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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Chemical composition of *Hypogymnia physodes* lichen and biological activities of some its major metabolites

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Abstract The aim of this study is to investigate chemical composition of acetone extract of the lichen *Hypogymnia physodes* and antioxidant, -microbial, and -cancer activities of some its major metabolites. Four depsidones (fumarprotocetraric-, 3-hydroxyphysodalic-, physodalic-, and physodic acids), two depsides (atranorin and chloroatranorin), and usnic acid were identified from the lichen *H. physodes* growing in Serbia using high-performance liquid chromatography with photodiode array detector. Antioxidant activity of isolated metabolites was evaluated by free radical scavenging, superoxide anion radical scavenging, and reducing power. As a result of this study physodic acid was found to have stronger antioxidant activities. The antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method. All isolated compounds were highly active with minimum inhibitory concentration values ranging from 0.0008 to 1 mg/ml. Anticancer activity was tested against FemX (human melanoma) and LS 174 (human colon carcinoma) cell lines using microculture tetrazolium method. Tested compounds were found have strong anticancer activity toward both cell lines with IC_{50} values ranging from 12.72 ± 0.35 to 24.63 ± 2.15 µg/ml. The present study shows that isolated lichen compounds demonstrated strong antioxidant, -microbial, and -cancer effects. The results suggest that this lichen can be used as new sources of the natural antimicrobial agents, antioxidants, and -cancer compounds.

Keywords *Hypogymnia physodes* · Lichen metabolites · Antioxidant activity · Antimicrobial activity · Anticancer activity

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Introduction

Lichens are complex symbiotic associations between a fungus (mycobiont) and photobiont which can be either an alga or cyanobacterium (Bates *et al.*, 2011). They have been proven to be the earliest colonizers of terrestrial habitats on the earth with a worldwide distribution from arctic to tropical regions and from the plains to the highest mountains. Their specific, even extreme, range of habitats, slow growth, and long life are the reason for them being able to produce numerous protective secondary metabolites against different physical and biological influences (Mitrović *et al.*, 2011a, b).

Lichens' secondary metabolites are synthesized mostly from fungal metabolism. They are crystal deposits on the surface of hiphes, which are badly dilutable in water and can usually be isolated from lichens by organic dilutants (Otzurk *et al.*, 1999). More than 100 secondary metabolites of lichen have been detected and isolated (Molnar and Farkaš, 2010). Chemical structures of classes of these compounds are similar and identification is often very difficult.

Lichen substances exert a wide variety of biological actions including antibiotic, -mycotic, -viral, -inflammatory, analgesic, antipyretic, -proliferative, and cytotoxic effects (Kosanić *et al.*, 2012a, b; Manojlović *et al.*, 2010).

Due to a relatively recent resurgence in lichen bioactivity, the therapeutic potential of many classes of lichen metabolites in medicine has largely remained unexplored. Thus, the aim of the present work was to present results of high-performance liquid chromatography (HPLC) analysis of the acetone extract of the lichen *Hypogymnia physodes* and evaluate the antioxidant capacity, antimicrobial and cytotoxic activities of its major secondary metabolites.

Results and discussion

This paper deals with the phytochemical analysis of acetone extract from the species *H. physodes*, lichen growing in Serbia. For the phytochemical analysis, a HPLC–UV method was used for the identification of phenolic compounds, specially depsides, depsidones, and usnic acid, in this lichen. Comparing the retention times (t_R) and UV spectra (200–400 nm) from HPLC–UV with those of authentic substances, it is readily confirmed that the fumarprotocetraric- ($t_R = 3.12 \pm 0.10$ min), 3-hydroxyphysodalic- ($t_R = 3.98 \pm 0.1$ min), physodalic- ($t_R = 4.31 \pm 0.10$ min), physodic- ($t_R = 5.91 \pm 0.10$ min), usnic acids ($t_R = 14.32 \pm 0.20$ min), atranorin ($t_R = 16.21 \pm 0.10$ min), and chloroatranorin ($t_R = 17.73 \pm 0.20$ min) are the metabolites present in acetone extract of *H. physodes*. The chromatograms for standards and *H. physodes* acetone extract eluted by HPLC are represented in Figs. 1 and 2. Table 1 shows the retention time of the detected lichen substances and their absorbance maxima (nm). Identification of these compounds was achieved by comparison of their retention times values with the standard substances previously isolated from lichens. Compounds identified in the extract mainly belong to depsidones (fumarprotocetraric-, 3-hydroxyphysodalic-, physodalic-, and physodic acids), some of them are depsides (atranorin and chloroatranorin), while usnic acid is well-known lichen metabolite with dibenzofuran structure which exhibits antibiotic activity. The structures of the detected compounds are shown in Fig. 3. After detection of the present compounds in lichens, major lichen metabolites in this species (usnic acid, atranorin, and physodic acid) were isolated by chromatographic column using different solvent systems and used for antioxidant, -microbial, and -cancer investigations.

The scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH)- and superoxide anion radicals by the tested compounds is shown in Table 2. There was a statistically significant difference between tested samples and control ($P < 0.05$). Physodic acid showed stronger DPPH- and superoxide anion radicals scavenging activity than usnic acid and atranorin. The inhibition concentration at 50 % inhibition (IC_{50}) values were 69.110 ± 0.35 and 169.65 ± 1.78 μg/ml for physodic acid, 130.73 ± 1.35 and 197.28 ± 1.82 μg/ml for usnic acid, and 131.48 ± 1.93 and 632.01 ± 2.13 μg/ml for atranorin (for DPPH- and superoxide anion radicals scavenging activity, respectively). As shown in Table 3, physodic acid also demonstrated the strongest reducing power.

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

The antimicrobial activity of the lichen components against the test microorganisms is shown in Table 4. The minimal inhibitory concentration (MIC) for components relative to the tested microorganisms ranged from 0.0008 to 1 mg/ml. Usnic- and physodic acids showed very strong and similar antimicrobial activity, followed by atranorin.

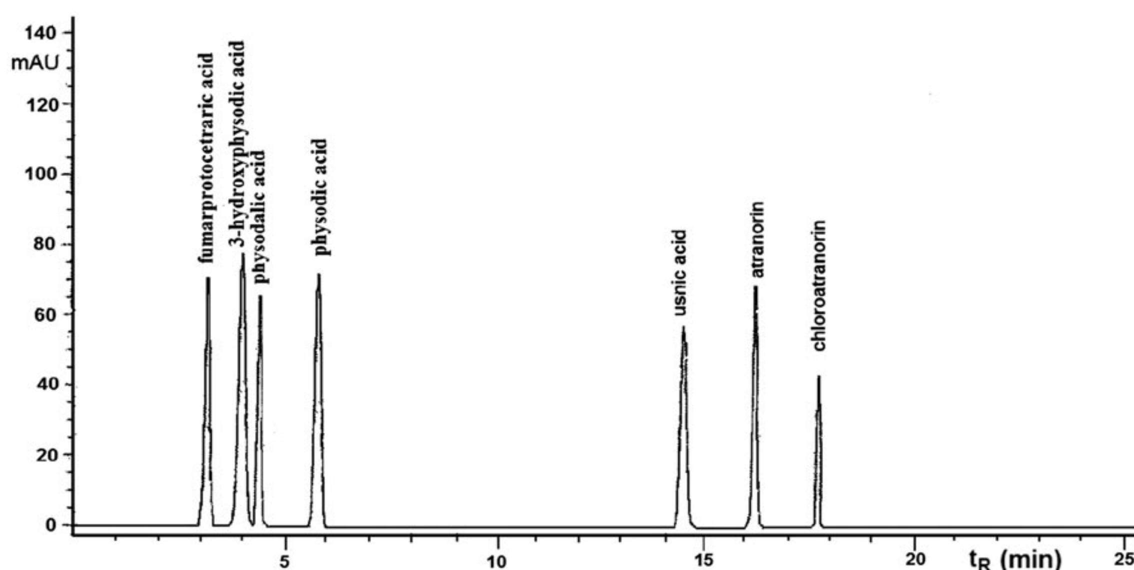

Fig. 1 Chromatogram of the standards used for identification of the compounds present in *Hypogymnia physodes*

