EXCLI Journal 2014;13:1226-1238 – ISSN 1611-2156
Received: June 25, 2014, accepted: September 25, 2014, published: November 18, 2014

Original article:

BIOLOGICAL ACTIVITIES AND CHEMICAL COMPOSITION OF LICHENS FROM SERBIA

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ABSTRACT

The aim of this study is to investigate chemical composition of acetone extracts of the lichens *Parmelia arseneana* and *Acarospora fuscata* and *in vitro* antioxidant, antimicrobial, and anticancer activities of these extracts and gyrophoric acid isolated from *A. fuscata*. The HPLC-UV method was used for the identification of secondary metabolites. Stictic acid, norstictic acid, gyrophoric acid, usnic acid, atranorin and chloroatranorin were identified in the *A. fuscata*. In *P. arseneana*, we detected stictic acid, norstictic acid, usnic acid and atranorin, while gyrophoric acid was not identified. Antioxidant activity was evaluated by measuring the scavenging capacity of tested samples on DPPH and superoxide anion radicals, reducing the power of samples and determination of total phenolic compounds in extracts. As a result of the study, gyrophoric acid was found to have the largest DPPH radical scavenging activity with an IC_{50} value of 105.75 μg/ml. Moreover, the tested samples had an effective superoxide anion radical scavenging and reducing power. The total content of phenol in extracts was determined as pyrocatechol equivalent. The antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method. The most active was also gyrophoric acid, with minimum inhibitory concentration values ranging from 0.019 to 1.25 mg/ml. Anticancer activity was tested against LS174 (human colon carcinoma cell line), A549 (human lung carcinoma cell line), Fem-x (malignant melanoma cell line), and a chronic myelogeneous leukaemia K562 cell line using the MTT method. Extract of *P. arseneana* expressed the strongest anticancer activity against all cell lines with IC_{50} values ranging from 11.61 to 47.06 μg/ml.

Keywords: HPLC analysis, biological activities, lichens, lichens compound

INTRODUCTION

Lichens are symbiotic organisms consisting of a fungus partner and a photosynthetic organism, either an alga or Cyanobacteria (Grube and Berg, 2010; Bates et al., 2011). More than 20,000 known species of lichens have been identified and inhabit diverse ecosystems ranging from Arctic tundra to desert

EXCLI Journal 2014;13:1226-1238 – ISSN 1611-2156
Received: June 25, 2014, accepted: September 25, 2014, published: November 18, 2014

climates (Oboh and Ademosun, 2006). They are ubiquitous on barks, stems, leaves and in soil but often grow in habitats that are less favourable for higher plants (Vrablikova et al., 2006). These organisms have historically been used as a cure for human diseases, food, dyes, in the production of alcohol and in the perfume industry (Bown, 2001).

New studies have revealed that these slow-growing organisms produce a diverse array of secondary metabolites with different biological activities (Johnson et al., 2011). Lichens are secondary metabolites, mostly synthesised from fungal metabolism. They are crystal deposits on the surface of hiphes, which poorly dilute in water and can usually be isolated from lichen by organic diluents (Otzurk et al., 1999).

The biological potential of many lichens and their metabolites has largely remained unexplored. Thus, the aim of the present work was to identify the secondary metabolites of *P. arseneana* and *A. fuscata* by HPLC-UV and to evaluate the antioxidant capacity, antimicrobial and anticancer activities of the acetone extracts from this lichen as well as isolated gyrophoric acid.

MATERIAL AND METHODS

Lichen samples

Lichen samples of *P. arseneana* Gyeln, and *A. fuscata* (Nyl.) Arnold., were collected from Kopaonik, Serbia, in September 2012. The voucher specimen of the lichens (Voucher No. 113 and 119) was deposited at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. The determination of the investigated lichens was accomplished using standard methods.

Preparation of the lichen extracts

Finely dry ground thalli of the examined lichens (100 g) were extracted using acetone 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 they were used in the tests. The extracts were dissolved in 5 % dimethyl sulphoxide (DMSO) for the experiments. DMSO was dissolved in sterile distilled water to the desired concentration.

