



Determination of chemical composition and antimicrobial, antioxidant and cytotoxic activities of lichens *Parmelia conspersa* and *Parmelia perlata*

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Abstract

The aim of this study is to investigate the chemical composition of extracts of the lichens *Parmelia conspersa* and *Parmelia perlata* and their antimicrobial, antioxidant, and anticancer activities. The phytochemical analysis of the acetone extracts of two *Parmelia* lichens was determined by (HPLC-UV) method. The predominant phenolic compounds in these extracts were norstictic acid and usnic acids in *P. conspersa*, while salazinic acid and stictic acid were the major metabolites detected in *P. perlata*. Besides these compounds, the tested extracts of these lichens contain atranorin and chloroatranorin. The lichen extracts showed comparable and strong antioxidant activity, exhibited higher DPPH and hydroxyl radical scavengings, chelating activity, and inhibitory activity towards lipid peroxidation. The lichen extracts demonstrated important antimicrobial activity against eight strains with MIC values from 19.53 to 312.5 µg/mL. Cytotoxic effects of lichens were tested against *Hep2c*, *RD* and *L2OB* cell lines using MTT method. Cytotoxic effects of *P. conspersa* and *P. perlata* extracts toward three cancer cell lines were in the range from 76.33 to 163.39 µg/mL. This is the first report of the detail chemical composition of the lichens *P. conspersa* and *P. perlata*. The present study showed that tested extracts of lichens demonstrated a important antimicrobial, antioxidant and anticancer effects. That suggests that these lichens can be used as new sources of the natural antimicrobial agents, antioxidants and anticancer compounds.

Keywords Lichen · Chemical composition · Antimicrobial activity · Antioxidant activity · Cytotoxic activity

Introduction

Lichens are complex associations composed of fungi (“mycobiont”) and one or more algae or cyanobacteria (“photobionts”) living in symbiosis [1]. Their use in medicine is based on the fact that they contain varied and unique biologically active substances. Lichen metabolites exhibit various biological activities such as antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties. Therefore, lichens are natural

antibiotics and potential drugs [2, 3]. They are very important in medicine because the resistance of microorganisms to many standard antibiotics pose serious threats to human health and creates a huge medical problem in the treatment of infectious diseases [4]. Lichens synthesize a variety of organic compounds as primary and secondary metabolites. They are produced by the fungal partners (mycobiont) of lichens and are deposited in crystal form on the outer surfaces of hyphae. And since these compounds are generally insoluble in water they can be isolated from the lichens using organic solvents [5]. It was found that secondary metabolites of lichens exhibit strong antioxidant activity due to consist of phenolic groups that have the ability to scavenge toxic free radicals. Depsides and depsidones are the largest classes of secondary metabolites of lichens. Depside molecules consist of two to four (2–4) hydroxybenzoic acid residues linked by ester groups, while molecules depsidones have an additional ether bond between aromatic rings, and is believed to be synthesized by oxidative cyclization of depsides. It has been found that depsidones are more efficient antioxidants than

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depsides [6]. So far more than 1000 primary and secondary metabolites, including phenolic compounds, dibenzofurans, depsides, depsidones, depsones, lactones, quinones and pulvinic acid derivatives, characteristic of lichens have been identified and some of them are isolated [7].

The aim of the present study was to identify the secondary metabolites of *Parmelia conspersa* from Serbia and *Parmelia perlata* from Tunisia using HPLC-UV analysis and to investigate the in vitro antimicrobial, antioxidant and anticancer activities of the acetone and methanol extracts from these lichens.

Materials and methods

Lichen material

Samples of the lichens were collected in various places during the summer 2012. *Parmelia conspersa* was collected from Mt. Boracki krs in Serbia and *Parmelia perlata* was collected from Mt. Atlas in Tunisia. The voucher specimen of each species are stored in the Mycological Herbarium of the Department of Biology and Ecology of Kragujevac, Faculty of Science.

Preparation of the lichen extracts

Extracts were prepared by macerating lichen samples with acetone and methanol. The first air-drying lichen material one week at room temperature (26 °C), after which it was ground to a uniform powder. Then, 500 g dry powdered lichen material was soaked in 2000 mL of an appropriate solvent (acetone and methanol) at room temperature for three days. After which extracts were filtered through a Whatman no. 42 (125 mm) filter paper and concentrated in a rotary evaporator. In this way, both extracts has been prepared.

High-performance liquid chromatography (HPLC) analysis

The extracts were dissolved in 1 mL of methanol and analyzed on an Agilent 1200 System HPLC (Agilent Technologies) instrument with C18 column (C18; L×I.D. 25 cm×4.6 mm, 10 μm particle size) and a UV spectrophotometric detector with methanol–water–phosphoric acid (80:20:0.9, v/v/v) solvent. The sample injection volume was 10 μL with a flow rate of 1.0 mL/min. Methanol was of HPLC grade and was purchased from Merck (Darmstadt, Germany). Phosphoric acid was analytical-grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The standards used were obtained from the following sources: stictic acid (a, $t_R = 2.75 \pm 0.10$ min)

from lichen *Xanthoparmelia conspersa*, norstictic acid (b, $t_R = 2.97 \pm 0.10$ min) was isolated from lichen *Ramalina furinacea*, salazinic acid (c, $t_R = 3.07 \pm 0.10$ min) was isolated from lichen *Parmelia saxatilis*, usnic acid was isolated from lichen *Evernia prunastri* (d, $t_R = 8.26 \pm 0.20$ min), atranorin (e, $t_R = 8.92 \pm 0.10$ min) and chloroatranorin (f, $t_R = 11.02 \pm 0.20$ min) from lichen *Parmelia sulcata*.