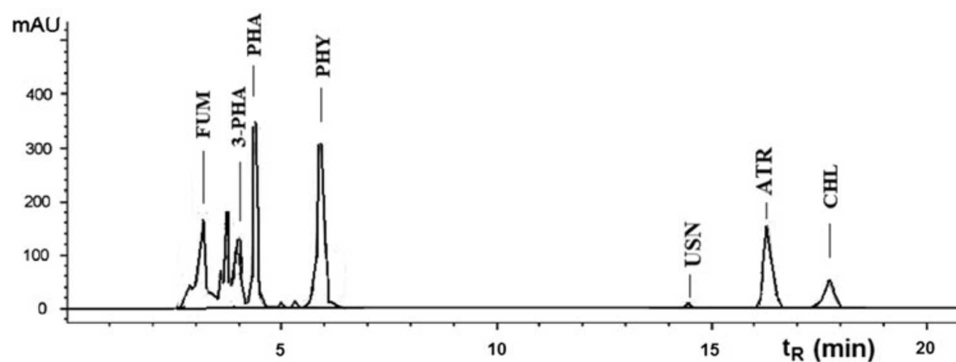

Fig. 2 HPLC chromatograms acquired at 254 nm of the acetone extract of *Hypogymnia physodes*. Chromatographic *peaks* identities are reported in Table 1

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

Symbols	Compounds	Substance classes	Retention time, $t_R \pm$ SD[a] (min)	Absorbance maxima (nm) UV spectrum
FUM	Fumarprotocetraric acid	Depsidone	3.12 ± 0.10	212, 240, 318
3-PHY	3-Hydroxyphysodalic acid	Depsidone	3.98 ± 0.10	205, 278, 308
PHA	Physodalic acid	Depsidone	4.31 ± 0.10	212, 242, 318
PHY	Physodic acid	Depsidone	5.91 ± 0.10	212, 263, 314
USN	Usnic acid	Dibenzofuran	14.32 ± 0.20	234, 282
ATR	Atranorin	Depside	16.21 ± 0.20	210, 252, 321[m]
CHL	Chloroatranorin	Depside	17.73 ± 0.20	213, 252, 315[m], 350

m Minor absorbance maximum

[a] Values are the means of three determinations ± SD

Antibacterial activity was stronger than antifungal activity for all compounds.

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

The cytotoxic activity of the tested 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 all compounds relative to the tested cells ranged from 12.72 ± 0.35 to 24.63 ± 2.15 μg/ml. The best cytotoxic activity was exhibited by usnic acid, followed by physodic acid and atranorin.

As a shown in the Table 5, positive control *cis*-diamminedichloroplatinum (*cis*-DDP) had slightly better cytotoxic activity than the tested components.

Some extracts from *H. physodes* lichen have been previously investigated for their biological activity. Mitrović *et al.* (2011a, b) published the results of antioxidant, -microbial, and -proliferative activities of methanol extract of *H. physodes*. Stojanović *et al.* (2010) also reported reducing power and radical scavenging activity for this species. Cansaran-Duman *et al.* (2010) found the antimicrobial effect by various *Hypogymnia* species.

Since in literature there is no data on biological activity of secondary metabolites from *H. physodes* lichen, here we report in vitro antioxidant, -microbial, and -cancer activities of compounds usnic acid, atranorin, and physodic acid isolated from this species.

The tested lichen compounds have a strong antioxidant activity against various oxidative systems in vitro. The isolated components belong to phenols, indicating an important role of phenol in the antioxidant activity for lichens. In fact, number of previous studies found that the lichens which were found to have higher content of phenols exert stronger antioxidant activity (Behera *et al.*, 2009; Kosanić *et al.*, 2012a). 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 *et al.*, 2012b). However, some authors believe that the antioxidant activity of lichens 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 lichen compounds was also studied by other researchers. For example, Luo *et al.* (2009) found antioxidant activity for lecanoric acid from *Umbilicaria antarctica*. Hidalgo *et al.* (1994) find