High Performance Liquid Chromatography (HPLC) analysis

The acetone extracts of the tested lichens were re-dissolved in 500 µl of acetone and analysed on an Agilent Technologies HPLC instrument 1200 Series with C18 column (250 mm x 4.6 mm, 5 µm) and a UV spectrophotometric detector with detection at three wavelengths. The same compounds were detected in all selected wavelengths (254, 280 and 320 nm). Methanol–water–phosphoric acid (90:10:0.9, v/v/v) was used as a solvent. Methanol was of HPLC grade and was purchased from Merck (Darmstadt, Germany). Deionised water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). Phosphoric acid was an analytical-grade reagent. The flow rate was 1.0 ml/min and the sample injection volume was 10 µl. The standards used were obtained from the following sources: stictic acid (t_R = 2.89 ± 0.20 min) was isolated from the lichen *Xanthoparmelia conspersa*, norstictic acid (t_R = 3.41 ± 0.10 min) from the lichen *Ramalina furinacea*, gyrophoric acid (t_R = 4.12 ± 0.10 min) from *Umbilicaria fructulosa*, and usnic acid (t_R = 7.96±0.20 min), atranorin (t_R = 8.56 ± 0.10 min) and chloroatranorin (t_R = 10.90 ± 0.10 min) from the lichen *Evernia prunastri*. The standard samples were isolated in our laboratory and their structures were confirmed by spectral data.

The isolation of gyrophoric acid from the lichen A. fuscata

The acetone extract of the lichen *A. fuscata* (500 mg) was dissolved in benzene and after filtration the residue was fractioned on a silica gel 60 column (under 0.063 mm; 230 mesh). The column was eluted with methanol-chloroform gradient solvent (20:1, 10:1 and 5:1) yielding nine fractions. The sixth eluted fraction of the lichen extract contained gyrophoric acid. This compound was used

EXCLI Journal 2014;13:1226-1238 – ISSN 1611-2156
Received: June 25, 2014, accepted: September 25, 2014, published: November 18, 2014

for the structural identification and determination of antioxidant, antimicrobial and cytotoxic activities. Gyrophoric acid was identified by the melting point, elemental analysis and ^{1}H and ^{13}C-NMR data (Huneck and Yoshimura, 1996). The purity of the isolated lichen acid was determined by HPLC-DAD and amounted to 97.8 %.

Antioxidant activity

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 (Ibanez et al., 2003; Dorman et al., 2004) but was modified. Two millilitres 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 spectrophotometer ("Jenway" UK). Ascorbic acid was used as a positive control. The DPPH radical concentration was calculated using the following equation:

DPPH scavenging effect (%) = [(A0 - A1) / A0] x 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.

Reducing power

The reducing power of samples was determined according to the method of Oyaizu (1986). One millilitre of test samples (1000, 500, 250, 125 and 62.5 µg/ml) was mixed with 2.5 ml of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1 %). The mixtures were incubated at 50 °C for 20 min. Then, trichloroacetic acid (10 %, 2.5 ml) was added to the mixture and the sample was centrifuged. Finally, the upper layer was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml; 0.1 %). The absorbance of the solution was measured at 700 nm in spectrophotometer ("Jenway" UK). Higher absorbance of the reaction mixture indicated that the reducing power was increased. Ascorbic acid was used as a positive control.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of samples was detected according to the method of Nishimiki et al. (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 µM in 0.1 M phosphate buffer, pH 7.4) and 1 ml nicotinamide adenine dinucleotide (NADH) solution (468 µM in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM 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 in spectrophotometer ("Jenway" UK) against blank samples. Decreased absorbance indicated increased superoxide anion radical scavenging activity. Ascorbic acid was used as a positive control. The percentage inhibition of superoxide anion generation was calculated using the following formula:

Superoxide anion scavenging activity (%) = [(A0 - A1) / A0] x 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.

