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ORIGINAL ARTICLE

Antioxidant properties of some lichen species

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Abstract Antioxidant activity of the acetone, methanol and aqueous extracts of the lichens *Cladonia furcata, Hypogymnia physodes, Lasallia pustulata, Parmelia caperata* and *Parmelia sulcata* has been screened in vitro by using different methods (DPPH radical scavenging, superoxide anion radical scavenging, reducing power, determination of total phenolic compounds and determination of total flavonoid content). Of the lichens tested, *Lasallia pustulata* had powerful antioxidant activities. Acetone, methanol and aqueous extracts of this lichen showed 90.93, 69.87 and 65.08% DPPH radical scavenging activities. Moreover, the tested extracts had effective reducing power and superoxide anion radical scavenging. Those various antioxidant activities were compared to standard antioxidants such as ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol. In addition, total content of phenol and flavonoid in extracts were determined as pyrocatechol equivalent, and as rutin equivalent, respectively. The strong relationships between total phenolic and flavonoid contents and the antioxidative activities of tested extracts suggest that these compounds play important role in antioxidant activity. The present stady shows that tested lichen species demonstrated a strong antioxidant activity and can be considered as good sources of natural antioxidants.

Keywords Lichens extract · Antioxidant activity

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Introduction

Oxidative stress is initiated by reactive oxygen species (ROS), such as superoxide anion radicals (O_2–), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$). At normal physiological concentrations ROS are required for cellular activities. They play a positive role in energy production, phagocytosis, regulation of cell growth intercellular signalling, and synthesis of biologically important compounds (Gulcin et al. 2004a, b). However, at higher concentrations, ROS can be toxic leading to the development in over a hundred of diseases which range from arthritis and connective tissue disorders to carcinogenesis, physical injury, infection and acquired immunodeficiency syndrome (Gulcin and Dastan 2007; Sangameswaran et al. 2009; Sachindra et al. 2010).

The most effective way to eliminate ROS which cause the oxidative stress is with the help of antioxidants. Antioxidants, both synthetic or natural, can be effective to help the human body in reducing oxidative damage by ROS. However, at the present time, suspected that synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have toxic and carcinogenic effects (Wei-Min Zhang et al. 2009). For this reason, much attention has been devoted to natural antioxidants for their capacity to protect organisms from damage induced by oxidative stress (Naveena et al. 2008). To find new natural sources of antioxidants, our attention was focused on lichens.

Various biological activities of some lichens are known, such as: antimicrobial, antiviral, anti-tumor, anti-inflammatory, analgesic, antipyretic, antiproliferative and antiprotozoal (Halama and Van Haluwin 2004; Huneck 1999; Lawrey 1986; Rankovic et al. 2010). However, very few researchers

proved that lichens have antioxidant activity (Rankovic et al. 2010; Silva et al. 2010). Hence, the aim of this study is to investigate in vitro antioxidant activity of the acetone, methanol and aqueous extract of the lichens *Cladonia furcata, Hypogymnia physodes, Lasallia pustulata, Parmelia caperata* and *Parmelia sulcata.*

Materials and methods

Lichen samples

Lichen samples of *Cladonia furcata* (Huds.) Schrad., *Hypogymnia physodes* (L.) Nyl., *Lasallia pustulata* (L.) Mérat., *Parmelia caperata* (L.) Ach. and *Parmelia sulcata* (Taylor) were collected from Kopaonik, Serbia, in September of 2009. Samples were preserved at the Department the Department of Biology and Ecology of Kragujevac, Faculty of Science. Determination of the investigated lichens was accomplished using standard keys (Purvis et al. 1992; Wirth 1995).

Cladonia furcata contains the "lichen substances" atranorin and fumarprotocetraric acid (Culberson 1969; Walker and Lintott 1997; Brodo et al. 2001). *Hypogymnia physodes* contains atranorin, chloroatranorin, physodic acid and physodalic acid (Culberson 1969; Hauck and Huneck 2007). *Lasallia pustulata* contains gyrophoric acid and umbilicarin (Culberson 1969). *Parmelia caperata* contains atranorin, protocetraric acid, usnic acid and caperatic acid (Culberson 1969; Rankovic et al. 2008), while *Parmelia sulcata* contains atranorin and salazinic acid (Culberson 1969; Brodo et al. 2001).

