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Cladonia lichens and their major metabolites as possible natural antioxidant, antimicrobial and anticancer agents

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ABSTRACT

The aim of this study is to investigate *in vitro* antioxidant, antimicrobial, and anticancer activities of the acetone extracts of the lichens *Cladonia furcata, Cladonia pyxidata* and *Cladonia rangiferina* and their atranorin and fumarprotocetraric acid constituents. Antioxidant activity was evaluated by free radical scavenging, superoxide anion radical scavenging, reducing power, and determination of total phenolic and flavonoid compounds. As a result of the study atranorin had largest free radical scavenging activity with IC_{50} values 131.48 μg/mL. Moreover, the tested samples had effective reducing power and superoxide anion radical scavenging. Total content of phenol and flavonoid in extracts was 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. The most active was fumarprotocetraric acid with minimum inhibitory concentration values ranging from 0.031 to 0.125 mg/mL. Anticancer activity was tested against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines using MTT method. All samples were found to be strong anticancer activity toward both cell lines with IC_{50} values ranging from 10.97 to 41.23 μg/mL.

1. Introduction

Lichens are symbiotic organisms consisting of a fungus partner and a photosynthetic organism, either an alga or Cyanobacteria (Bates, Cropsey, Caporaso, Knight, & Fierer, 2011; Grube & Berg, 2010). More than 20,000 known species of lichens have been identified and inhabit diverse ecosystems ranging from arctic tundra to desert climates (Oboh & Ademosun, 2006). They are ubiquitous on barks, stems, leaves and in soil but often grow in habitats less favorable for higher plants (Vrablikova, McEvoy, Solhaug, Bartak, & Gauslaa, 2006). These organisms have historically been used as food, dyes, in production of alcohol and perfume industry. Lichens have also, for hundreds of years, been used in many countries as a cure for diseases of humans. Namely, *Cladonia rangiferina* was used to cure colds, arthritis, fever, jaundice, constipation, convulsions, cough, and tuberculosis. Similarly, *Cladonia pyxidata* was used in the treatment of pertussis (Bown, 2001).

In recent years, there has been a renewed interest in lichens as a potential source for bioactive compounds with therapeutic properties. New studies have revealed that these slow growing organisms produce a diverse array of secondary metabolites with different biological activities (Johnson et al., 2011). Lichen secondary metabolites are from derived mycobiont metabolism organized into several distinct chemical classes such as depsides, depsidones, dibenzofurans, xanthones, terpene derivatives, etc. (Johnson et al., 2011; Manojlović, Vasiljević, Masković, Jusković, & Bogdanović-Dusanović, 2012). In addition, lichens have also been identified as a source of biologically active enzymes, polysaccharides and fatty acids that may have pharmacological potential (Huneck & Yoshimura, 1996; Johnson et al., 2011). Due to a relatively recent resurgence in lichen bioactivity, therapeutic potential of many classes of lichens and their metabolites in medicine has largely remained unexplored. Here, we report the antioxidant, antimicrobial and anticancer activity of the acetone extracts of the lichens

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Cladonia furcata, C. pyxidata and *C. rangiferina* and their atranorin and fumarprotocetraric acid constituents.

2. Material and methods

2.1. Chemicals and reagents

Media for microorganisms (Müller-Hinton agar, Müller-Hinton broth, Sabourad dextrose agar and Sabourad dextrose broth) were purchased from Torlak, Belgrade, Serbia. The microorganisms, human melanoma FemX and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Acetone was obtained from Kefo, Belgrade, Serbia. All other reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Lichen samples

Lichen samples of *C. furcata* (Huds.) Schrad., *C. pyxidata* (L.) Hoffm., and *C. rangiferina* (L.) Weber ex F.H. Wigg., were collected from Kopaonik, Serbia, in September of 2011. The demonstration samples are preserved in facilities of the Department of Biology and Ecology of Kragujevac, Faculty of Science. Determination of the investigated lichens was accomplished using standard methods.

2.3. Preparation of the lichen extracts and isolation of compounds

Finely dry ground thalli of the investigated lichens (100 g) were extracted using acetone in a Soxhlet extractor (IKA, Werke, Staufen, Germany). The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator (IKA RV 10, Werke, Staufen, Germany). The dry extracts were stored at −18 °C until they were used in the tests. The extracts were dissolved in 5 mL/100 mL dimethyl sulphoxide (DMSO) for the experiments.