Determination of total phenolic compounds

Total soluble phenolic compounds in the acetone extracts 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

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Received: June 25, 2014, accepted: September 25, 2014, published: November 18, 2014

volumetric flask was diluted with distilled water (46 ml). One millilitre of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 ml of sodium carbonate (2 %) was added and then allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in in spectrophotometer ("Jenway" UK). The total concentration of phenolic compounds in the extract was determined as micrograms of pyrocatechol equivalent (PE) per milligram of dry extract by using an equation that was obtained from a standard pyrocatechol graph as follows:

Absorbance = 0.0057 x total phenols [μg PE/mg of dry extracts] - 0.1646 (R^2 = 0.9203)

Antimicrobial activity

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 of 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 purpurescens* (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). Fungal cultures were maintained on potato dextrose (PD) agar and Sabourad dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4 °C and sub-cultured 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 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 (1998).

Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method with using 96-well micro-titre plates (Sarker et al., 2007). A series of dilutions with concentrations ranging from 40 to 0.0047 mg/ml for extracts and component was used in the experiment against every microorganism tested. The starting solutions of test samples were obtained by measuring a certain quantity of samples and dissolving this 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 colour 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, and 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.

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Cytotoxic activity

Cell culture

Human colon carcinoma LS174 cells, human lung carcinoma A549 cells, malignant melanoma Fem-x cells and chronic myelogeneous leukaemia K562 cells (American Type Culture Collection, USA) were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10 % (inactivated at 56 °C) FBS, 3 mM of L-glutamine, and antibiotics, at 37 °C in humidified air atmosphere with 5 % CO_2. For the growth of MDA-MB-361 and MDA-MB-453 cells, complete medium was enriched with 1.11 g/l glucose.

In vitro cytotoxic assay

In vitro assay for cytotoxic activity of investigated extract was performed when the cells reached 70-80 % confluence. Stock solution (50 mg/ml) of extract was dissolved in corresponding medium to the required working concentrations. Neoplastic LS174 cells (7000 cells per well), A549 cells (5000 cells per well), Fem-x cells (5000 cells per well) and K562 cells (5000 cells per well) were seeded into 96-well micro-titre plates, and 24 h later, after cell adherence, 5 different, double-diluted concentrations of investigated extract were added to the wells. Final concentrations of the extract were 200, 100, 50, 25, and 12.5 μg/ml, except for the control wells, where only nutrient medium was added. The cultures were incubated for the next 72 h. The effect on cancer cell survival was determined 72 h after the addition of extract, by the MTT test (Mosmann, 1983). Briefly, 20 μl of MTT solution (5 mg/ml PBS) was added to each well and incubated for a further 4 h at 37 °C in 5 % CO_2 and humidified air. Subsequently, 100 μl of 10 % SDS was added to solubilise the formazan crystals formed from MTT after the conversion by mitochondrial dehydrogenases of viable cells. Absorbencies proportional to the number of viable cells were measured using a microplate reader (Multiskan EX, Thermo Scientific, Finland) at 570 nm. Each experiment was performed in triplicate and independently repeated at least four times.

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 extract for 24 h. After treatment, the cells were collected by trypsinisation, and fixed in ice-cold 70 % ethanol at −20 °C overnight. After fixation, the cells were washed in PBS and pellets obtained by centrifugation were treated with RNase (100 μg/ml) at 37 °C temperature for 30 min and then incubated with propidium iodide (PI) (40 μg/ml) for at least 30 min. DNA content and cell cycle distribution were analysed using a Becton Dickinson FAC-Scan flow cytometer. Flow cytometry analysis was performed using a CellQuestR (Becton Dickinson, San Jose, CA, USA), on a minimum of 10,000 cells per sample (Clothier, 1995).

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. All values are expressed as mean ± SD of three parallel measurements.