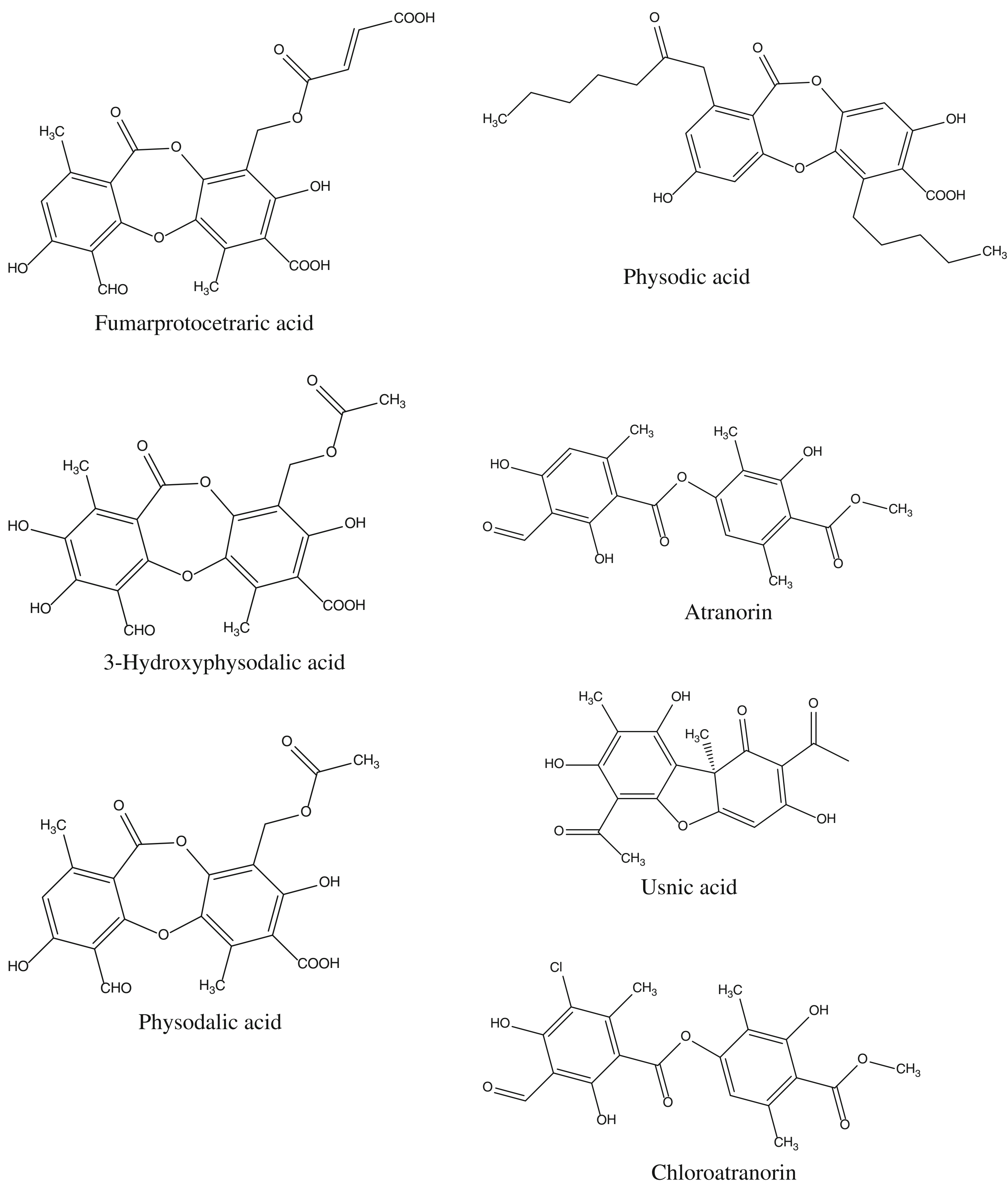

Fig. 3 Structures of the identified compounds

antioxidant activity of atranorin isolated from *Placopsis* sp. and divaricatic acid isolated from *Protousnea malaceae*. Amo de Paz *et al.* (2010) explored antioxidant properties of salazinic-, stictic-, and usnic acids from *Xanthoparmelia* sp.

In our experiments, the tested lichen compounds show very strong antimicrobial activity. 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 *et al.*, 2012b). However, it is necessary understand that lichens contain a large number of natural compounds, and their antimicrobial activity is not only a result of the different activities of individual components but may also be the result of their interactions, which can have different effects on the overall activity of lichens.

