BIOACTIVE CONSTITUENTS FROM LICHENS

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Abstract: Here we investigate antioxidant and antimicrobial activities of the acetone extract of the *Cladonia rangiferina* and their fumarprotocetraric acid. Antioxidant activity was evaluated by free radical scavenging, superoxide anion radical scavenging, reducing power and determination of total phenolics. As a result, fumarprotocetraric acid had larger free radical scavenging activity with (IC_{50} = 228.46 μg/mL). Tested samples also had effective reducing power and superoxide anion radical scavenging. Total phenolic content was determined as pyrocatechol equivalent. The antimicrobial activity was estimated by determination of the MIC, where most active was fumarprotocetraric acid with MIC values ranging from 0.03 to 0.25 μg/mL.

Key words: anticancer activity; antimicrobial activity; antioxidant activity; lichens

Introduction

Lichens are symbiotic organisms consisting of a fungi and a photosynthetic organism, either an alga or Cyanobacteria (Grube and Berg, 2010). These organisms have historically been used as food, dyes, in production of alcohol and perfume industry. Lichens have also, for hundreds of years, been used in many countries as a cure for diseases of humans. Namely, *C. rangiferina* was used to cure colds, arthritis, fever, jaundice, constipation, convulsions and tuberculosis (Bown, 2001).

In recent years, there has been a renewed interest in lichens as a potential source for bioactive compounds with therapeutic properties. Lichen secondary metabolites are from derived mycobiont metabolism organized into several distinct chemical classes such as depsides, depsidones, dibenzofurans, xanthones, terpene derivatives, etc (Manojlović et al., 2012). Here, we report the antioxidant, antimicrobial and anticancer activity of the acetone extracts of the lichen *C. rangiferina* and their fumarprotocetraric acid constituent.

Material and methods

Preparation of the lichen extracts and isolation of compounds

Lichen sample of *C. rangiferina* (L.) Weber ex F.H. Wigg., was collected from Kopaonik, Serbia, in September of 2014. 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.

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

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

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

Antioxidant activity

Antioxidant activity was evaluated by free radical scavenging, reducing power and determination of total phenolic compounds. The free radical scavenging activity of lichen extracts was measured by1,1-diphenyl-2-picryl-hydrazil (DPPH) according to the Dorman et al's method (2004). The Oyaizu method (1986) was used to determine the reducing power. The superoxide anion radical scavenging was evaluated by Nishimiki et al's method (1972). The amount of total phenols was determined as a pyrocatechol equivalent using Folin-Ciocalteu reagent according to the Slinkard and Singleton's method (1997).

Antimicrobial activity

The following bacteria were used as test organisms in this study: *Bacillus mycoides* (ATCC 6462), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 13883). All the bacteria used were obtained from the American Type Culture Collection (ATCC). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Candida albicans* (ATCC 10231), *Penicillium 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 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 spectrophotometriacally at 530 nm, and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by NCCLS (1998).

The MIC was determined by the broth microdilution method using 96-well micro-titer plates (Sarker et al., 2007). The MIC was determined by establishing visible growth of microorganisms. The boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration.

Statistical analyses

Statistical analyses were performed with the SPSS software packages. All values are expressed as mean ± SD of three parallel measurements.

Results and discussion

Fumarprotocetraric acid (Fig. 1) was isolated from the acetone extract of *C. rangiferina* using the same method but different solvent system (methanol-water, 4:1, v/v).