RESULTS

The present paper deals with the phytochemical analysis of acetone extracts from the species *A. fuscata* and *P. arseneana*, and their antioxidant, antimicrobial and anticancer activities. Figures 1 and 2 show the HPLC chromatograms of the standards and the acetone extracts from the species *A. fuscata* and *P. arseneana*. Six metabolites, stictic acid, norstictic acid, gyrophoric acid, usnic acid atranorin and chloroatranorin, were identified in *A. fuscata*. On the other hand, the HPLC chromatogram for *P. arseneana* showed the presence of stictic acid, norstictic

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acid, usnic acid and atranorin, with gyrophoric acid not being identified. Identification of these compounds was achieved by comparison of their t_R values with the standard substances previously isolated from lichens. Table 1 shows the retention time of the detected lichen substances and their absorbance maxima (nm). Gyrophoric acid (4-[4-(2,4-dihydroxy-6-methylbenzoyl)oxy-2-hydroxy-6-methylbenzoyl]oxy-2-hydroxy-6-methylbenzoic acid (4-[4-(2,4-dihydroxy-6-methylbenzoyl)oxy-2-hydroxy-6-methylbenzoyl]oxy-2-hydroxy-6 methylbenzoic acid) is very interesting because the activity of tridepsides has not been well studied. Because of that, after HPLC analysis of the tested lichen extracts, gyrophoric acid (t_R = 4.18 min) was isolated from the acetone extracts of *A. fuscata* and used for antioxidant, antimicrobial and anti-tumour activities. Gyrophoric acid has specific UV spectra with absorption maxima at 214, 270 and 304 nm (Figure 3).

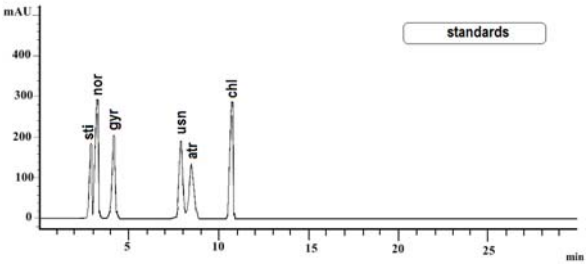

Figure 1: Chromatogram of the standards used for the identification of compounds present in *Acarospora fuscata* and *Parmelia arseneana*

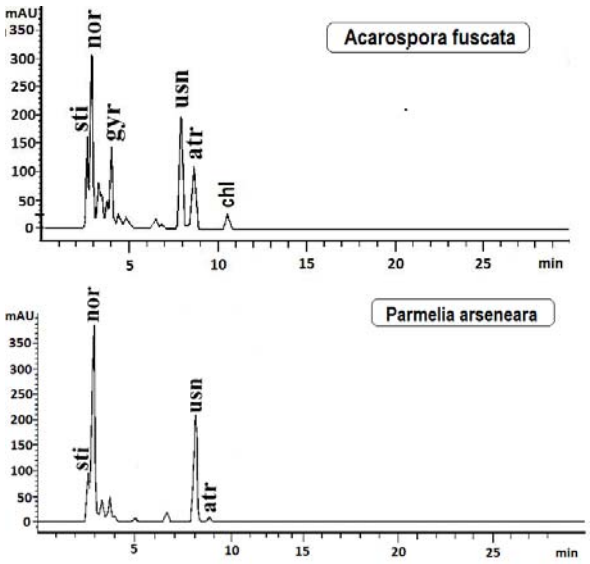

Figure 2: The HPLC chromatogram acquired at 254 nm of the extracts from *Acarospora fuscata* and *Parmelia arseneana*

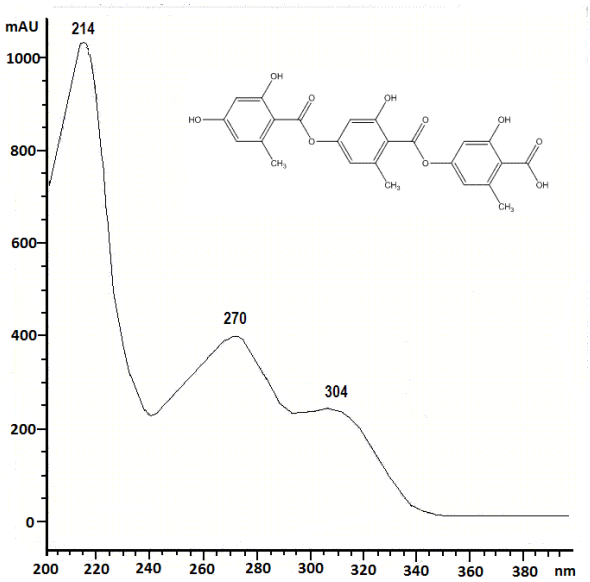

Figure 3: UV spectrum of gyrophoric acid

Table 1: Retention time of the examined lichen substances and their absorbance maxima (nm)