The intensity of the antimicrobial effect depended on the species of organism tested. The compounds used in this study had a stronger antibacterial than -fungal activity. Also, the tested compounds showed more potent inhibitory effects on Gram-positive bacteria than on -negative. This observation is in accordance with other studies (Yang and Anderson, 1999; Kosanić *et al.*, 2012b; Agrawal and Talele, 2013) focused on the antimicrobial activity which have demonstrated that Gram-positive bacteria are more sensitive to the antimicrobial activity than the Gram-negative bacteria and fungi due to differences in the composition and permeability of the cell wall. The cell wall of Gram-positive bacteria is made of peptidoglycans and teichoic acids, while the cell wall of Gram-negative bacteria is made of peptidoglycans, lipopolysaccharides, and proteins (Heijenoort, 2001; Kosanić *et al.*, 2012a). The cell wall of fungi is poorly permeable and it consists of polysaccharides such as hitchin and glucan (Farkaš, 2003).

Numerous lichen compounds were screened for antimicrobial activity in search of the new antimicrobial agents. Candan *et al.* (2006) find an antimicrobial activity for gyrophoric acid from *Xanthoparmelia pokornyi*. Similar results were reported by Turk *et al.* (2006) for atranorin from *Pseudoevernia furfuraceae*. Kosanić and Ranković (2011) found out that fumarprotocetraric acid from *Cladonia furcata* had a strong antimicrobial influence.

Table 2 DPPH radical and superoxide anion scavenging activities of isolated compounds

Lichen compounds	DPPH radical scavenging IC_{50} (μg/ml)	Superoxide anion scavenging IC_{50} (μg/ml)
Usnic acid	130.73 ± 1.35	197.28 ± 1.82
Atranorin	131.48 ± 1.93	632.01 ± 2.13
Physodic acid	69.110 ± 0.35	169.65 ± 1.78
Ascorbic acid	6.42 ± 0.18	115.61 ± 1.16

Table 5 Growth inhibitory effects of acetone extracts of isolated compounds on FemX and LS 174 cell lines

Lichen compounds	FemX IC_{50} (μg/ml)	LS 174
Usnic acid	12.72 ± 0.35	15.66 ± 1.45
Atranorin	20.91 ± 1.98	24.63 ± 2.15
Physodic acid	19.52 ± 0.87	17.89 ± 0.73
Cis-DDP	0.94 ± 0.35	2.3 ± 0.31

Table 3 Reducing power of acetone extracts of isolated compounds

Lichen compounds	Absorbance (700 nm)				
	1000 μg/ml	500 μg/ml	250 μg/ml	125 μg/ml	62.5 μg/ml
Usnic acid	0.6723 ± 0.015	0.5468 ± 0.011	0.0692 ± 0.008	0.0249 ± 0.004	0.0101 ± 0.003
Atranorin	0.1523 ± 0.009	0.0830 ± 0.006	0.0493 ± 0.006	0.0467 ± 0.007	0.0397 ± 0.005
Physodic acid	0.8773 ± 0.028	0.6641 ± 0.024	0.0870 ± 0.009	0.0422 ± 0.006	0.0275 ± 0.003
Ascorbic acid	2.113 ± 0.032	1.654 ± 0.021	0.0957 ± 0.008	0.0478 ± 0.008	0.0297 ± 0.004

Table 4 Minimum inhibitory concentration (MIC) of acetone extracts of isolated compounds

Lichen compounds	Usnic acid	Atranorin	Physodic acid	*S*	*K*
B. mycoides	0.0008	0.015	0.0016	7.81	–
B. subtilis	0.0008	0.0312	0.0008	7.81	–
E. coli	0.25	1	0.5	31.25	–
K. pneumoniae	0.0625	0.5	0.0075	1.95	–
S. aureus	0.125	0.25	0.0312	31.25	–
A. flavus	0.5	1	1	–	3.9
A. fumigatus	0.25	0.5	0.5	–	3.9
C. albicans	0.125	0.25	0.25	–	1.95
P. purpurescens	0.5	1	1	–	3.9
P. verrucosum	0.5	1	1	–	3.9

Values are given as mg/ml for tested samples and as μg/ml for antibiotics

K ketoconazole, *S* streptomycin

In present study, the results clearly demonstrated that isolated compounds from *H. physodes* induced significant cytotoxic effect on the tested cancer cell lines. Until now, only few researchers proved that lichen compounds have anticancer activity. Einarsdottir *et al.* (2010) reported significant anticancer effect for (+)-usnic acid from *Cladonia arbuscula* and (−)-usnic acid from *Alectoria ochroleuca*. Bogo *et al.* (2010) explored anticancer properties of lecanoric acid from *Parmoterma tinctorum*. Burlando *et al.* (2009) found anticancer activity for several lichen compounds. Some literature data reported that lichen components are responsible for overall 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 lichens may be the result of synergistic or antagonistic effect of several compounds.

