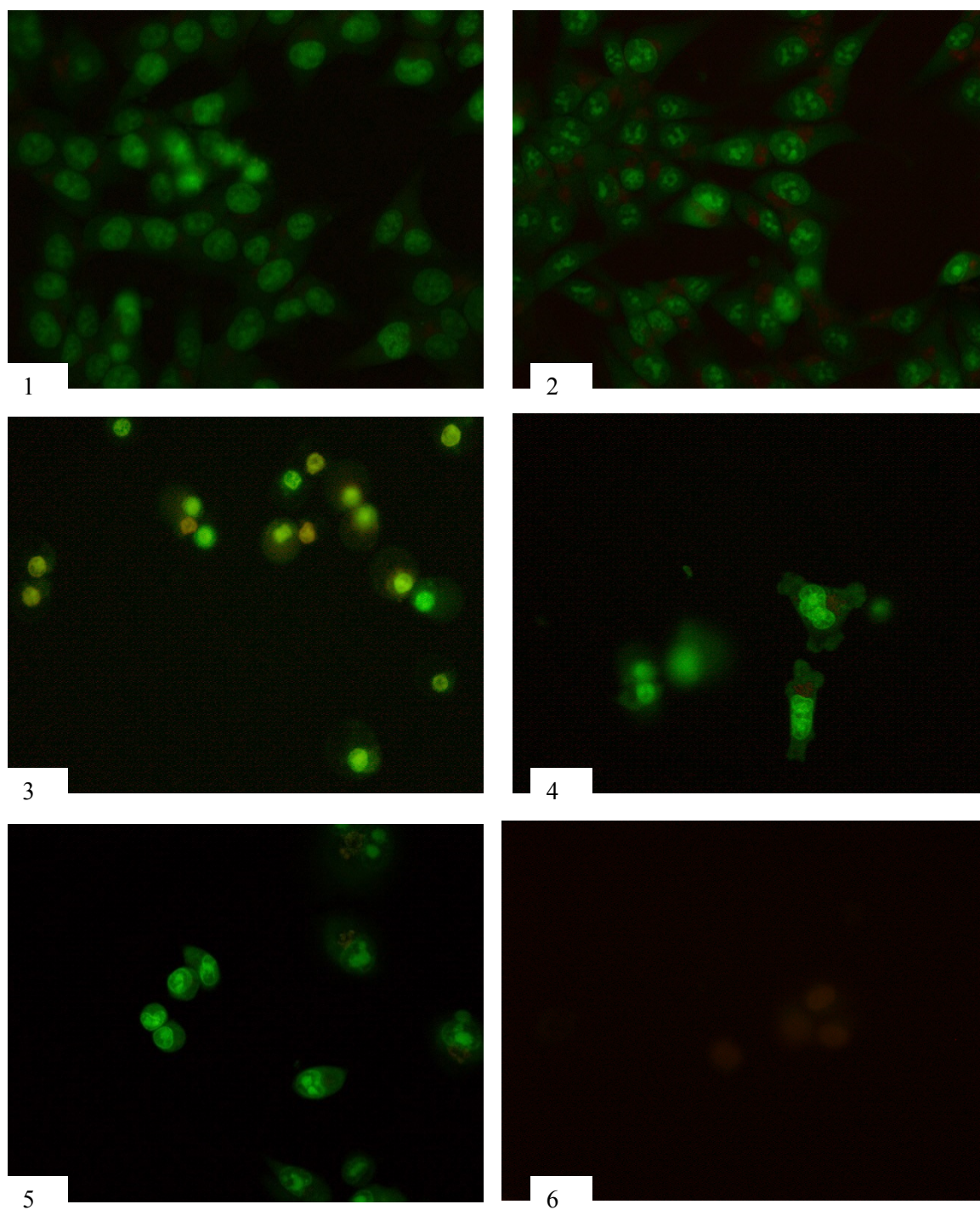


Fig. 5. AO/EB staining of HCT-116 cells to detect apoptosis and necrosis induced by methanolic extracts of *U. crustulosa* (2), *P. ambigua* (3), *U. polyphylla* (4), *L. muralis* (5), *P. saxatilis* (6) in concentration of 250 $\mu\text{g/mL}$, after 72 hours of exposure. Untreated cells were observed as a control cells (1). Magnification on fluorescent microscope was 400 \times .

Our results in AO/EB apoptotic test showed condensation of chromatin and nuclear fragmentation and cell shrinkage which were clearly observed on fluorescence microscopy.

4. Discussion

In the present study, *in vitro* antioxidant, antimicrobial and cytotoxic activities of methanol extract from the lichens *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla* were examined.