2.4. Isolation of atranorin

The acetone extract of the lichen *C. furcata* (100 mg) was fractionated on a silica gel column (0.149–0.074 mm). The column was eluted with hexane–ethyl acetate (6:1, 3:1 and 1:1, v/v), as eluent, collecting 20 mL in each fraction (fifteen fractions). The fractions were monitored by TLC and the obtained spots comapared with the spots of standards previously isolated from lichens. TLC results indicated which fractions contain the compounds we are interested in isolating. Based on these facts, it is concluded that the first eluted fraction of the lichen extract contains atranorin (21 mg), which was further purified by rechromatography and preparative layer chromatography (hexane–ethyl acetate, 6:1) and used for structure identification and antioxidant, antimicrobial and anticancer activities. The atranorin was dissolved in 5 mL/100 mL DMSO for further testing.

Atranorin (colorless crystalline substance) was identified by its melting point and spectroscopic data (Huneck & Yoshimura, 1996). The purity of the isolated compound was determined by HPLC-DAD and amounted to 98.8%.

2.5. Isolation of fumarprotocetraric acid

The acetone extract of the lichen *C. rangiferina* (100 mg) was chromatographed on a silica gel column (0.149–0.074 mm) and eluted with hexane–ethyl acetate (4:1, v/v) yielding ten fractions (20 mL of each fractions). The fractions were monitored by TLC and the obtained spots comapared with the spots of standards previously isolated form lichens. The last two eluted fraction of the lichen extract contain depsidone derivative fumarprotocetraric acid. This compound (18 mg) was purified by recrystalisation.

After purification fumarprotocetraric acid was used for structure identification and antioxidant, antimicrobial and anticancer studies. The fumarprotocetraric acid was dissolved in 5 mL/100 mL DMSO for further testing. Fumarprotocetraric acid (colorless crystalline substance) was identified by its melting point and spectroscopic data (Huneck & Yoshimura, 1996). The purity of the isolated compound was determined by HPLC-DAD and amounted to 97.3%.

2.6. Antioxidant activity

2.6.1. Scavenging DPPH radicals

The free radical scavenging activity of samples was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH). The method used is similar to the method previously used by some authors (Dorman, Bachmayer, Kosar, & Hiltunen, 2004; Ibanez et al., 2003) but was modified in details. Two milliliters of methanol solution of DPPH radical in the concentration of 0.05 mg/mL and 1 mL of test samples (1000, 500, 250, 125 and 62.5 μg/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 in a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK). Ascorbic acid was used as positive control. The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scavenging effect}(\%) = [(A0 - A1)/A0] \times 100$$

where $A0$ is the absorbance of the negative control and $A1$ is the absorbance of reaction mixture or standard.

The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity.

2.6.2. Reducing power

The reducing power of samples was determined according to the method of Oyaizu (1986). One milliliter of test samples (1000, 500, 250, 125 and 62.5 μg/mL) were mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL of 1 g/100 mL). The mixtures were incubated in an incubator (Sutjeska, Zagreb, Croatia) at 50 °C for 20 min. Then, 2.5 mL of 10 g/100 mL trichloroacetic acid was added to the mixture and centrifuged at 3000 *g* for 10 min using a centrifuge (HermLe, Wehingen, Germany). Finally, the upper layer was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL of 0.1 g/100 mL). The absorbance of the solution was measured at 700 nm in a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK). Higher absorbance of the reaction mixture indicated that the reducing power is increased. Ascorbic acid was used as positive control.

2.6.3. Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of samples was detected according to the method of Nishimiki, Rao, and Yagi (1972). Briefly, 0.1 mL of test samples (1000, 500, 250, 125 and 62.5 μg/mL) was mixed with 1 mL nitroblue tetrazolium (NBT) solution (156 μmol/L in 0.1 mol/L phosphate buffer, pH 7.4) and 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 μmol/L in 0.1 mol/L phosphate buffer, pH 7.4). The reaction was started by adding 100 μL of phenazine methosulphate (PMS) solution (60 μmol/L in 0.1 mol/L phosphate buffer, pH 7.4). The mixture was incubated at room temperature for 5 min, and the absorbance was measured at 560 nm in a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK) against blank samples. Decreased absorbance indicated increased superoxide anion radical scavenging activity. Ascorbic acid was used as positive control. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging activity}(\%) = [(A0 - A1)/A0] \times 100$$

where $A0$ is the absorbance of the negative control and $A1$ is the absorbance of reaction mixture or standards.