Conclusions

In conclusion, it can be stated that tested lichen compounds have a strong antioxidant, -microbial, and -cancer activity in vitro. On the basis of these results, lichens appear to be good natural antioxidant, -microbial, and -cancer 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 other lichens that exhibit strong antioxidant, -microbial, and -cancer activity.

Experimental

Identification of sample

Lichen samples of *H. physodes* (L) Nyl. were collected from Kopaonik, Serbia, in September of 2011. The voucher specimen of the lichen (Voucher No. 92) was deposited at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. Determination of the investigated lichens was accomplished using standard methods.

Preparation of the lichen extracts

Finely dry ground thalli from the lichen (100 g) were extracted using acetone in a Soxhlet extractor. The extract was filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extract was stored at −18 °C until it was used for phytochemical screening and process of isolation of secondary metabolites.

HPLC analysis

The extract of the lichen *H. physodes* was redissolved in 500 µl of acetone and analyzed on an Agilent HPLC instrument 1200 Series with C18 column (C18; 25 cm × 4.6 mm, 10 m) and a UV spectrophotometric detector with methanol–water–phosphoric acid (80:20:0.9, v/v/v) solvent. Phosphoric acid was analytical grade reagent. Methanol was of HPLC grade and was purchased from Merck (Darmstadt, Germany). 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: 3-hydroxyphysodalic acid ($t_R = 3.98 \pm 0.10$), physodalic acid ($t_R = 4.31 \pm 0.10$), physodic acid ($t_R = 5.91 \pm 0.10$), atranorin ($t_R = 16.21 \pm 0.20$), and chloroatranorin ($t_R = 17.73 \pm 0.20$) were isolated from lichen *P. furfuraceae* and usnic acid ($t_R = 14.32 \pm 0.20$) from *Usnea barbata*.

Isolation of lichen metabolites

Isolation of usnic acid

The dried acetone extract of the lichen *H. physodes* (500 mg) was dissolved in benzene. The precipitate which formed on cooling was collected and HPLC analyzed. HPLC analysis showed that precipitate contains, besides usnic acid, a small amount of atranorin and chloroatranorin. Therefore, precipitate was fractioned on a silica gel column (0.149–0.074 mm; 100–200 mesh) using cyclohexane–ethyl acetate (75:25, v/v). The first eluted compound was usnic acid, which was further recrystallized from chloroform to ethanol, yielding 95 mg pure yellow compound. This compound was further purified by co-chromatography and used for structure identification and determination of antioxidant, -microbial, and cytotoxic activities. Usnic acid was identified by its melting point and spectroscopic data (Huneck and Yoshimura, 1996).

Isolation of atranorin

The acetone extract of the lichen *H. physodes* (100 mg) was fractionated on a silica gel column (0.149–0.074 mm; 100–200 mesh). The column was eluted with methanol–water gradient solvent (6:1, 3:1, and 1:1, v/v) yielding 15 fractions. The last eluted fraction of the lichen extract contains atranorin (21 mg), which was further purified by co- and preparative layer chromatography and used for structure identification and antioxidant, -microbial, and -cancer activities. Atranorin (colorless crystalline substance) was identified by its melting point and spectroscopic data (Huneck and Yoshimura, 1996).

Isolation of physodic acid

The dried acetone extract of the lichen *H. physodes* (500 mg) was dissolved in benzene. After filtration, the solution was concentrated using an evaporator under reduced pressure. The residue was fractioned on a silica gel column (0.149–0.074 mm; 100–200 mesh). The column was eluted with methanol–chloroform gradient solvent (10:1 and 5:1) yielding seven fractions. The fourth eluted fraction of the lichen extract contains physodic acid (142 mg). This compound was used for structure identification and determination of antioxidant, -microbial, and cytotoxic activities. Physodic acid was identified by its melting point and spectroscopic data (Huneck and Yoshimura, 1996).

Isolated lichen compounds were used for antioxidant, -microbial, and -cancer investigations. The isolated components were dissolved in 5 % DMSO for the experiments. The DMSO was dissolved in sterile distilled water to the desired concentration.