Compound	Substance class	Retention time (t_R ± SD)* (min)	Absorbance maxima (nm) UV spectrum
Stictic acid	depsidone	2.89±0.20	212, 236, 310m
Norstictic acid	depsidone	3.41±0.10	212, 239, 310m
Gyrophoric acid	tridepside	4.12±0.10	214, 270, 304
Usnic acid	dibenzofurane	7.96±0.20	234, 282
Atranorin	depside	8.56±0.10	210, 252, 321^m
Chloroatranorine	depside	10.90±0.10	213, 252, 315^m, 350

* Values are the means of three determinations ± SD, m – minor absorbance maximum

EXCLI Journal 2014;13:1226-1238 – ISSN 1611-2156
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The scavenging DPPH radicals of the studied samples are shown in Table 2. The IC_{50} values of both extracts and compounds ranged from 105.75 to 742.89 µg/ml. There was a statistically significant difference between samples and control ($P<0.05$). Among the tested extracts, acetone extract from *P. arseneana* showed the largest DPPH radical scavenging activity (IC_{50} = 612.75 µg/ml). The isolated lichen component demonstrated very strong DPPH radical scavenging activity, greater than those from extracts. The IC_{50} value for gyrophoric acid was 105.75 µg/ml.

The results of the reducing power assay of the tested extracts and compound are summarised in Table 3. Higher absorbance indicates higher reducing power. As shown in Table 3, gyrophoric acid showed the highest reducing power.

The scavenging of superoxide anion radicals by the tested lichen extracts and compounds is shown in Table 2. There was a statistically significant difference between samples and control ($P<0.05$). The isolated lichen component showed the highest superoxide anion radical scavenging activity (the IC_{50} was 196.62 µg/ml). The scavenging activities of tested extracts were somewhat lower.

The total phenolic contents of the tested lichen extracts are given in Table 4. The total phenolics contents of the acetone extracts of *P. arseneana* and *A. fuscata* were 54.38 and 47.59 µg PE/mg, respectively.

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

The antimicrobial activities of the lichen extracts and isolated component against the test microorganisms are shown in Table 5. The extract from *P. arseneana* showed moderate antibacterial and antifungal activity. They inhibited the microorganisms tested at concentrations from 0.078 to 10 mg/ml. Extracts from *A. fuscata* also inhibited all of the tested microorganisms, but at higher concentrations. The strongest antimicrobial activity was found in gyrophoric acid, which inhibited all of the species of bacteria and fungi in extremely low amounts (Table 5).

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 5. In the negative control, DMSO had no inhibitory effect on the tested organisms.

Table 2: DPPH radical scavenging activity and superoxide anion scavenging activity of acetone extracts of *Parmelia arseneana* and *Acarospora fuscata* and isolated gyrophoric acid

Lichen species	DPPH radical scavenging IC_{50} (µg/ml)	Superoxide anion scavenging IC_{50} (µg/ml)
Parmelia arseneana	612.75	783.94
Acarospora fuscata	742.89	957.46
Gyrophoric acid	105.75	196.62
Ascorbic acid	6.42	115.61

Table 3: Reducing power of acetone extracts of *Parmelia arseneana* and *Acarospora fuscata* and isolated gyrophoric acid

	Absorbance (700 nm)				
Lichen species	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml
Parmelia arseneana	0.5326	0.3996	0.2895	0.1748	0.0904
Acarospora fuscata	0.5015	0.3247	0.2245	0.1374	0.0752
Gyrophoric acid	1.3211	1.1243	0.7126	0.3923	0.2014
Ascorbic acid	2.113	1.654	0.0957	0.0478	0.0297

EXCLI Journal 2014;13:1226-1238 – ISSN 1611-2156
Received: June 25, 2014, accepted: September 25, 2014, published: November 18, 2014

Table 4: Total phenolics content of acetone extracts of *Parmelia arseneana* and *Acarospora fuscata*