Fourier transform-infrared spectroscopy (FTIR)

The IR spectra were recorded with on Nicolet iS10 FT-IR spectrometer (Thermo Scientific, USA) equipped with Smart iTR attenuated total reflectance (ATR) accessory. Dry powdery sample was placed on a horizontal ATR crystal of spectrometer applying constant pressure. The sample undergoing a 32-scan and data of infrared absorbance is collected in the range from 4000 to 400 cm^{-1} and resolution of 4.0 cm^{-1} . Prior to each repeated exposure of the sample were obtained reference spectra from cleaned blank crystal. For analysis of spectral data was used Omnic software (version 8.1), and all samples were analyzed in triplicates.

FT-IR analysis

Analysis of the IR spectrum of norstictic acid showed absorption bands at the following frequencies (cm^{-1}): $\nu\text{O-H}$ (3420 cm^{-1}), ν_{as} and ν_{s} of CH_3 (2982 cm^{-1} and 2925 cm^{-1}), δ_{as} and δ_{s} of CH_3 (1442 and 1380 cm^{-1}), lactol carbonyl $\nu\text{C=O}$ (1760 cm^{-1}), aldehyde carbonyl $\nu\text{C=O}$ (1650 cm^{-1}), aromatic $\nu\text{C=C}$ (1573 cm^{-1}), $\nu\text{C-O-C}$ (1286 cm^{-1} , 1242 cm^{-1} , 1152 cm^{-1} , 1086 cm^{-1} and 1058 cm^{-1}). IR spectrum of salazinic acid demonstrated absorption bands at the following frequencies (cm^{-1}): $\nu\text{O-H}$ (3293 cm^{-1}), ν_{as} and ν_{s} of CH_3 (2978 cm^{-1} and 2901 cm^{-1}), δ_{as} and δ_{s} of CH_3 (1434 cm^{-1} and 1375 cm^{-1}), lactol carbonyl $\nu\text{C=O}$ (1769 cm^{-1}), aldehyde carbonyl $\nu\text{C=O}$ (1659 cm^{-1}), aromatic $\nu\text{C=C}$ (1552 cm^{-1}), $\nu\text{C-O-C}$ (1291 cm^{-1} , 1254 cm^{-1} , 1136 cm^{-1} and 1078 cm^{-1}) [8–11].

Isolation of lichen metabolites

Norstictic acid and salazinic acid were isolated from the acetone extracts of *P. conspersa* and *P. perlata* by chromatographic column using benzene – acetone mixtures (20:1, 10:1 and 5:1 v/v), as solvent systems. The dried acetones extracts of the lichen *P. conspersa* and *P. perlata* were dissolved in benzene. After filtration, norstictic acid and salazinic acid were isolated by chromatographic column (silica gel, 0.149–0.074 mm; 100–200 mesh) with benzene – acetone mixtures (20:1, 10:1 and 5:1 v/v), as solvent systems. Norstictic acid and salazinic acid were identified by spectroscopic data and their melting points [8].

Antimicrobial activity

Preparation of test microorganisms

To assess the antimicrobial activity of lichen extracts were used the following bacteria: *Staphylococcus aureus* ATCC 25,923, *Klebsiella pneumoniae* ATCC 13,883, *Escherichia coli* ATCC 25,922, *Proteus vulgaris* ATCC 13,315, *Proteus mirabilis* ATCC 14,153, *Bacillus subtilis* ATCC 6633, and fungi: *Candida albicans* ATCC 10,231 and *Aspergillus niger* ATCC 16,404. The bacteria were cultured on Muller–Hinton agar, while the fungi were cultured on potato-glucose agar for 7 days at room temperature of 20 °C under alternating light and dark conditions. The fungi were recultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed four times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Torlak Institute, Belgrade, Serbia.

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentrations (MIC) of the lichen extract and cirsimarin against test microorganisms were determined by microdilution method using 96 multi-well microtiter plates [12]. All testing of bacterial and fungal cultures were performed in Mueller–Hinton broth and Sabouraud dextrose broth, respectively. In the first row of the plate was pipetted into a volume of 100 µL stock solutions of oil (in methanol, 200 µL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL). In the other wells was added 50 µL of Mueller–Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for analysis of oil). A volume of 50 µL from the first test wells was pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. Then to each well were added 10 µL of resazurin indicator solution (prepared by dissolution of a 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth. Finally, in each well was added 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL). Plates were wrapped in foil to prevent dehydration bacteria and prepared in triplicate, and then they were placed in an incubator at 37 °C for 24 h for the bacteria, and at 28 °C for 48 h for the yeast. Subsequently, color change was assessed visually. MIC values were determined using resazurin. Resazurin is oxidation-reduction indicator that changes color when reduced to resorufin by oxidoreductases within viable cells. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. As MIC value tested compounds was taken average of the three values. Standard antibiotic

amracin was used as a positive control of growth inhibition tested bacteria and ketoconazole was used as control against the tested fungi.