Preparation of the lichen extracts

The lichen samples was dried in the shade in an airy place and then stored in paperbags and kept at room temperature. Then the lichen material was milled by an electrical mill. Finely dry ground thalli of the investigated lichens (50 g) were extracted with acetone, methanol and water (250 ml) 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) at a concentration of 1 mg/ml for the experiments.

Antioxidant activity

Scavenging DPPH radicals

The free radical scavenging activity of lichen extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH). The method used is similar to the method previously used by some authors (Gadow et al. 1997; Ibanez et al. 2003; Dorman et al. 2004), but was modified in details. Two ml of methanol solution of DPPH radical in the concentration of 0.05 mg/ml and 1 ml of plant extract 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 against methanol as blank in spectrophotometer ("Janway" GBR). 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 (2 ml of methanol solution of DPPH radical + 1 ml of 5% DMSO) and A_1 is the absorbance of reaction mixture or standards. Ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol were used as standards.

Reducing power

The reducing power of extracts was determined according to the method of Oyaizu (1986). One mL of extracts were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (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 (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml; 0.1%). The absorbance of the solution was measured at 700 nm in spectrophotometer ("Janway" GBR). Blank was prepared with all the reaction agents without extract. Higher absorbance of the reaction mixture indicated that the reducing power is increased. Ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol were used as standards.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of lichen extracts was detected according to the method of Nishimiki et al. (1972). Briefly, 0.1 ml of extracts was mixed with 1 ml nitroblue tetrazolium (NBT) solution (156 μM in 0.1 M phosphate buffer, pH 7.4) and 1 ml 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 ("Janway" GBR) against blank sample (phosphate buffer). Decreased absorbance indicated increased superoxide anion radical scavenging activity. 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 (consisting of all the reaction agents except the extract) and A_1 is the absorbance of reaction mixture or standards. Ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol were used as standards.

Determination of total phenolic compounds

Total soluble phenolic compounds in the lichen extracts were determined with Folin- Ciocalteu reagent according to the method of Slinkard (Slinkard and Slingleton 1997) using pyrocatechol as a standard phenolic compound. Briefly, 1 ml of the lichen extract in a volumetric flask diluted with distilled water (46 ml). One milliliter of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min 3 ml of Na_2CO_3 (2%) was added and then was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in spectrophotometer ("Janway" GBR) against blank consisting of all the reaction agents except the extract. The total concentration of phenolic compounds in the extract determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph as

$$\text{Absorbance} = 0.0021 \times \text{total phenols}[\mu\text{g pyrocatechol}] - 0.0092$$

$$(R^2 = 0.9934)$$

Total flavonoid content

The total flavonoid content was determined using the Dowd method (Meda et al. 2005). Two ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution. The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in spectrophotometer ("Janway" GBR). Negative control, without extract was used as the blank. The total flavonoid content was determined as microgram of rutin equivalent by using an equation that was obtained from standard rutin graph as

$$\text{Absorbance} = 0.0144 \times \text{total flavonoid}[\mu\text{g rutin}] + 0.0556$$

$$(R^2 = 0.9992)$$

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 were used. Pearson's bivariate correlation test was carried out to calculate correlation coefficients (r) between the content of total phenolic and flavonoid and the DPPH radical scavenging activity, reducing power and superoxide anion radical scavenging. All values are expressed as mean ± SD of three parallel measurements.

Results and discussion

The antioxidant activity of the tested lichen extracts was shown in Fig. 1 (for DPPH radical scavenging, superoxide anion radical scavenging and reducing power) and Table 1 (for determination of total phenolic and flavonoid content).