Lichen species	Phenolics content (µg PE/mg of extract)
Parmelia arseneana	54.38 ± 1.065
Acarospora fuscata	47.59 ± 1.013

Table 5: Minimum inhibitory concentration (MIC) of acetone extracts of *Parmelia arseneana* and *Acarospora fuscata* and isolated gyrophoric acid

Lichen species	*Parmelia arseneana*	*Acarospora fuscata*	Gyrophoric acid	S - K
B. mycoides	0.156	0.625	0.039	7.81 -
B. subtilis	0.078	0.312	0.019	7.81 -
E. coli	2.5	5	0.312	31.25 -
K. pneumoniae	0.625	2.5	0.156	1.95 -
S. aureus	0.312	1.25	0.078	31.25 -
A. flavus	5	10	1.25	- 3.9
A. fumigatus	2.5	10	0.625	- 3.9
C. albicans	1.25	2.5	0.156	- 1.95
P. purpurescens	10	10	1.25	- 3.9
P. verrucosum	10	5	0.625	- 3.9

Values given as mg/ml for tested samples and as µg/ml for antibiotics. Values are the mean of three replicate. Antibiotics: K – ketoconazole, S – streptomycin

The cytotoxic activity of the tested lichen extracts and compounds against the target cell lines is shown in Table 6. The *P. arseneana* extract exhibited remarkable cytotoxic effect against LS174, K562 and Fem-x cells (IC_{50} 11.61, 13.84 and 16.86 µg/ml, respectively) and moderate cytotoxic effect when screened against A549 cells (IC_{50} 47.06 µg/ml). In contrast, the extract of the *A. fuscata* displayed some mild effect against K562 cells, very weak to LS174 cells and no activity against the A549 and Fem-x cells (IC_{50}> 200 mg/ml). Also, gyrophoric acid, as a typical secondary metabolite of lichens, showed very weak activity against A549 and 174 cells and exerted a moderate cytotoxic effect against Fem-x and K562 cells (IC_{50} 64.01 and 78.45 µg/ml, respectively). As shown in the table, the positive control (cis-DDP) had a significantly better cytotoxic activity than the tested samples.

Flow cytometry analysis performed on Fem-x and K562 cells after 24 h of incubation with investigated extracts or gyrophoric acid (IC_{50} (µg/ml)) indicated an alteration in the percentage of cells in each stage of the cell cycle as compared to the control. The results show a typical pattern of DNA content, reflecting marked apoptosis of sub-G1 phase in malignant cells and the corresponding change in reducing the percentage of cells in G0/G1 and S-G2/M phases of the cell cycle, as shown in Figure 4.

Table 6: Growth inhibitory effects of acetone extracts of *Parmelia arseneana* and *Acarospora fuscata* and isolated gyrophoric acid on FemX, A549, LS174 and K562 cell lines

Cell lines	FemX	A549	LS174	K562
Lichen species	IC_{50} (µg/ml)			
Parmelia arseneana	16.86 ± 1.63	47.06 ± 1.41	11.61 ± 0.48	13.84 ± 0.47
Acarospora fuscata	>200	>200	183.37 ± 2.39	116.22 ± 2.94
Gyrophoric acid	64.01 ± 2.38	151.51 ± 0.57	151.65 ± 1.74	78.45 ± 1.51
Cis-DDP	0.86 ± 0.33	4.91 ± 0.42	3.18 ± 0.29	2.22 ± 0.08

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Received: June 25, 2014, accepted: September 25, 2014, published: November 18, 2014