Antioxidant activity

Determination of the total phenolics

Determination of total phenolics content was performed using the Folin-Ciocalteu method [13]. The lichen extract was diluted to the concentration of 1 mg/mL, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of NaHCO₃ (7.5%). The resulting mixture was staying 15 min at the 45 °C, after which absorbance was measured at 765 nm on spectrophotometer against blank sample. Total phenolic content in the extracts were expressed in the form of gallic acid equivalents (mg GA/g extract). The values are presented as means of triplicate analyses.

Determination of total antioxidant capacity

The total antioxidant activity of the lichen extracts was determined using the phosphomolybdenum method [14]. This test is based on the reduction of Mo (VI)-Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 mL of sample extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes with reaction solution were incubated at 95 °C for 90 min. After which the absorbance of the solution was measured at 695 nm using spectrophotometer versus blank after cooling to room temperature. Methanol in the place of extract was used as the blank. As standard was used ascorbic acid (AA). The total antioxidant capacity was determined as milligrams of ascorbic acid per gram of the dry extract (mg AA/g extract).

Determination of DPPH free radical scavenging activity

The free radical scavenging activity of extracts was measured using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazil) according to modificate method from Kumarasamy [15]. 8 mg DPPH was dissolved in 100 mL methanol to obtain a concentration of 80 µg/mL. Then serial dilutions were carried out with the stock solution (1 mg/mL) of the extract. The resulting solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. As reference standards were used ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) and dissolved in methanol were used to make the stock solution with the same concentration (1 mg/mL). Control sample

was prepared containing the same volume without test compounds or reference antioxidants. Methanol 95% was used as blank. Inhibition DPPH free radical scavenging activity (%) of lichen extract was calculated using the following equation:

$$\%inhibition = Ac - As / Ac \times 100$$

where Ac was the absorbance of the control (containing DPPH of the stock solution and methanol), and As was the absorbance of the sample (containing sample extract solution or standard solution without DPPH of the stock solution).

Results are presented as the IC₅₀ values, which is the concentration of the test material that reduces 50% of the free radical concentration, was calculated as µg/mL through sigmoidal dose–response curve.

Determination of the inhibitory activity toward lipid peroxidation

The antioxidant activity of extracts was determined using the thiocyanate method [16]. Serial dilutions were carried out with the stock solution (1 mg/mL) of the extracts, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 mL 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 mL with 40 mM phosphate buffer, pH 7.0. After incubation at 37 °C in the dark for 72 h, a 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL FeCl₂ (20 mM) and 0.1 mL ammonium thiocyanate (30%). The absorbance of this mixture was measured at 500 nm, after it was stirred for 3 min. As reference compounds were used ascorbic acid, gallic acid, α-tocopherol and BHT. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition of linoleic acid peroxidation (%) was calculated using following formula:

$$\%inhibition = Ac - As / Ac \times 100$$

where Ac was the absorbance of the control and As was the absorbance of the sample.

Measurement of ferrous ion chelating ability

Based by decrease in absorbance at 562 nm of the iron (II)-ferrozine complex was measured by ferrous ion chelating ability [17]. One milliliter of 0.125 mM FeSO₄ was added to 1.0 mL sample (with different dilutions), followed by 1.0 mL of 0.3125 mM ferrozine. Before measuring the absorbance,

mixture was allowed to equilibrate for 10 min. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

$$chelating\ effect(\%) = Ac - As / Ac \times 100$$

where Ac was the absorbance of the control and As was the absorbance of the sample.

Determination of hydroxyl radical scavenging activity

The ability of lichen *P. conspersa* and *P. perlata* to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according method described by [18]. The reaction mixture contained 100 µL of extract dissolved in water, 500 µL of 5.6 mM 2-deoxy-D-ribose in KH₂PO₄-NaOH buffer (50 mM, pH 7.4), 200 µL of premixed 100 µM FeCl₃ and 104 mM EDTA (1:1 v/v) solution, 100 µL of 1.0 mM H₂O₂ and 100 µL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50 °C for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (Ac) and of the sample (As), using following formula:

$$\%inhibition = Ac - As / Ac \times 100$$

where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses.

Cytotoxic activity

Measurement of cytotoxic activity by MTT assay

The influence of *P. conspersa* and *P. perlata* extracts on growth of malignantly transformed cell lines was evaluated by MTT assay according to Itharat et al. [19]. The following cell lines have been used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma - HeLa derivative) and L2OB (cell line derived from murine fibroblast). Cells were seeded (2×10^5 cell/mL; 100 µL/well) in 96-well cell culture plates (NUNC) in nutrient medium (MEM Eagle supplemented with 5% (for Hep2c) or 10% (for RD and L2OB) FCS) and grown at 37 °C in humidified atmosphere for 24 h. Then, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) or absolute ethanol (control) diluted with nutrient medium to desired concentrations were added (100 µL/well) and cells were grown at 37 °C in humidified

atmosphere for 48 h. Positive control for each cell line were wells where 100 μL of pure nutrient medium were added. After incubation period, supernatants were discarded, MTT (stock solution: 5 mg/mL in PBS) dissolved in D-MEM medium to final concentration 500 $\mu\text{g}/\text{mL}$ was added in each well (100 $\mu\text{L}/\text{well}$) and plates were incubated at 37 $^{\circ}\text{C}$ in humidified atmosphere for 4 h. Reactions were halted by the addition of 10% SDS/10 mM HCl (100 $\mu\text{L}/\text{well}$). After overnight incubation at 37 $^{\circ}\text{C}$, absorbances were measured at 580 nm using a spectrophotometer (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22,182, USA). The number of viable cells per well (NVC) was calculated from a standard curve drawn as cell numbers plotted against A_{580} . Corresponding cells (grown in flasks), after cell count by hemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (*ut supra*). IC_{50} value 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), and all experiments were done in triplicate.