Antioxidant activity

Scavenging DPPH radicals

The free radical scavenging activity of isolated compounds was measured by DPPH. The method used was similar to the method previously used by some authors (Ibanez *et al.*, 2003; Dorman *et al.*, 2004) but was modified in its 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. The absorbance was then measured at 517 nm in spectrophotometer (“Jenway” UK). Ascorbic acid was used as positive control. The DPPH radical concentration was calculated using the following equation:

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

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

The IC_{50} was the parameter used to compare the radical scavenging activity.

Reducing power

The reducing power of isolated compounds 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. Trichloroacetic acid (10 %, 2.5 ml) was then added to the mixture, which 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 a spectrophotometer (“Jenway” UK). A 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 isolated compounds 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 solution (156 μM in 0.1 M phosphate buffer, pH 7.4) and 1 ml nicotinamide adenine dinucleotide solution (468 μM in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 μl of phenazine methosulfate 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:

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

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

The 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 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 ATCC and the mycological collection maintained by the Mycological Laboratory within the Department of Biology of Kragujevac University’s Faculty of Science (DBFS). The bacterial cultures were maintained on Müller–Hinton agar substrates (Torlak, Belgrade). The fungal cultures were maintained on potato dextrose (PD) and

Sabouraud dextrose (SD) agars (Torlak, Belgrade). All of the cultures were stored at 4 °C and subcultured every 15 days.

The sensitivity of microorganisms to tested samples was tested by determining the 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 freshly mature (3- to 7-day-old) cultures that grew at 30 °C on a PD agar substrate. The spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and were then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by NCCLS (National Committee for Clinical Laboratory Standards) (1998).

Minimal inhibitory concentration (MIC)

The MIC was determined by the broth microdilution method using 96-well microtiter plates (Sarker *et al.*, 2007). A series of dilutions with concentrations ranging from 4 to 0.00181 mg/ml for isolated compounds was used in the experiment against every microorganism tested. The starting solutions of the test samples were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Twofold dilutions of the test samples were prepared in a Müller–Hinton broth for bacterial cultures and a SD broth for fungal cultures. The MIC 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 change in color of resazurin was defined as the MIC for the tested microorganism at a 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.

Cytotoxic activity

Cell lines

The human melanoma FemX and human colon carcinoma LS 174 cell lines were obtained from the ATCC (Manassas, VA, USA). Both cancer cell lines were maintained in the recommended RPMI 1640 medium supplemented with 10 % heat-inactivated (56 °C) fetal bovine serum (FBS), L-glutamine (3 mM), streptomycin (100 mg/ml), penicillin (100 IU/ml), and 25 mM HEPES and was adjusted to pH 7.2 by bicarbonate solution. The cells were grown in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

Treatment of cell lines

A stock solution of isolated compounds, made in DMSO, was dissolved in corresponding medium to the required working concentrations. Neoplastic FemX- (5,000 cells/well) and neoplastic LS 174 cells (7,000 cells/well) were seeded into 96-well microtiter plates, and 24 h later, after 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 the nutrient medium was added to the cells. Nutrient medium was RPMI 1640 medium, supplemented with L-glutamine (3 mM), streptomycin (100 lg/ml), and penicillin (100 IU/ml), 10 % heat-inactivated (56 °C) FBS and 25 mM HEPES, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 h.

Determination of cell survival (MTT test)

The effect of isolated compounds on cancer cell survival was determined by the MTT test (microculture tetrazolium test), according to Mosmann (1983) with modification by Ohno and Abe (1991), 72 h after the addition of the compounds, as it was described earlier. Briefly, 20 µl of MTT solution (5 mg/ml PBS) was added to each well. The samples were incubated for further 4 h at 37 °C in 5 % CO_2 in a humidified air atmosphere. Then, 100 µl of 10 % SDS was 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 light absorbance, which was then read in an ELISA plate reader at 570 nm. Absorbance (A) at 570 nm was measured 24 h later. To get cell survival (%), A of a sample 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 grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. The 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 *cis*-DDP was used. All of the experiments were done in triplicate.

Statistical analyses

Statistical analyses were performed with the EXCEL and SPSS software package. To determine the statistical

significance of antioxidant activity, Student's t test was used. All values are expressed as mean $\pm$ SD of three parallel measurements.

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