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Chemical composition of three *Parmelia* lichens and antioxidant, antimicrobial and cytotoxic activities of some their major metabolites

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ABSTRACT

The aim of this study is to investigate chemical composition of acetone extracts of the lichens *Parmelia caperata*, *P. saxatilis* and *P. sulcata* and antioxidant, antimicrobial and anticancer activities of some their major metabolites. The phytochemical analysis of acetone extracts of three *Parmelia* lichens were determined by HPLC-UV method. The predominant phenolic compounds in these extracts were protocetraric and usnic acids (*P. caperata*) and depsidone salazinic acid (other two species). Besides these compounds, atranorin and chloroatranorin, were also detected in some of these extracts. Antioxidant activity of their isolated metabolites was evaluated by free radical scavenging, superoxide anion radical scavenging and reducing power. As a result of the study salazinic acid had stronger antioxidant activity than protocetraric acid. The antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method. Both compounds were highly active with minimum inhibitory concentration values ranging from 0.015 to 1 mg/ml. Anticancer activity was tested against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines using MTT method. Salazinic acid and protocetraric acid were found to be strong anticancer activity toward both cell lines with IC_{50} values ranging from 35.67 to 60.18 μg/ml. The present study shows that tested lichen compounds demonstrated a strong antioxidant, antimicrobial, and anticancer effects. That suggest that these lichens can be used as new sources of the natural antimicrobial agents, antioxidants and anticancer compounds.

Introduction

Lichens are complex symbiotic associations between a fungus (mycobiont) and photobiont which can be either an alga or cyanobacteria (Bates et al. 2011). They are proven as the earliest colonizers of terrestrial habitats on the earth with a worldwide distribution from artic to tropical regions and from the plains to the highest mountains. Their specific, even extreme, conditions existence, slow growth and long life are the reason for producing of numerous protective compounds against different physical and biological influences (Mitrović et al. 2011).

Lichens synthesize a variety of secondary metabolites, mostly from fungal metabolism. They are crystals deposited on the surface of hiphes. They are poorly soluble in water and can usually be isolated from a lichen by organic dilutants (Otzurk et al. 1999). More than one hundred secondary metabolites, mainly monoaromatics, depsides, depsidones, pulvinates, dibenzofurans, anthraquinones and xanthones, characteristic of lichen have been detected and isolated (Molnar and Farkaš 2010). Chemicals structures of these classes of compounds are similar and identification is often very difficult.For a long time, some lichen species have been used in traditional medicine in the treatment of numerous infectious diseases (Bown 2001). The use of lichens in medicine is based on the fact that they contain unique and varied biologically active substances. Lichen substances exert a wide variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects (Kosanić et al. 2012a; Manojlović et al. 2010). Thus, the aim of the present work was to identify of secondary metabolites of *P. caperata*, *P. saxatilis* and *P. sulcata* by HPLC-UV and to evaluate the antioxidant capacity, antimicrobial and anticancer activities of the acetone extracts from this lichen as well as their major secondary metabolites.

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Material and methods

Lichen samples

Lichen samples of *P. caperata* (L.) Ach. *P. sulcata* (Taylor) and *P. saxatilis* (L.) Ach. 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.

Preparation of the lichen extracts

Finely dry ground thalli of the investigated 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% dimethylsulfoxide (DMSO) for the experiments.

High performance liquid chromatography (HPLC) analysis

Dry lichen extracts were redissolved in 500 μl of acetone and analyzed on an 1200 Series HPLC (Agilent Technologies) instrument with C18 column (C18; 25 cm × 4.6 mm, 10 m) and a UV spectrophotometric detector with methanol–water–phosphoric acid (75:25:0.9, v/v/v) solvent. 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 flow rate was 1.0 ml/min. The sample injection volume was 10 μl. The standards used were obtained from the following sources: salazinic acid (SAL, $t_R = 3.01 \pm 0.20$ min) was isolated from lichen *Lobaria pulmonaria*, protocetraric acid (PRO, $t_R = 3.62 \pm 0.20$ min) isolated from lichen *Toninia candida*, usnic acid (USN, $t_R = 10.53 \pm 0.30$ min), atranorin (ATR, $t_R = 11.72 \pm 0.10$ min) and chloroatranorin (CHL, $t_R = 13.23 \pm 0.20$ min) from lichen *Evernia prunastri*.

Isolation of lichen metabolites

Usnic acid (190 mg) was isolated from the acetone extract of *P. caperata* (500 mg) by precipitation in benzene, and in this study it has been used as a standard compound. Protocetraric acid was isolated from the filtrate residue using a silica gel column (0.149–0.074 mm; 100–200 mesh) with methanol–chloroform gradient solvent (10:1 and 5:1). Salazinic acid (300 mg) was isolated from acetone extract of *P. saxatilis* (500 mg) by precipitation in acetone. All isolated compounds were identified by their melting points and spectroscopic data (Huneck and Yoshimura 1996).

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 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 vigorosly and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in spectrophotometer ("Jenway", UK). Free radical scavenging activity was compared to ascorbic acid and usnic acid. The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scavenging effect}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

where A_0 is the absorbance of the negative control and A_1 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 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 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 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 is increased. Reducing power was compared to ascorbic acid and usnic acid.

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 methosulfate (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. Superoxide anion scavenging activity was compared to ascorbic acid and usnic acid. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging activity}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

where A_0 is the absorbance of the negative control and A_1 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.

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 the bacteria used were obtained from the American Type Culture Collection (ATCC). Their identification was confirmed at the Microbiological Laboratory of Kragujevac, University of Kragujevac, Department of Biology. 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 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).