Statistical analysis

All computations were made by employing the statistical software (SPSS, version 11.0). Experimental results are presented as mean \pm standard deviations of three measurements. Statistical analyses were performed using Student's *t*-test and one way analysis of variance while the probability value of 0.05 was considered significant.

Results and discussion

The HPLC chromatogram for standards and the acetone extracts of the species *P. conspersa* and *P. perlata* are represented in Figs. 1 and 2. In the investigated extracts, depsidones, depsides and dibenzofuran were present as the most

abundant substances classes. In addition to the major secondary metabolites norstictic acid (NOR, $t_{\text{R}} = 2.97 \pm 0.10$) and usnic acid (USN, $t_{\text{R}} = 8.26 \pm 0.20$), stictic acid (STI, $t_{\text{R}} = 2.75 \pm 0.10$) and atranorin (ATR, $t_{\text{R}} = 8.92 \pm 0.10$) were also identified in acetone extract of *P. conspersa*. The acetone extract of *P. perlata* showed that the most intense peaks belong to the salazinic acid (SAL, $t_{\text{R}} = 3.07 \pm 0.10$) and stictic acid (STI, $t_{\text{R}} = 2.75 \pm 0.10$), in addition to the peaks of atranorin (ATR, $t_{\text{R}} = 8.92 \pm 0.10$) and chloroatranorin (CHL, $t_{\text{R}} = 11.02 \pm 0.20$). Identification of these compounds was achieved by comparison of their t_{R} values with the standard substances previously isolated from lichens. The UV absorbance spectral data (200–400 nm) also corresponded with those of standards and found in Yoshimura et al. [20]. Table 1 showed the retention time of the detected lichen substances and their absorbance maxima (nm). The UV spectra of depsidones have 3 absorption maxima and are dissimilar from those of depsides and monocyclic compounds. Stictic, norstictic and salazinic acid belong to the depsidones, while atranorin and chloroatranorin are depside. Usnic acid is antibiotic with dibenzofuran structure and its UV spectrum (absorbance maxima at 234 and 282 nm) is very specific compared with many other lichen substances. Chromatographic peaks identities are reported in Table 1.

Figure 3 showed IR spectra of the lichen acids, norstictic and salazinic, isolated from lichens *P. conspersa* and *P. perlata*. In Table 2 are reported the IR peak values and functional groups of the isolated compounds. The structures of the detected compounds are shown in Fig. 4. Some of the lichen compounds have been previously identified [21–23], while salazinic acid and chloroatranorin are identified for the first time in lichen *P. perlata*. Norstictic acid and atranorin were identified for the first time in lichen *P. conspersa*.

The results of the antimicrobial activity of the lichen extracts against the tested microorganisms are reported in Table 3. Minimum inhibitory concentrations were determined for eight selected microorganisms, of which six bacteria and two fungi. The MIC for the tested extracts of lichens related to the tested bacteria and fungi were ranged from 19.53 to 312.5 $\mu\text{g}/\text{mL}$. The lowest measured MIC value

Fig. 1 HPLC chromatogram of mixed standards used for identification of the lichen compounds. (a = Stictic acid; b = Norstictic acid; c = Salazinic acid; d = Usnic acid; e = Atranorin; f = Chloroatranorin)

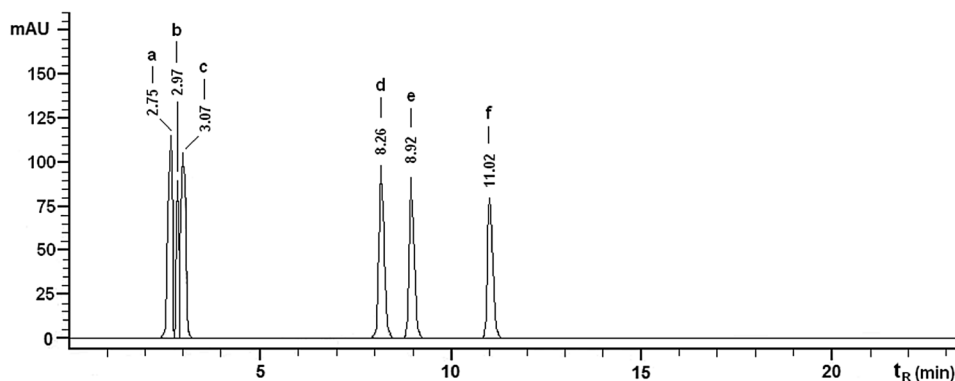


Fig. 2 HPLC chromatograms of the acetone extracts of *P. conspersa* and *P. perlata* at 254 nm