DPPH radical scavenging

The scavenging DPPH radicals of the studied lichen extracts is shown in Fig. 1. Acetone, methanol and aqueous extracts of the tested lichen showed a good scavenging activity on DPPH radical. There was a statistically significant difference between extracts and control ($P<0.05$). The scavenging effects of all lichen extracts were 29.99–90.93%. Extracts from lichen *Lasallia pustulata* showed largest DPPH radical scavenging activities than those from the other samples. The percentage inhibition on DPPH radical of acetone, methanol and aqueous extracts of this lichen were 90.93, 69.87 and 65.08% and greater than ascorbic acid, BHA and α-tocopherol (86.58, 79.78 and 63.99%), respectively. The scavenging activity was also good for the lichen *Hypogymnia physodes* (60.18% for the acetone, 73.18% for the methanol and 30.98% for the aqueous extracts). The remaining tested species showed a lower DPPH radical scavenging activities.

Free radical scavenging action is considered to be one among the various mechanisms for antioxidation (Sini and Devi 2004). This assay is being used widely as a preliminary test which provides information on the reactivity of test compound with a stable free radical since odd electron of DPPH gives strong absorption band at 517 nm (purple colour) and when it is quenched by the extract, there is a decrease in absorbance and discoloration from purple to yellow. This method is rapid, no expensive reagents or sophisticated instruments are required. (Sheetal et al. 2008).

Ferric reducing power capacity

The results of the reducing power assay of lichen extracts are summarized in Fig. 1. High absorbance indicates high reducing power. Measured values of absorbance varied from 0.012 to 0.086. Among the tested lichen species,

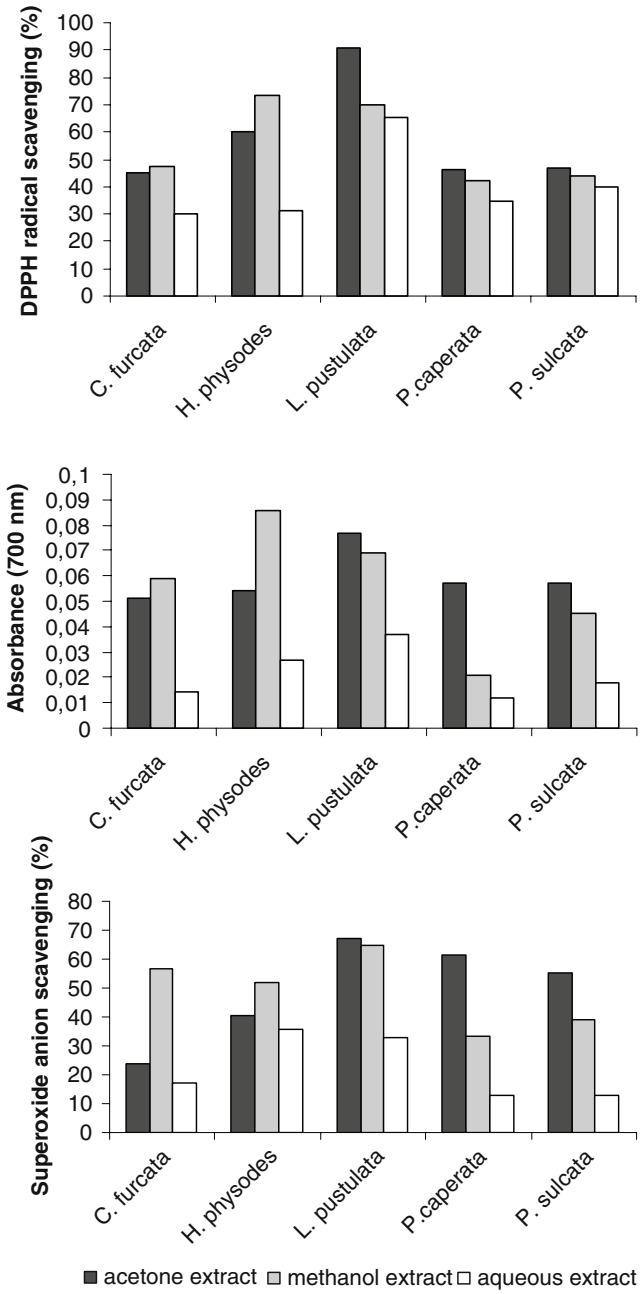

Fig. 1 DPPH radical scavenging, reducing power and superoxide anion scavenging of different extracts of *Cladonia furcata, Hypogymnia physodes, Lasallia pustulata, Parmelia caperata* and *Parmelia sulcata*. (n=3)