The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity.

2.6.4. Determination of total phenolic compounds

Total soluble phenolic compounds in the extract were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton (1997) using pyrocatechol as a standard phenolic compound. Briefly, 1 mL of the extract (1 mg/mL) in a volumetric flask 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 2 g/100 mL sodium carbonate was added and then was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK). The total concentration of phenolic compounds in the extract determined as microgram of pyrocatechol equivalent (PE) per milligram of dry extract by using an equation that was obtained from a standard pyrocatechol graph as follows:

$$\text{Absorbance} = 0.0057 \times \text{total phenols [}\mu\text{g PE/mg of dry extracts]} - 0.1646\left(R^2 = 0.9203\right)$$

2.6.5. Total flavonoid content

The total flavonoid content was determined using the Dowd method (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). Two milliliters of 2 g/100 mL aluminum 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 a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK) against blank samples. The total flavonoid content determined as microgram of rutin equivalent (RE) per milligram of dry extract by using an equation that was obtained from a standard rutin graph as follows:

$$\text{Absorbance} = 0.0296 \times \text{total flavonoid [}\mu\text{g RE/mg of dry extracts]} + 0.0204\left(R^2 = 0.9595\right)$$

2.7. Antimicrobial activity

2.7.1. Microorganisms and media

The following bacteria were used as test organisms in this study: *Bacillus mycoides* (ATCC 6462), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 13883). All the bacteria used were obtained from the American Type Culture Collection (ATCC). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Candida albicans* (ATCC 10231), *Penicillium purpurascens* (DBFS 418) and *Penicillium verrucosum* (DBFS 262). They were from the American Type Culture Collection (ATCC) and 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, Serbia). Fungal cultures were maintained on potato dextrose (PD) agar and Sabourad dextrose (SD) agar (Torlak, Belgrade, Serbia). All cultures were stored at 4 °C and subcultured every 15 days.

The sensitivity of microorganisms to tested samples was tested by determining the minimal inhibitory concentration (MIC).

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37 °C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard (bioMérieux, Marcy l'Etoile, France) 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 (Jenway, Bibby Scientific Limited, Stone, UK) at 530 nm and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS (1998).

2.7.2. Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined using 96-well micro-titer plates (Spektar, Čačak, Serbia), by the broth microdilution method (Sarker, Nahar, & Kumarasamy, 2007). A series of dilutions with concentrations ranging from 50 to 0.195 mg/mL for extracts and 4 to 0.00181 mg/mL for compounds was used in the experiment against every microorganism tested. The starting solutions of test samples were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of test samples 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. All experiments were performed in triplicate.

2.8. Cytotoxic activity

2.8.1. Cell lines

The human melanoma Fem-x and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10 mL/100 mL heat-inactivated (56 °C) fetal bovine serum, (Sigma Chemical Co. St Louis, MO, USA) supplemented with 3 mmol/L L-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 10 mL/100 mL heat inactivated fetal bovine serum (FBS-Sigma Chemical Co.). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.8.2. Treatment of cell lines

Stock solutions (100 mg/mL) of test samples, made in dimethylsulfoxide (DMSO), were dissolved in corresponding medium to the required working concentrations. Neoplastic FemX cells (5000 cells per well) and neoplastic LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates, and 24 h later, after the cell adherence, five different, double diluted, concentrations of investigated compounds, were added to the wells. Final concentrations applied to target cells were 200, 100, 50, 25 and 12.5 μg/mL,

except to the control wells, where only nutrient medium was added to the cells. Nutrient medium was RPMI 1640 medium, supplemented with L-glutamine (3 mmol/L), streptomycin (100 μg/mL), and penicillin (100 IU/mL), 10 mL/100 mL heat inactivated (56 °C) fetal bovine serum (FBS) and 25 mMol/L Hepes, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 h.