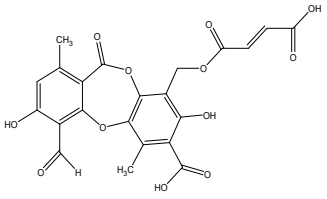

Fig. 2. Structure of fumarprotocetraric acid

The scavenging DPPH radicals of the studied samples are shown in Table 1. The IC_{50} values of extract and compound were 987.64 – 228.46 μg/mL. The results of the reducing power assay of the tested extract and compounds are summarised in Table 2. The isolated compounds showed higher reducing power than extract. The scavenging of superoxide anion radicals is shown in Table 1. The IC_{50} was 1595.12 μg/mL for extract and 389.57 μg/mL for fumarprotocetraric acid. The total amount of phenolics was determined as the pyrocatechol equivalent using an equation obtained from a standard pyrocatechol graph. The total phenolics contents of the acetone extracts of *C. rangiferina* were 22 μg PE/mg (Table 3). The stronger antimicrobial activity was found in fumarprotocetraric acid, which in extremely low amounts (0.031 to 0.25 mg/mL) inhibited all microorganisms (Table 4).

The tested samples have a strong antioxidant activity against various oxidative systems *in vitro*. In fact, it was observed that lichen extracts where found the higher content of phenols exert stronger radical scavenging effect, suggesting that phenolics are the main agents for their antioxidant activity. These results mostly agree with the literature, where we can find a number of reports for the antioxidant activity of extracts with high content of phenolic compounds (Behera et al., 2009).

Table 1. DPPH radical scavenging activity and superoxide anion scavenging activity of acetone extract of C. rangiferina and their compound

Lichen species and compounds	DPPH radical scavenging IC_{50} (μg/mL)	Superoxide anion scavenging IC_{50} (μg/mL)
C. rangiferina	987.64	1595.12
Fumarprotocetraric acid	228.46	389.57
Ascorbic acid	6.42	115.61

Table 2. Reducing power of acetone extract of C. rangiferina and their compound

Lichen species and compounds	Absorbance (700 nm)				
	1000	500	250 (μg/mL)	125	62.5
C. rangiferina	0.0441	0.0312	0.0201	0.0102	0.0079
Fumarprotocetraric acid	0.0975	0.0521	0.0312	0.0275	0.0194
Ascorbic acid	2.113	1.654	0.0957	0.0478	0.0247

Table 3. Total phenolics of acetone extract of C. rangiferina

Lichen species and compounds	Phenolics content (μg PE/mg of extract)
C. rangiferina	22.00 ± 1.065

Table 4. Minimum inhibitory concentration (MIC) of acetone extracts of C. rangiferina and their compound.Values given as mg/mL for tested samples and as μg/mL for antibiotics. Antibiotics: K – ketoconazole, S – streptomycin

Lichen species and compound	*Cladonia rangiferina*	Fumarprotocetraric acid	S - K
B. mycoides	0.78	0.062	7.81 -
B. subtilis	0.78	0.062	7.81 -
E. coli	-	0.125	31.25 -
K. pneumoniae	0.78	0.03	1.95 -
S. aureus	0.78	0.125	31.25 -
A. flavus	12.5	0.25	- 3.9
A. fumigatus	12.5	0.25	- 3.9
C. albicans	12.5	0.125	- 1.95
P. purpurescens	12.5	0.25	- 3.9
P. verrucosum	12.5	0.25	- 3.9

Antioxidant effect of some other lichens was also studied by other researchers. For example, Luo et al. (2006) found antioxidant activity for methanol extracts from the lichen *Thamnolia vermicularis*. Praveen Kumar et al. (2010) find an antioxidant activity for the extracts of the lichen *Ramalina hossei* and *R. conduplicans.*

In our experiments, the tested lichen extract 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. However, it is necessary understand that extracts are mixtures of natural compounds, and their antimicrobial activity is not only a result of the different activities of individual components but may be the result of their interactions, which can have different effects on the overall activity of extracts.

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

Numerous lichens were screened for antimicrobial activity in search of the new antimicrobial agents. Kosanić et al. (2012) find an antimicrobial activity for the acetone extract of the lichens *Umbilicaria crustulosa*, *U. cylindrica*, and *U. polyphylla*. Similar results were reported by Karthikai Devi et al. (2011) for different extracts extracted from the lichen *Roccella belangeriana*.

Conclusion

Based on these results, lichen apear to be good natural antioxidant and antimicrobial agents and could be of significance in food and pharmaceutical industry.

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