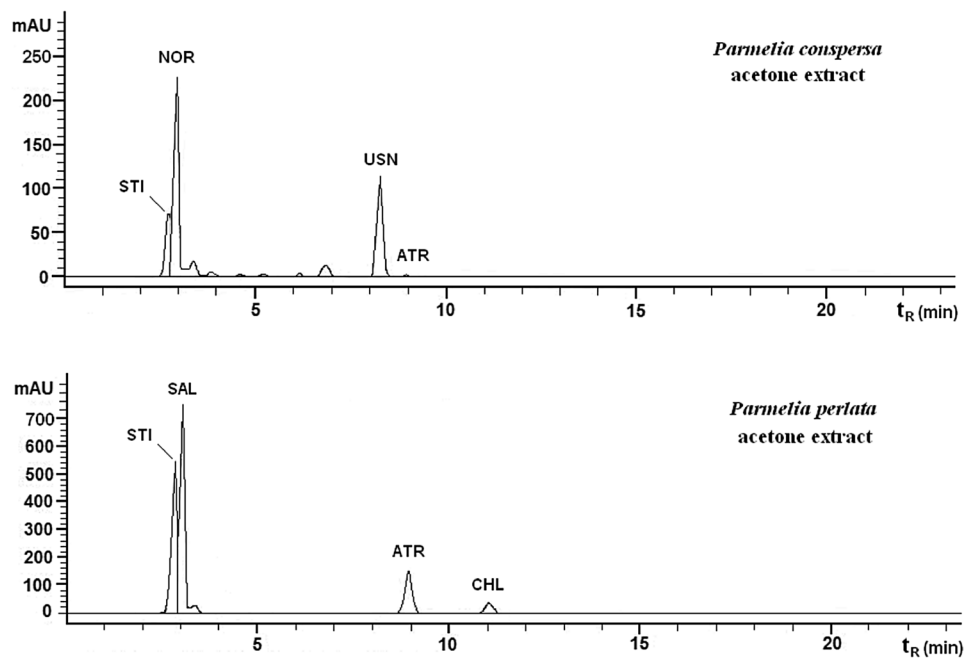


Table 1 Retention time of the examined lichen substances and their absorbance maxima (nm)

Peaks	Compound	Substance class	Retention time ($t_R \pm SD$) ^a (min)	Absorbance maxima (nm) UV spectrum
STI	Stictic acid	Depsidone	2.75 ± 0.10	212, 236, 310 ^m
NOR	Norstictic acid	Depsidone	2.97 ± 0.10	212, 239, 310 ^m
SAL	Salazinic acid	Depsidone	3.07 ± 0.10	213, 238, 312
USN	Usnic acid	Dibenzofurane	8.26 ± 0.20	234, 282
ATR	Atranorin	Depside	8.92 ± 0.10	210, 252, 321 ^m
CHL	Chloroatranorin	Depside	11.02 ± 0.20	213, 252, 315 ^m , 350

^aValues are the means of three determinations $\pm SD$,^m minor absorbance maximum

was $19.53 \mu\text{g/mL}$ related to fungi *Aspergillus niger* for acetone extract of *P. perlata*, while the highest measured MIC value was $312.5 \mu\text{g/mL}$ related to bacteria *Staphylococcus aureus* for acetone extracts of *P. conspersa* and *P. perlata*. The antimicrobial activity was compared with the standard antibiotics amracin and ketoconazole for bacteria and fungi, respectively. *Parmelia perlata* is used in traditional medicine treatment for a number of ailments and as a food supplement. So far there are reports of antimicrobial activity of *P. perlata* but it differs from our study, because it has been used different test microorganisms for examination samples [24]. There are also studies on antibacterial activity of *P. perlata* extracts on *Staphylococcus aureus* [25, 26]. The relevant antimicrobial activity of these lichens probably depends on the presence of secondary metabolites, such as stictic acid, norstictic acid, usnic acid and atranorin at *P. conspersa*, and such as stictic acid, salazinic acid, atranorin and chloroatranorin at *P. perlata*. There are some studies about the antimicrobial activity of these compounds [3, 27–29].

Until now, many researchers have been investigated the antioxidant properties of many lichens extracts and some of them showed very good antioxidant activity [30–32]. Secondary metabolites that have been identified and isolated from some *Parmelia* species manifested high antioxidant activity [3]. Table 4 shows the results of determination of total phenols and antioxidant capacity of the examined lichen extracts. The total phenolic contents expressed as gallic acid equivalents of the methanol and acetone extracts were amounted to 93.12 and 90.03 mg GA/g for *P. conspersa* and 94.26 and 89.26 mg GA/g for *P. perlata*, respectively. These results showed that the methanol extracts of both lichens had a higher total phenolic content than the acetone extracts. Results for total antioxidant capacity showed that methanol extracts of *P. conspersa* and *P. perlata* with antioxidant capacity of 90.26 and 94.66 $\mu\text{g AA/g}$ possess a greater antioxidant activity than the acetone extracts of these lichens with antioxidant capacity of 81.11 and 83.09 $\mu\text{g AA/g}$, respectively.