2.8.3. Determination of cell survival (MTT test)

The effect of test samples on cancer cell survival was determined by MTT test (microculture tetrazolium test), according to Mosmann (1983) with modification by Ohno and Abe (1991) 72 h upon addition of the compounds, as it was described earlier. Briefly, 20 μl of MTT solution (5 mg/mL PBS) were added to each well. Samples were incubated for further 4 h at 37 C in 5% CO_2 and humidified air atmosphere. Then, 100 μl of 10 g/L SDS were added to extract the insoluble product formazan, resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 570 nm. Absorbance (A) at 570 nm was measured 24 h later (Mosmann, 1983; Ohno & Abe, 1991). To get cell survival S (%), was used the following equation: $S(\%) = (As - Ab) \times 100/(Ac - Ab)$. Therefore, A of a sample (As) with cells grown in the presence of various concentrations of the investigated test samples was divided with control optical density (the A of control cells *(Ac)* grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank *(Ab)* was always subtracted from A of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. As a positive control was used *cis*-diamminedichloroplatinum (Cis-DDP), which was dissolved in RPMI-1640 medium to the final concentrations. All experiments were done in triplicate.

2.8.4. Flow cytometry analysis

Cellular DNA content and cell distribution were quantified by flow cytometry using propidium iodide (PI). Cells (3×10^5 cells/well) were seeded in 6-well plates and incubated with or without IC_{50} concentration of investigated compounds for 24 h. After treatment, the cells were collected by trypsinization, and fixed in ice-cold 70 mL/100 mL ethanol at −20 °C overnight. After fixation, the cells were washed in PBS and pellets obtained by centrifugation was treated with RNase A (100 μg/mL) at 37 °C for 30 min, and then incubated with 40 μg/mL of propidium iodide (PI) for at least 30 min. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences Franklin Lakes, NJ, USA), equipped with a 15 mW, air-cooled 488 nm argon ion laser for excitation of PI. The PI fluorescence (FL2) was collected after passing a 585/42-nm band pass filter. FACSCalibur flow cytometer equipped with an FL2 upgraded doublet discrimination module (DDM), allows screening, and then excluding possible occurrence of cell doublets, clumps and debris, by plotting FL2-area versus FL2-width signals (Carbonari, Tedesco, & Fiorilli, 2001). PI fluorescence data was collected using linear amplification. For the each sample 10,000 events was collected (Clothier, 1995). Finally, data were analyzed using CELLQuest 3.2.1.f1 software (BD Biosciences).

2.8.5. Statistical analyses

Statistical analyses were performed with the EXCEL and SPSS softwares package. 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 and superoxide anion radical scavenging. All values are expressed as mean ± SD of three parallel measurements.

3. Results

From the obtained lichen extracts, were isolated lichen compounds atranorin and fumarprotocetraric acid. Atranorin (Fig. 1) was isolated from the acetone extract of *C. furcata* by column chromatography on silica gel using solvent systems methanol–water (6:1, 3:1 and 1:1, v/v). Fumarprotocetraric acid (Fig. 2) was isolated from the acetone extract of *C. rangiferina* using the same method but different solvent system (methanol–water, 4:1, v/v). Atranorin belonging to the depsides while fumarprotocetraric acid is depsidone.

The scavenging DPPH radicals of the studied samples are shown in Table 1. The IC_{50} values of all extracts and compounds ranged from 131.48 to 987.64 μg/mL. There was a statistically significant difference between samples and blank ($p < 0.05$). Among the tested extracts, acetone extract from *C. pyxidata* showed largest DPPH radical scavenging activity ($IC_{50} = 461.72$ μg/mL). The lichen components isolated demonstrated very strong DPPH radical scavenging activity, greater than those from extracts. Fumarprotocetraric acid showed weaker DPPH radical scavenging activity than atranorin. The IC_{50} values for atranorin and fumarprotocetraric acid were 131.48 and 228.46 μg/mL respectively.

The results of the reducing power assay of the tested extracts and compounds are summarized in Table 2. Higher absorbance indicates higher reducing power. Among the lichen extracts, *C. pyxidata* showed the highest reducing power, followed by *C. furcata* and *C. rangiferina*. The isolated compounds showed very high reducing power, higher than those from extracts.

The scavenging of superoxide anion radicals by the tested lichen extracts and compounds is shown in Table 1. There was a statistically significant difference between samples and control ($p < 0.05$). The lichen components isolated showed the highest superoxide anion radical scavenging activity (the IC_{50} was 169.65 μg/mL for atranorin and 389.57 μg/mL for fumarprotocetraric acid). The scavenging activities of tested extracts were somewhat lower.