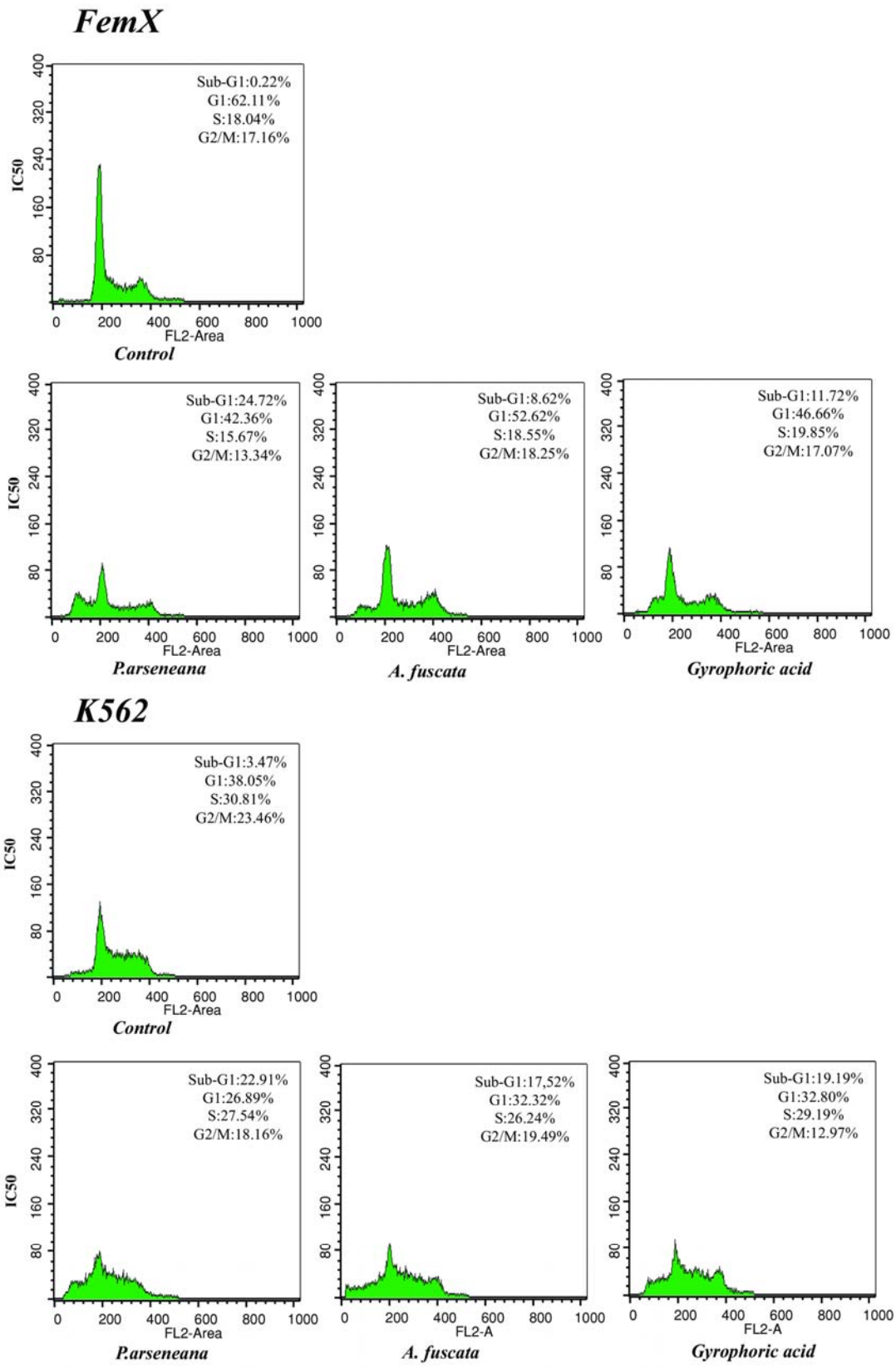

Figure 4: Cell-cycle distribution after 24 h of continuous action of (IC_{50}) *P. arseneana, A. fuscata* and Gyrophoric acid in FemX and K562 cell lines. Representative histograms of cell-cycle distribution of malignant cells measured by flow cytometric analysis of DNA content after treatment.

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DISCUSSION

Antioxidant, antimicrobial and anticancer activities of some lichen extracts and lichen metabolites were previously shown in our papers (Kosanić et al., 2013; Manojlović et al., 2012a; Ranković et al., 2012). In the present study, the chemical composition of the acetone extracts of the lichens *P. arseneana* and *A. fuscata* and *in vitro* antioxidant, antimicrobial, and anticancer activities of these extracts and gyrophoric acid from *A. fuscata* were examined.