Hypogymnia physodes and *Lasallia pustulata* give highest reducing power, although the activity were lower than ascorbic acid, BHA and α-tocopherol. The reducing power in the lichen extracts decreased in the following order: *H. physodes* (methanol) > *L. pustulata* (acetone) > *L. pustulata* (methanol) > *C. furcata* (methanol) > *P. caperata* (acetone) > *P. sulcata* (acetone) > *Hypogymnia physodes* (acetone) > *C. furcata* (acetone) > *P. sulcata* (methanol) > *L. pustulata* (aqueous) > *H. physodes* (aqueous) > *P. caperata* (methanol) > *P. sulcata* (aqueous) > *C. furcata* (aqueous) > *P. caperata* (aqueous).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing properties are generally associated with the presence of reductones. Gordan (1990) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom. The reduction of ferrous ion (Fe3+) to ferric ion (Fe2+) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm. The result presented here indicates that the marked ferric reducing power activity of extract to be due to presence of polyphenols which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them into more stable products and terminate free radical chain reactions (Sasikumar et al. 2010).

Superoxide anion scavenging activity

Results of superoxide anion scavenging activities of tested extracts are shown in Fig. 1. All extracts revealed a good superoxide anion scavenging activity, although the activity was lower than ascorbic acid, BHA and α-tocopherol (91.19, 91.32 and 98.87%). The superoxide anion scavenging activity for different extracts was within the range 12.74–67.37%. There was a statistically significant difference between extracts and control (P<0.05). Maximum scavenging activity (67.37%) was in the acetone extracts of the lichen *Lasallia pustulata.* Aqueous extract of lichen *Parmelia sulcata* demonstrated weakest superoxide anion scavenging activity (12.74%).

Numerous biological reactions generate superoxide radical which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide anion radical are potential precursors of damaging oxygen species and thus the study of the scavenging of this radical is important (Jayasri et al. 2009). In the PMS/NADH-NBT system, superoxide anion is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen (Robak and Gryglewski 1998). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture (Gulcin 2004). The superoxide radical scavenging activities of extracts were evaluated based on their ability to quench the superoxide radical generated from the PMS/NADH reaction.

Total phenolics and flavonoid content

Total phenolic and flavonoid constituents of tested extracts are given in Table 1.

The amount of total phenolic compounds was determined as the pyrocatechol equivalent using an equation obtained

Table 1 Total phenolics and flavonoid content of different extracts of *Cladonia furcata, Hypogymnia physodes, Lasallia pustulata, Parmelia caperata* and *Parmelia sulcata*

Lichen species	Extracts	Phenolics content μg of pyrocatechol equivalent	Flavonoid content μg of rutin equivalent
C. furcata	A[a]	12.1±1.06	16.5±1.09
	B	52.7±1.21	24.9±1.11
	C	5.8±1.31	5.8±1.08
H. physodes	A	30.1±1.21	30.1±1.19
	B	86.8±1.31	32.1±1.12
	C	6.3±1.02	11.3±1.09
L. pustulata	A	84.3±1.34	49.6±1.21
	B	49.6±1.21	28.7±1.11
	C	23.9±1.07	27.9±1.12
P. caperata	A	40.4±1.09	15.3±1.09
	B	15.3±1.03	12.9±1.11
	C	14.9±1.02	9.9±1.09
P. sulcata	A	38.2±1.27	25.8±1.11
	B	25.1±1.11	9.6±1.09
	C	9.6±1.19	2.5±1.05