The total phenolic and flavonoid contents of the tested lichen extracts are given in Table 3. The total amount of phenolic compounds was determined as the pyrocatechol equivalent using an equation obtained from a standard pyrocatechol graph. The total phenolics contents of the acetone extracts of *C. pyxidata, C. furcata* and *C. rangiferina* were 35.35, 28 and 22 μg PE/mg respectively. The total amount of flavonoid compounds was determined as the rutin equivalent using an equation obtained from a standard rutin graph. The total flavonoid contents of the acetone extracts of *C. pyxidata, C. furcata* and *C. rangiferina* were 11.31, 10.55 and 5.13 μg RE/mg respectively.

The tested extracts exhibited the highest radical scavenging activity with the greatest amount of phenolic contents. Correlation coefficient between phenolic compounds of the tested extracts and

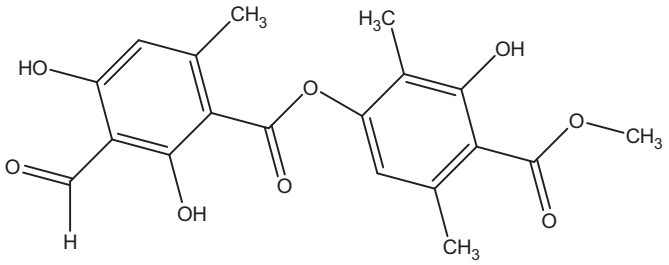

Fig. 1. Structures of atranorin.

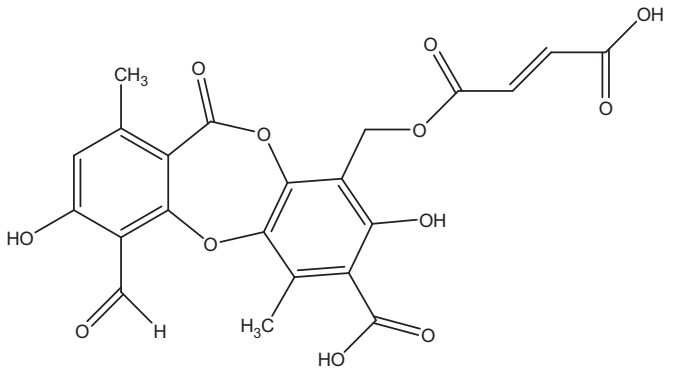

Fig. 2. Structures of fumarprotocetraric acid.

Table 1
DPPH radical scavenging activity and superoxide anion scavenging activity of acetone extracts of *Cladonia furcata, C. pyxidata* and *C. rangiferina* and their compounds.

Lichen species and compounds	DPPH radical scavenging IC_{50} (μg/mL)	Superoxide anion scavenging IC_{50} (μg/mL)
C. furcata	967.97 ± 2.35***	1579.30 ± 3.65***
C. pyxidata	461.72 ± 2.93***	666.80 ± 2.74***
C. rangiferina	987.64 ± 2.02***	1595.12 ± 3.22***
Atranorin	131.48 ± 1.53***	169.65 ± 1.58**
Fumarprotocetraric acid	228.46 ± 1.96***	389.57 ± 2.07***
Ascorbic acid	6.42 ± 0.18	115.61 ± 1.16

Data are the average of three replicates.
The extracts and pure compounds were dissolved in 5 mL/100 mL DMSO.
Significance was determined by using student's *t*-test: $^{**}p < 0.01^{*}$; $^{***}p < 0.001$; compared to control.

their DPPH radical and superoxide anion radical scavenging activity were $r = 0.908$ and $r = 0.900$, respectively.

Various antioxidant activities were compared to ascorbic acid. The results showed that standard antioxidant had stronger activity than tested samples.

The antimicrobial activities of the lichen extracts and lichen components against the test microorganisms are shown in Table 4. Extracts from *C. furcata* and *C. rangiferina* showed similar antibacterial and antifungal activity. They inhibited the microorganisms tested at concentrations from 0.78 to 25 mg/mL. Extracts from *C. pyxidata* inhibited all the tested microorganisms, but at higher concentrations. The lichen components isolated demonstrated very strong antimicrobial activity. The MIC for different components relative to the tested microorganisms ranged from 0.015 to 1 mg/mL. The strongest antimicrobial activity was found in fumarprotocetraric acid, which in extremely low amounts (0.031–0.25 mg/mL) inhibited all the species of bacteria and fungi (Table 4).

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 samples as shown in Table 4. In a negative control, DMSO had no inhibitory effect on the tested organisms.