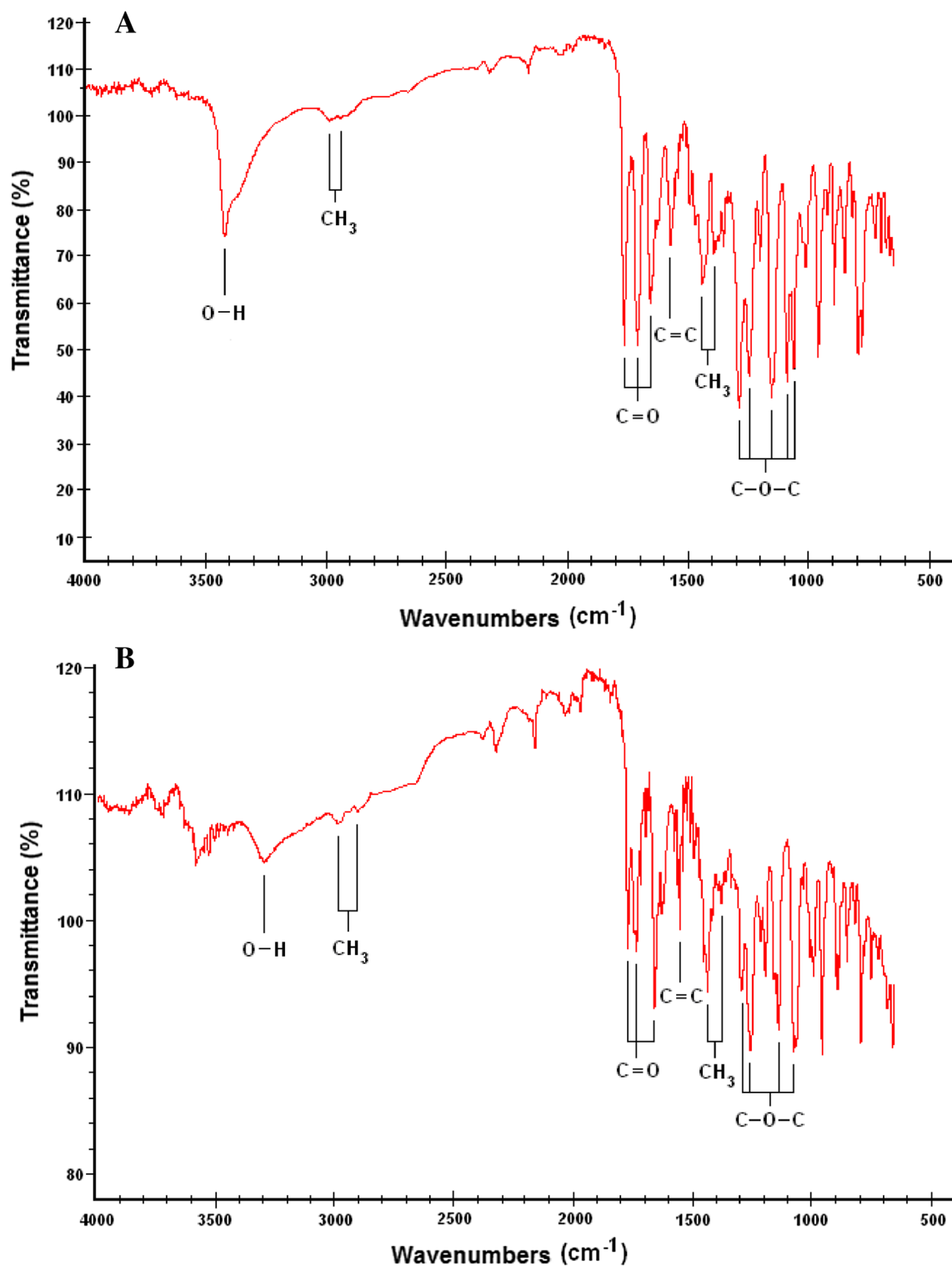
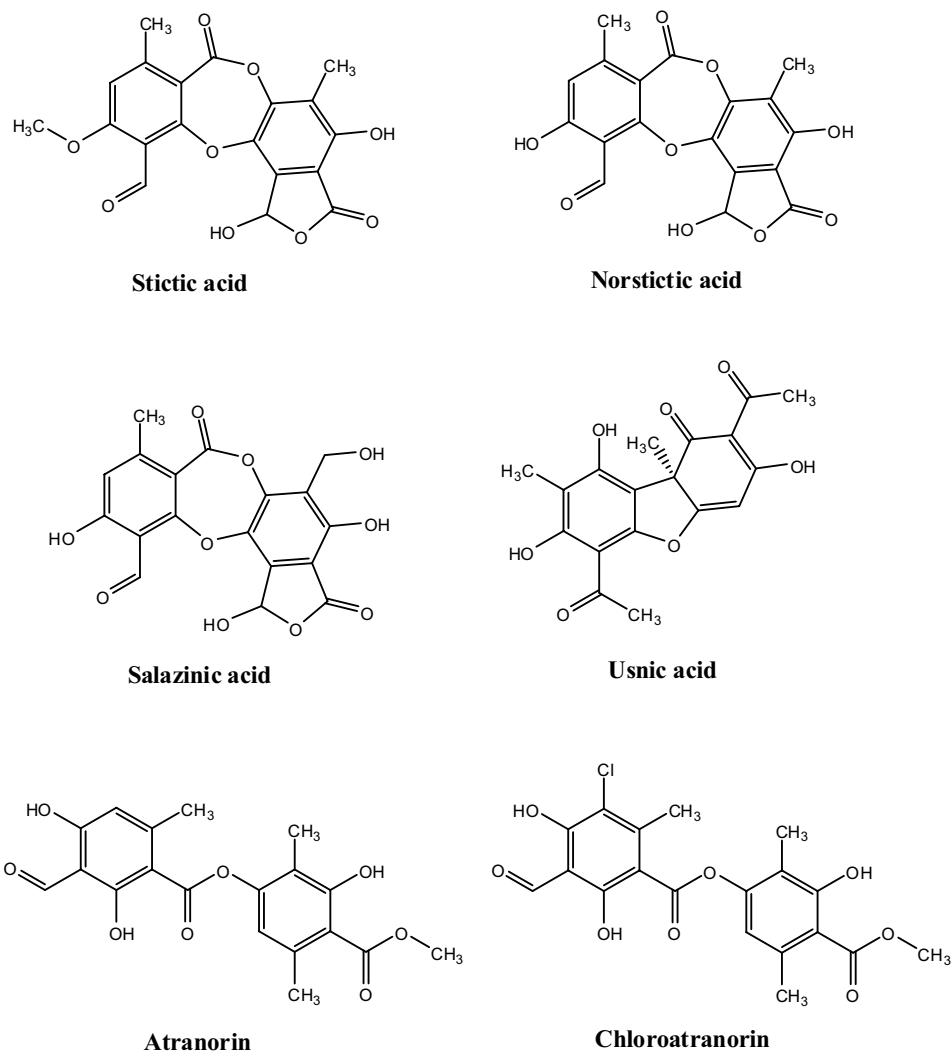