[a] A—acetone extract; B—methanol extract; C—aqueous extract

(n=3)

from a standard pyrocatechol graph (y=0.0021x - 0.0092, R^2=0.9934). Results of the study showed that the phenolic compound of the tested extracts varied from 5.81 to 86.76 μg of pyrocatechol equivalent. Highest phenolic compounds was identified in methanol extract of *Hypogymnia physodes* at a 86.76 μg of pyrocatechol equivalent while aqueous extracts of *Cladonia furcata* showed the lowest content at 5.81 μg of pyrocatechol equivalent. High phenolic contents were also found in acetone, methanol and aqueous extract of *Lasallia pustulata* with 84.33, 49.62 and 23.90 μg of pyrocatechol equivalent, respectively.

The amount of total flavonoid compounds was determined as the rutin equivalent using an equation obtained from a standard rutin graph (y=0.0144x+0.0556, R^2=0.9992). As a shown in the Table 1, excellent flavonoid content was found n extracts of the lichen *Lasallia pustulata* (49.62 μg of rutin equivalent for the acetone, 28.67 μg for the methanol and 27.87 μg for the aqueous extract). Good flavonoid content was also found in the methanol extract of lichen *Hypogymnia physodes* (32 μg of rutin equivalent). Other lichen extracts showed lower flavonoid content.

Phenolic components are potential antioxidants, free radical terminators (Shahidi and Wanasundara 1992; Kaushik et al. 2010). These compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sawa et al. 1999). Flavonoids are the most important natural phenolics and they possess a broad spectrum of chemical and biological activities including radical scavenging properties (Mohammed et al. 2010).

Correlations between total phenolic content and antioxidative function

The tested extract exhibited the highest radical scavenging activity, ferric reducing power and superoxide anion radical scavenging with the greatest amount of phenolic content. Content of total phenolic of the extracts was strongly related with DPPH radical scavenging activity (r=0.84), with reducing power (r=0.89) and with superoxide anion radical scavenging (r=0.80). Also, there is a good correlation between flavonoid compounds of the tested extracts and free radical scavenging activity, reducing power and superoxide anion radical scavenging (r=0.92, r=0.81 and r=0.74). Previous researches also showed a high correlations between antioxidative activities and phenolic content (Odabasoglu et al. 2004; Hodzic et al. 2009). The strong relationships between total phenolic contents of tested extracts and the antioxidative activities suggest that phenolics might be the major antioxidant compounds in studied extracts.

The tested lichen extracts have a strong antioxidant activity against various oxidative systems in vitro. The intensity of antioxidant activity depended on the tested lichen species and the solvent that used for extraction. The differences in the antioxidant activity of various solvents might be due to their different capabilities to extract bioactive substances (Behera et al. 2005). The aqueous extracts of the tested lichens

showed the weakest antioxidant effect. That's probably because the active components produced by lichens are poorly soluble in water (Kinoshita et al. 1994).

Numerous studies showed that environmental factors play an important role in antioxidant activity of lichens. Extreme environmental factors such as high light, air pollution, desiccation, rehydration and high temperature affecting decreased antioxidant activity and reducing synthesis of antioxidants by lichens (Bartak et al. 2004; Weissman et al. 2005, 2006).

Antioxidant activity of some other lichen was studied by other researchers. For example, Gulcin et al. (2002) found that the aqueous extracts of *Cetraria islandica* had a strong antioxidant activity. Similar results were reported by Behera et al. (2005) for different extracts from the lichen *Usnea ghattensis*. Prashith et al. (2009) find an antioxidant activity for the extracts of the lichen *Parmotrema pseudotinctorum* and *Ramalina hossei*. Manojlović et al. (2010) explored antioxidant properties of *Laurera benguelensis*.

Conclusion

It conclusion, it can be stated that the tested lichen extracts exhibited potent antioxidant activities. On the basis these results, lichen appear to be good and safe natural sources of antioxidants and could be helpful as to increase the nutritional value of different foods. Further work should be focused on the isolation of pure compounds of the crude extracts of the studied lichen and investigation of their antioxidant activity.

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