The cytotoxic activity of the tested lichen extracts and compounds against the tested cell lines is shown in Table 5. The tested samples exhibited high cytotoxic activity against the target cells *in vitro*. The IC_{50} value for different samples relative to the tested cells ranged from 10.97 to 41.23 μg/mL. The best cytotoxic activity was exhibited the extract from *C. rangiferina*. The IC_{50} against FemX and LS174 cell lines was very low (19.97 and 10.97 μg/mL, respectively).

As shown in table, positive control (Cis-DDP) had slightly better cytotoxic activity than tested samples.

Therefore, we chose to examine the mechanism of action of atranorin and fumarprotocetraric acid by cytofluorimetric analysis, using propidium iodide to label DNA. The effect of compounds upon the cell cycle was assessed after incubating FemX and LS174 cells with the compounds at their IC_{50} μg/mL. Table 6 shows a representative cell-cycle distribution of FemX and LS174 cells incubated in the absence or presence of atranorin and fumarprotocetraric acid for 24 h, the approximate doubling time of this cell line. We observed that *in vitro* antiproliferative activity was accompanied by an important sub-G1 fraction and concomitant decrease in G2/M of FemX and LS174 cell lines after treatment with both compounds. As expected, based on MTT results, the atranorin showed the strongest increase in the percentage of the sub-G1 population in the both cell line, and led to a *G0/G1* cell cycle block. This was consistent with the compounds inducing apoptosis in a cell cycle-dependent manner.

4. Discussion

Antioxidant, antimicrobial and anticancer activities of *C. furcata* lichen were previously shown in our paper (Ranković, Kosanić, & Stanojković, 2011). In order to obtain complete results and to compare the activity of different lichen species, now we expand our research. We compared different biological activities between three *Cladonia* species and their isolated metabolites, in order to determine importance of isolated components for the activity of the extracts. Since we find relatively good results, in future research we plan to isolate more components of these species to find more active compounds.

In the present study, *in vitro* antioxidant, antimicrobial and cytotoxic activities of acetone extract from the lichens *C. furcata, C. pyxidata* and *C. rangiferina* and their atranorin and fumarprotocetraric acid, were examined.

The tested lichen extracts have a strong antioxidant activity against various oxidative systems *in vitro*. Strong antioxidant activity of tested lichen extracts is correlated with a high content of

Table 2
Reducing power of acetone extracts of *Cladonia furcata, C. pyxidata* and *C. rangiferina* and their compounds.

Lichen species and compounds	Absorbance (700 nm)				
	1000 μg/mL	500 μg/mL	250 μg/mL	125 μg/mL	62.5 μg/mL
C. furcata	0.0518 ± 0.002***	0.0321 ± 0.001***	0.0213 ± 0.001**	0.0114 ± 0.003**	0.0082 ± 0.001**
C. pyxidata	0.0884 ± 0.004***	0.0487 ± 0.002***	0.0310 ± 0.005**	0.0224 ± 0.006**	0.0157 ± 0.004**
C. rangiferina	0.0441 ± 0.003***	0.0312 ± 0.002***	0.0201 ± 0.002**	0.0102 ± 0.004**	0.0079 ± 0.001***
Atranorin	0.1523 ± 0.021***	0.0830 ± 0.004***	0.0493 ± 0.006**	0.0467 ± 0.007	0.0397 ± 0.007**
Fumarpr. acid	0.0975 ± 0.008***	0.0521 ± 0.003***	0.0312 ± 0.004**	0.0275 ± 0.005**	0.0194 ± 0.006**
Ascorbic acid	2.113 ± 0.032	1.654 ± 0.021	0.0957 ± 0.008	0.0478 ± 0.008	0.0247 ± 0.004**

Data are the average of three replicates.
The extracts and pure compounds were dissolved in 5 mL/100 mL DMSO.
Significance was determined by using student's *t*-test: $^{**}p < 0.01^{*}$; $^{***}p < 0.001$ compared to control.

Table 3
Total phenolics and flavonoid content of acetone extracts of *Cladonia furcata, C. pyxidata* and *C. rangiferina.*

Lichen species and compounds	Phenolics content (μg PE/mg of extract)	Flavonoid content (μg RE/mg of extract)
C. furcata	28.00 ± 1.065	10.55 ± 1.099
C. pyxidata	35.35 ± 1.013	11.31 ± 1.078
C. rangiferina	22.00 ± 1.065	5.13 ± 1.099

Data are the average of three replicates.