Fig. 3 IR spectrum of norstictic acid (a) and salazinic acid (b)

In Table 5 are given the results of DPPH scavenging activity for the examined *P. conspersa* and *P. perlata* extracts. The IC₅₀ values of all tested extracts ranged from 47.11 to 57.11 µg/mL. Among the examined extracts, the

methanol extract from *P. conspersa* showed the largest DPPH scavenging activity (47.11 ± 0.23 µg/mL). Also, the methanol extract of *P. conspersa* displayed a higher antioxidant activity compared to methanol, chloroform and

Fig. 4 Chemical structures of the identified lichen metabolites**Table 2** IR peak values and functional groups of isolated compounds

Compound	Functional group	Peak values ν_{\max} (cm ⁻¹)
Norstictic acid	O-H	3420 m
	CH ₃	2982w, 2925w, 1442 m, 1380 m
	C=O	1760s, 1707s, 1650s
	C=C	1573 m
	C-O-C	1286s, 1242s, 1152s, 1086s, 1058s
Salazinic acid	O-H	3293 m
	CH ₃	2978w, 2901w, 1434s, 1375 m
	C=O	1769s, 1732s, 1659s
	C=C	1552 m
	C-O-C	1291s, 1254s, 1136s, 1078s

s - strong, m - medium, w - weak

petrol ether extracts of *Toninia candida* [33]. However, the methanol and acetone extracts of *P. conspersa* and *P. perlata* showed stronger antioxidant activity than the acetone extract of *Toninia candida* and *Usnea barbata* [34], and many other lichen species [32].

The results of inhibitory activity towards lipid peroxidation (Table 5) demonstrated that all examined extracts of *P. conspersa* and *P. perlata* exhibited significant inhibitory activity (from 22.29 µg/mL to 35.87 µg/mL).

Also in Table 5 are shown IC₅₀ values for the metal chelating activity and these values were very similar for examined extracts of *P. conspersa* and *P. perlata*.

The hydroxyl radical scavenging activity of the examined extracts are given in Table 5. The IC₅₀ value of the methanol and acetone extracts were 55.52 and 64.17 µg/mL for *P. conspersa*, and 59.41 and 62.34 µg/mL for *P. perlata*, respectively. The results suggest that the methanol and acetone extracts of *P. conspersa* and *P. perlata* were free radical scavengers, acting possibly as primary antioxidants.

Table 3 Antimicrobial activity of the extracts of *P. conspersa* and *P. perlata*

Microorganisms	<i>Parmelia conspersa</i>		<i>Parmelia perlata</i>		Standard antibiotic	
	Methanol extract	Acetone extract	Methanol extract	Acetone extract	Amracin	Ketoconazole
<i>Staphylococcus aureus</i>	78.125	312.5	156.25	312.5	0.97	–
<i>Klebsiella pneumoniae</i>	156.25	156.25	156.25	156.25	0.49	–
<i>Escherichia coli</i>	39.1	78.125	39.1	39.1	0.97	–
<i>Proteus vulgaris</i>	78.125	78.125	78.125	78.125	0.49	–
<i>Proteus mirabilis</i>	39.1	156.25	156.25	78.125	0.49	–
<i>Bacillus subtilis</i>	78.125	78.125	78.125	156.25	0.24	–
<i>Candida albicans</i>	39.1	39.1	78.125	78.125	–	1.95
<i>Aspergillus niger</i>	39.1	39.1	39.1	19.53	–	0.97

^aMinimum inhibitory concentration (MIC); the values are presented as means of triplicate analyses

Table 4 Total phenolic and total antioxidant capacity of the examined *P. conspersa* and *P. perlata* extracts

Lichen species	Extracts	Total phenolic (mg GA/g)	Total antioxidant capacity (µg AA/g)
<i>Parmelia conspersa</i>	Methanol	93.12 ± 0.58	90.26 ± 0.69
	Acetone	90.03 ± 1.09	81.11 ± 0.93
<i>Parmelia perlata</i>	Methanol	94.26 ± 0.69	94.66 ± 0.78
	Acetone	89.26 ± 0.93	83.09 ± 0.65

The tested extracts of *P. conspersa* and *P. perlata* show strong antioxidant activity against different oxidative systems. The strong antioxidant activity is the result of high total phenolic content of tested extracts, because between the total phenolic content and the antioxidant activity is a positive correlation, and there are reports about it [35, 36]. Nevertheless, it should be taken into consideration that individual phenolics may have distinct antioxidant activities;

there may be antagonistic or synergistic interactions between phenolics and other compounds like carbohydrates and proteins [37]. Mainly, the antioxidant activity of phenolic compounds is affected by their chemical structure. The antioxidant activity of phenols also depends on the type and polarity of the extracting solvent, the extraction procedures, as well as the test system and substrate to be protected by the antioxidant [38].