The tested lichen extracts and isolated compounds have a strong antioxidant activity against various oxidative systems *in vitro*. The strong antioxidant activity of tested samples is correlated with a high content of total phenols. In fact, it was observed that the examined lichen extracts containing the higher content of phenols exerted a stronger radical scavenging effect, suggesting that phenolics are the main agents responsible 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 et al., 2009). In most lichens, phenols are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide, and hydroxyl radicals (Kosanić et al., 2012a). However, some authors believe that the antioxidant activity of extracts may not necessarily be correlated with the content of polyphenolics, suggesting that the antioxidant activity of different lichens may also depend on other, non-phenol components (Odabasoglu et al., 2004).

The antioxidant effect of some other lichens was also studied by other researchers. For example, Kosanić et al. (2013) found an antioxidant activity for the acetone extracts of the lichen *Evernia prunastri* and *Pseudoevernia furfuraceae* and their evernic acid and physodic acid constituents. Manojlović et al. (2012b) explored the antioxidant properties of *Umbilicaria cylindrica*. Praveen Kumar et al. (2010) found an antioxidant activity for the extracts of the lichen *Ramalina hossei* and *Ramalina conduplicans*. Compared with their results, the results of this research suggest that the tested samples showed a relatively powerful antioxidant activity.

In our experiments, the tested lichen extracts show a relatively strong antimicrobial activity but the antimicrobial activity of their component 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 (Ranković et al., 2012). However, it is necessary to 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 (Yang and Anderson, 1999; Kosanić et al., 2012b; Ličina et al., 2013) focused on 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, lipopolysaccharides and lipoproteins (Heijenoort, 2001; Kosanić et al., 2012b). The cell wall of fungi is poorly permeable and consists of polysaccharides such as hitchin and glucan (Farkaš, 2003).

Numerous lichens and lichen compounds were screened for antimicrobial activity in search of the new antimicrobial agents (Ranković et al., 2012; Goel et al., 2011), but in this study, for the first time, the antimi-

crobial activity of the acetone extracts of *P. arseneana* and *A. fuscata* was investigated. In correlation with the results obtained in experiments with other lichens, we noticed that the studied samples showed relatively strong antimicrobial activity.

In the present study, the results clearly demonstrate that tested samples showed a significant cytotoxic effect on the tested cancer cell lines. Some literature data reported that lichen components are responsible for the anticancer activities of lichens (Bucar et al., 2004; Burlando et al., 2009). 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. Our results are in accordance with the previously obtained data on gyrophoric acid cytotoxic activity *in vitro* (Bačkorová et al., 2012).

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 compound is yet to be tested. Thus, further research will be necessary for fractionation in order to identify compounds responsible for the observed anti-tumour effects, and to establish the opportunities for reinforcement activities as well as to improve the selectivity.

Until now, only a few researchers proved that lichens have anticancer activity. Kosanić et al. (2013) reported a significant anticancer effect for *Evernia prunastri* and *Pseudoevernia furfuraceae*. Manojlović et al. (2010) explored anticancer properties of *Thamnolia vermicularis*. Triggiani et al. (2009) found strong anticancer activity for *Xanthoria parietina.*

The obtained data indicate that *P. arseneana* extract has significant important biological properties and remarkable cytotoxic activity, since they display IC_{50} values in a range comparable to that of the antitumour drug cisplatin. Finally, *P. arseneana* extracts are considered as agent with potential anti-tumour activity, and could therefore be a candidate for further stages of screening *in vitro* and/or *in vivo.*

In conclusion, it can be stated that tested lichen extracts and their compound had a certain level of antioxidant, antimicrobial and anticancer activities *in vitro.* On the basis of these results, lichens appear to be good natural antioxidant, antimicrobial and anticancer agents. Furthermore, it could be of significance in the food industry as well as to control various human, animal and plant diseases. Further studies should be performed to search new compounds from lichens that exhibit strong antioxidant, antimicrobial and anticancer activities.

ACKNOWLEDGEMENTS

This work was financed in part by the Ministry of Science, Technology, and Development of the Republic of Serbia and was carried out within the framework of projects no. 173032, 175011 and 172015.

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