Table 5
Growth inhibitory effects of acetone extracts of *Cladonia furcata, C. pyxidata* and *C. rangiferina* and their compounds on FemX and LS174 cell lines.

Lichen species and compounds	IC_{50} (μg/mL)	
	FemX	LS174
C. furcata	23.52 ± 1.63**	40.22 ± 3.32***
C. pyxidata	34.19 ± 0.69***	24.52 ± 1.45**
C. rangiferina	19.97 ± 0.05**	10.97 ± 0.96**
Antranorin	20.91 ± 1.98**	24.63 ± 2.15**
Fumarprotocetraric acid	30.67 ± 0.87**	41.23 ± 0.38***
cis-diamminedichloroplatinum	0.94 ± 0.35	2.3 ± 0.31

Data are the average of three replicates.
The extracts and pure compounds were dissolved in 5 mL/100 mL DMSO.
Significance was determined by using student's *t*-test: $^{**}p < 0.01^{*}$; $^{***}p < 0.001$ compared to control.

total phenols. In fact, it was observed that the examined lichen extracts where found the higher content of phenols and flavonoids exert stronger radical scavenging effect, suggesting that phenolics are the main agents for their antioxidant activity. These results mostly agree with the literature, where we can find a number of reports for the antioxidant activity of extracts with high content of phenolic compounds (Behera, Verma, Sonone, & Makhija, 2009). In most lichens, phenols, including depsides, depsidones, and dibenzofurans, are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide, and hydroxyl radicals (Kosanić, Ranković, & Stanojković, 2012a). As well as, in our experiment the isolated components fumarprotocetraric acid and atranorin belonging phenols also exhibited significantly strong antioxidant activity than extracts, which indicates an important role of phenol in the antioxidant activity. However, some authors believe that the antioxidant activity of extracts may not be necessarily correlated with the content of polyphenolics Odabasoglu et al. (2004), suggesting that the antioxidant activity of different lichens may also depend on other, non-phenol components.

Antioxidant effect of some other lichens was also studied by other researchers. For example, Luo et al. (2006) found antioxidant activity for methanol extracts from the lichen *Thamnolia vermicularis*. Praveen Kumar, Prashith Kekuda, Vinayaka, and Sudharshan (2010) find an antioxidant activity for the extracts of the lichen *Ramalina hossei* and *Ramalina conduplicans.* Manojlović, Vasiljević, Gritsanapan, Supabphol, and Manojlović (2010) explored antioxidant properties of *Laurera benguelensis*.

In our experiments, the tested lichen extracts show a relatively strong antimicrobial activity but the antimicrobial activity of their components was much stronger. This means that lichen components are responsible for the antimicrobial activity of lichens. Differences in antimicrobial activity of different species of lichens are probably a consequence of the presence of different components with antimicrobial activity (Kosanić, Ranković, & Stanojković, 2012b). However, it is necessary understand that extracts are mixtures of natural compounds, and their antimicrobial activity is not only a result of the different activities of individual components but may be the result of their interactions, which can have different effects on the overall activity of extracts.

The intensity of the antimicrobial effect depended on the species of organism tested. The extracts and compounds used in this study had a stronger antibacterial than antifungal activity. This observation is in accordance with other studies (Kosanić et al., 2012b; Yang & Anderson, 1999), focused on the antimicrobial activity which have demonstrated that bacteria are more sensitive to the antimicrobial activity 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 peptidoglucanes and teichoic acids, while the cell wall of Gram-negative bacteria is made of peptidoglucanes, lipopolysacharides and lipoproteins (Heijenoort, 2001; Kosanić et al., 2012a). The cell wall of fungi is poorly permeable and it consists of polysaccharides such as chitin and glucan (Farkaš, 2003).

Numerous lichens were screened for antimicrobial activity in search of the new antimicrobial agents. Kosanić et al. (2012a) find an antimicrobial activity for the acetone extract of the lichens *Umbilicaria crustulosa*, *Umbilicaria cylindrica*, and *Umbilicaria polyphylla*. Similar results were reported by Karthikai Devi, Anantharaman, Kathiresan, and Balasubramanian (2011) for different extracts extracted from the lichen *Roccella belangeriana*. Goel, Dureja, Rani, Uniyal, and Laatsch (2011) found out that lichen *Parmelia reticulata* had a strong antimicrobial influence.