Phenolic compounds express the antioxidant effects mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [39]. Some metabolites of lichens in their structure contain phenolic groups which are considered to be a key element for the antioxidative efficiency [40]. Salazinic and norstictic acid possesses two phenolic groups in their molecules, while stictic acid has one phenolic group.

Table 6 shows the IC₅₀ values of cytotoxic activity of the tested extracts on Hep2c, RD and L2OB cells, determined

Table 5 The antioxidant activity of the examined *P. conspersa* and *P. perlata* extracts

Lichen/extracts/standards		^a IC ₅₀ (µg/mL)			
		DPPH scavenging activity	Inhibitory activity against lipid peroxidation	Metal chelating activity	Hydroxyl radical scavenging activity
<i>Parmelia conspersa</i>	Methanol	47.11 ± 0.23	22.29 ± 1.25	33.45 ± 1.54	55.52 ± 1.34
	Acetone	53.23 ± 0.59	35.87 ± 1.03	33.23 ± 1.09	64.17 ± 0.92
<i>Parmelia perlata</i>	Methanol	53.45 ± 1.45	26.46 ± 1.58	39.91 ± 0.68	59.41 ± 0.43
	Acetone	57.11 ± 0.88	31.30 ± 1.23	43.23 ± 1.03	62.34 ± 0.84
Gallic acid		3.79 ± 0.69	255.43 ± 11.68	–	59.14 ± 1.10
Ascorbic acid		6.05 ± 0.34	> 1000	–	160.55 ± 2.31
BHT		15.61 ± 1.26	1.00 ± 0.23	–	33.92 ± 0.79
α-Tocopherol		–	0.48 ± 0.05	–	–

^aIC₅₀ values were determined by nonlinear regression analysis. Results are mean values ± SD from three experiments

Table 6 Growth inhibitory effects of *P. conspersa* and *P. perlata* extracts on Hep2c, RD and L2OB cell lines assessed by MTT assay

Lichen /extracts /control	Time (h)	IC ₅₀ (µg/mL)		
		Cell lines		
		Hep2c	RD	L2OB
<i>Parmelia conspersa</i>				
Methanol	48	94.67 ± 5.67	76.53 ± 0.94	102.28 ± 7.10
Acetone		163.39 ± 0.46	88.59 ± 2.34	150.34 ± 0.90
<i>Parmelia perlata</i>				
Methanol	48	98.34 ± 3.59	76.33 ± 0.44	105.54 ± 4.19
Acetone		135.44 ± 0.50	87.49 ± 4.36	147.17 ± 0.27
<i>Cis-DDP</i>	48	9.43 ± 0.72	1.94 ± 0.75	7.1 ± 0.94

by MTT assay. The examined extracts of *P. conspersa* and *P. perlata* expressed significant cytotoxic activity against the three cell lines. The IC₅₀ values of all tested extracts ranged from 76.33 to 163.39 µg/mL. The methanol extracts of both lichens showed better cytotoxic effect towards the tested cell lines than their acetone extracts, while the tested extracts showed the best cytotoxic activity towards RD cells. The positive control (Cis-DDP) had slightly better cytotoxic activity than tested extracts.

There are previous reports on examination cytotoxic activity of many lichens [41, 42], but in this study first time was explored cytotoxic activity of *P. conspersa*. The molecular mechanism of cell death by lichen compounds includes cell cycle arrest, apoptosis, necrosis, and inhibition of angiogenesis [43]. Bačkorová and colleagues reported that antiproliferative/ cytotoxic effects of atranorin efficiently induced apoptosis and inhibited cell proliferation in various cancer cell lines tested. This study has confirmed a differential sensitivity of cancer cell lines to lichen secondary metabolites [44]. Depsidones, salazinic acid and stictic acid were evaluated for their cytotoxic activity towards hepatocytes from rat and lymphocytes from rat spleens. The research has shown that salazinic acid and stictic acid, showed apoptosis of hepatocytes in a dose dependent manner with stictic acid showing the strongest apoptotic activity [45]. Also, in the work of Pejin and associates, it is shown that the results suggest a moderate anticancer activity towards malignant HT-29. This may indicate that norstictic acid can be considered as a promising lead compound for the design of novel human colon adenocarcinoma drugs [46].

Conclusions

This is the first report of salazinic acid and chloroatranorin in *P. perlata* and norstictic acid and atranorin in *P. conspersa*. In addition, salazinic acid and norstictic acid are isolated from these lichens. All compounds that are present in these lichens have already been identified in other lichen

species. The extracts of these lichens showed significant antimicrobial, antioxidant and anticancer activities in vitro. There seems to have been a recent upsurge in the recognition of the potential of lichens as useful source of secondary metabolites with varied biological activities. And this is partly due to the varied and fairly documented biological properties of lichen secondary metabolites. In this study antioxidant activity of *P. perlata* and anticancer activity of *P. conspersa* were tested for the first time. This study suggests that lichens can be a new source of natural antimicrobial, antioxidant and anticancer agents. Future investigation will be focused on isolation of new compounds from lichens and determination of their biological activities in vitro and in vivo.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JT, AR and NM. The first draft of the manuscript was written by JT and NM. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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