The tested lichen extracts have a strong antioxidant activity against various oxidative systems *in vitro*. We found that the tested extracts with the highest radical scavenging activity also had the greatest amount of phenolic content. The highest value of phenols was seen in the methanol extract of *Parmeliopsis ambigua* which exhibited the strongest radical scavenging activity. Based on these results, it could be concluded that antioxidative nature of the extracts might depend on their phenolics. Phenolic components are potential antioxidants, free radical terminators [18,19]. These compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals such as singlet oxygen, superoxide and hydroxyl radicals may be explained by their phenolic hydroxyl groups [20]. Flavonoids are also the most important natural phenolics and they possess a broad spectrum of chemical and biological activities including radical scavenging properties [21]. Numerous studies have found a high correlations between antioxidative activities and phenolic content [22,23]. Interestingly, Odabasoglu et al. [23] reported that in some lichens extracts no correlation was found between the total phenol and the antioxidant activity, suggesting that the antioxidant activity of different lichens may also depend on other, non-phenol components. Antioxidant effect of some other lichens has also been studied. For example, Gulcin et al. [24] found that the aqueous extracts of *Cetraria islandica* had a strong antioxidant activity. Similar results were reported by Behera et al. [25] for different extracts from the lichen *Usnea ghattensis*. Kekuda et al. [26] find an antioxidant activity for the extracts of the lichen *Parmotrema pseudotinctorum* and *Ramalina hossei*. Manojlović et al. [27] explored antioxidant properties of *Laurera benguelensis*.

In our experiments, the tested lichen extracts show a relatively strong antimicrobial activity. The intensity of the antimicrobial effect depended on the species of lichen, its concentration and the tested organism. The extract of *Umbilicaria polyphylla* had the strongest antimicrobial activity among the tested species in this study, inhibiting the tested bacteria and fungi at low concentrations, while the *Parmeliopsis ambigua* and *Umbilicaria crustulosa* showed a little weaker activity. Differences in antimicrobial activity of different species of lichens are probably a consequence of the presence of different components with antimicrobial activity [28,29,22].

The extracts used in this study, had a stronger antibacterial than antifungal activity. This observation is in accordance with other studies [30,22] focused on the antimicrobial activity which have demonstrated that bacteria are more sensitive than the fungi due to differences in the composition and permeability of the cell wall. The cell wall of Gram-positive bacteria is made of peptidoglucones and teichoic acids, while the cell wall of Gram-negative bacteria is made of peptidoglucones, lipopolysaccharides and lipoproteins [31,32]. The cell wall of fungi is poorly permeable and it consists of polysaccharides such as chitin and glucan [33].

Numerous taxonomically similar lichens have been screened for antimicrobial activity in search of the new antimicrobial agents. Ranković et al. [22] found that extract of the lichen *Parmelia centrifuga* conferred antimicrobial activity. Similar results were reported by Candan et al. [34] for different extracts extracted from the lichen *Parmelia sulcata*. Goel et al. [35] found out that lichen *Parmelia reticulata* had a strong antimicrobial influence.

In present study, the results clearly demonstrate that extracts of the five lichens induced a cytotoxic effect on the tested cancer cell lines. Until now, only few researchers proved that lichen extracts have anticancer activity. Bezivin et al. [36] reported significant anticancer effect for *Parmelia caperata*, *Cladonia convoluta*, *Cladonia rangiformis*, *Platisma glauca* and *Ramalina*

cuspidata. Manojlović et al. [37] explored anticancer properties of *Thamnolia vermicularis*. Trigiani et al. [38] found strong anticancer activity for *Xanthoria parietina*.

Some literature data reported that lichen components are responsible for anticancer activities of lichens. Anticancer activity of various lichens components are known, such as: usnic acid, lecanoric acid, gyrophoric acid, salazinic acid, lobaric acid, evernic acid, vulpinic acid, protolichesterinic acid [39,40]. However, it is difficult to determine the contribution of individual components to the overall effect. Often, the activity of the extracts may be the result of a synergistic effect of several compounds. More works needs to be done to elucidate which compounds may be responsible for the anticancer activity in the extracts from the five lichens in the current study.

5. Conclusion

In conclusion, it can be stated that tested lichen extracts have a strong antioxidant, antimicrobial and cytotoxic activity *in vitro*. On the basis of these results, the five lichens appear to be good and safe natural antioxidant, antimicrobial and anticancer agents and also, could be of significance in the food industry and to control various human, animal and plant diseases. Further studies should search for new compounds from lichens that exhibit strong antioxidant, antimicrobial and anticancer activity.

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