In present study, the results clearly demonstrate that acetone extracts of studied lichens induced significant cytotoxic effect on the tested cancer cell lines some are even stronger than the isolated components. Some literature data reported that lichen components are responsible for anticancer activities of lichens (Bucar, Schneider, Ogmundsdottir, & Ingolfsdottir, 2004; Burlando et al., 2009).

Table 4
Minimum inhibitory concentration (MIC) of acetone extracts of *Cladonia furcata, C. pyxidata* and *C. rangiferina* and their compounds.

Lichen species and compounds	*Cladonia furcata*	*Cladonia pyxidata*	*Cladonia rangiferina*	Atranorin	Fumarproto. acid	S	K
B. mycoides	0.78	12.5	0.78	0.015	0.062	7.81	–
B. subtilis	0.78	12.5	0.78	0.0312	0.062	7.81	–
E. coli	1.56	25	–	1	0.125	31.25	–
K. pneumoniae	0.78	6.25	0.78	0.5	0.03	1.95	–
S. aureus	0.78	12.5	0.78	0.25	0.125	31.25	–
A. flavus	25	25	12.5	1	0.25	–	3.9
A. fumigatus	12.5	25	12.5	0.5	0.25	–	3.9
C. albicans	6.25	12.5	12.5	0.25	0.125	–	1.95
P. purpurescens	25	25	12.5	1	0.25	–	3.9
P. verrucosum	25	25	12.5	1	0.25	–	3.9

Values given as mg/mL for tested samples and as μg/mL for antibiotics.
Values are the mean of three replicates.
The extracts and pure compounds were dissolved in 5 mL/100 mL DMSO.
Antibiotics: K – ketoconazole, S – streptomycin.

Table 6
Effect of isolated compounds on cell cycle progression.

	Apoptotic cells sub-G1	G1	S	G2/M
FemX				
Control	0.30 ± 0.016	58.19 ± 1.232	15.42 ± 1.471	22.87 ± 1.343
Atranorin	28.27 ± 1.213**	35.34 ± 1.139**	13.06 ± 1.092	17.15 ± 1.641*
Fumarpr. acid	18.05 ± 1.082**	47.84 ± 1.752*	15.90 ± 1.155	19.31 ± 1.284*
LS174				
Control	2.55 ± 0.102	49.53 ± 1.562	12.06 ± 1.089	32.21 ± 1.123
Atranorin	20.88 ± 1.257**	41.37 ± 1.854	14.27 ± 1.112	15.90 ± 1.094**
Fumarpr. acid	17.11 ± 1.174**	43.01 ± 1.693	16.51 ± 1.213	24.33 ± 1.153*

Effect of compounds on cell cycle phase distribution. FemX and LS174 cell lines were exposed to compounds (IC_{50} μg/mL) for 24 h and then collected for analysis of cell cycle phase distribution using flow cytometry. Percentage of cells under different stages of cell cycles (sub-G1, G1, S, G2/M) is shown. Data are the average of three replicates. Controls are untreated cells incubated for 24 h only in a nutrient medium. The pure compounds were dissolved in 5 mL/100 mL DMSO. Significance was determined by using student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control.

However, it is difficult to determine the contribution of individual components for the overall anticancer effect. Often, the activity of the extracts may be the result of synergistic or antagonistic effect of several compounds.

The importance of lichens as anticancer agents is confirmed in recent years, which suggests that lichens can be used as biological agents in the treatment of cancer. The mechanism of action of the tested extracts and their compounds need to be tested. The further research will be necessary to fractionation in order to identify compounds responsible for the observed anti-tumor effect and to establish the opportunities reinforcement activities and to improve the selectivity.

Until now, only few researchers proved that lichen have anticancer activity. Bezivin, Tomasi, Lohezic-Le Devehat, and Boustie (2003) reported significant anticancer effect for *Parmelia caperata, Cladonia convoluta, Cladonia rangiformis, Platisma glauca* and *Ramalina cuspidata*. Manojlović et al. (2010) explored anticancer properties of *T. vermicularis*. Trigiani et al. (2009) found strong anticancer activity for *Xanthoria parietina.*

In conclusion, it can be stated that tested lichen extracts and their compounds have a strong antioxidant, antimicrobial and anticancer activity *in vitro.* On the basis of these results, lichen appear to be good 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 be done to search new compounds from lichens that exhibit strong antioxidant, antimicrobial and anticancer activity.

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