

LICHENS AS POSSIBLE SOURCES OF ANTIOXIDANTS

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ABSTRACT

Acetone, methanol and aqueous extracts of the lichen *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudoevernia furfuraceae* and *Umbilicaria cylindrica* were investigated for antioxidant activity by five different methods: DPPH radical scavenging, superoxide anion radical scavenging, reducing power, determination of total phenolic compounds and determination of total flavonoid content. Different antioxidant activities of the tested extracts were studied in comparison to known antioxidants such as ascorbic acid, butylated hydroxyanisole (BHA) and α -tocopherol. The tested extracts had strong antioxidant activity. The DPPH radical scavenging activity ranging from 32.68-94.70%. For reducing power, measured values of absorbance varied from 0.016 to 0.109. The superoxide anion scavenging activity for different extracts was 7.31-84.51%. In addition, the high contents of total phenolic compounds (12-76.42 μ g of pyrocatechol equivalent) and total flavonoids (1.37-54.77 μ g of rutin equivalent) suggests that phenols and flavonoids might be the major antioxidant compounds in studied extracts. Tested lichen species were found to possess effective antioxidant activities and can be used as good natural sources of antioxidants.

Keywords: Lichens extract; antioxidant activity.

INTRODUCTION

Reactive oxygen species (ROS), which include superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen (1O_2), are different forms of activated oxygen (Halliwell, 1995; Squadriato and Pelor, 1998; Huda-Faujan *et al.*, 2009). In normal values, ROS are necessary for cell function, but in high concentrations leads to oxidative stress and to the development a large number of diseases such as arthritis, carcinogenesis, aging (Sangameswaran *et al.*, 2009).

Antioxidants, both synthetic and natural, are substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Souri *et al.*, 2008). 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 (Zhang *et al.*, 2009). Because of that, are needed the development and use of natural antioxidants for their capacity to protect organisms without any negative effects from damage induced by oxidative stress (Naveena *et al.*, 2008; Gulcin *et al.*, 2004). Natural antioxidants should be required from various sources such as plants, macromicetes and lichens. In the search for new natural antioxidant sources, 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; Ranković *et al.*, 2007). However, very few researchers proved that lichens have antioxidant activity (Gulluce *et al.*, 2006; Ranković *et al.*, 2010). For this reason, the purpose of this research is to evaluate *in vitro* antioxidant activity of the acetone, methanol and aqueous extract of the lichens *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudoevernia furfuraceae* and *Umbilicaria cylindrica*.

MATERIALS AND METHODS

Lichen samples

Lichen samples of *Cetraria islandica* (L.) Ach., *Lecanora atra* (Hudson) Ach., *Parmelia pertusa* (Schränk.) Schaer., *Pseudoevernia furfuraceae* (L.) Zopf., and *Umbilicaria cylindrica* (L.) Delise ex Duby were collected from Kopaonik, Serbia, in September of 2009. The investigated samples are stored at the premises of the Department of Biology, Faculty of Science, Kragujevac. Determination of the tested lichens was carried out with standard keys (Purvis *et al.*, 1992; Wirth, 1995; Dobson, 2000).

Preparation of the lichen extracts

Finely fragmented dry lichens thalli were extracted using acetone, methanol and water in a Soxhlet extractor (50 g of dry lichens thalli with 250 ml of each solvent separately). The extracts were filtered and then concentrated in a rotary evaporator. The crude extracts were kept at -18°C until they were used in the experiment. For the test, the extracts were dissolved in 5% dimethyl sulfoxide in concentration of 1 mg/ml.

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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 was almost the same as that used by other authors (Gadow *et al.*, 1997; Ibanez *et al.*, 2003; Dorman *et al.*, 2004), but was modified in details. 2 ml of methanol mixture of DPPH radical in the concentration of 0.05 mg/ml and 1 ml of plant extract were placed in cuvettes. The solution was stirred vigorously and left to stand for 30 min at room temperature. After that was measured absorbance at 517 nm in spectrophotometer ("Janway" GBR). The following equation was used to calculate the DPPH radical concentration:

$$\text{DPPH radical scavenging (\%)} = (A_0 - A_1/A_0) \times 100$$

(A_0 is the absorbance of the negative control,

A_1 is the absorbance of reaction mixture or standards)

Reducing power

The method of Oyaizu (1986) was used to determine the reducing power of extracts. 1 mL of tested extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixtures were incubated for 20 min at 50°C. Then, to the mixture was added TCA (10%, 2.5 mL) and centrifuged. At the end, the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%). The absorbance of the solution was measured at 700 nm in spectrophotometer ("Janway" GBR). The reducing power is increased if absorbance of the reaction mixture increased.

Superoxide anion radical scavenging activity

The method of Nishimiki *et al.*, (1972) was used to measure the superoxide anion radical scavenging activity of lichen extracts. 0.1 ml of extracts was mixed with 1 ml nitroblue tetrazolium (NBT) solution (156 μ M in 0.1 M phosphate buffer, pH 7.4) and 1 mL NADH solution (468 μ M in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 μ L of phenazine methosulphate (PMS) solution (60 μ M 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 samples. The following formula was used to calculate the percentage inhibition of superoxide anion generation

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

(A_0 is the absorbance of the negative control, A_1 is the absorbance of reaction mixture or standards).

Determination of total phenolic compounds

For determination of total soluble phenolic compounds in the lichen extracts was used Folin- Ciocalteu reagent according to the method of Slinkard and Slingleton (1997). Briefly, 1ml of the lichen extract was diluted with

46 ml of distilled water. Then, one milliliter of Folin-Ciocalteu reagent was added and the mixture was stirred vigorously. 3 ml of Na_2CO_3 (2%) was added after 3 min and then was allowed to stand for 2h with intermittent shaking. After that, absorbance was measured at 760 nm in spectrophotometer ("Janway" GBR). The total phenolic content in the extract determined as microgram of pyrocatechol equivalent according to the equation that was obtained from standard pyrocatechol graph as

$$\text{Absorbance} = \frac{0.0021 \times \text{total phenols } [\mu\text{g pyrocatechol}] - 0.0092}{(R^2 = 0.9934)}$$

Total flavonoid content

Dowd method (Meda *et al.*, 2005) was used to measure the total flavonoid content in lichen extracts. 2 mL of the extract solution was mixed with 2 mL of 2 % aluminium trichloride ($AlCl_3$) in methanol. The mixture was incubated for 10 min at room temperature, and the absorbance was measured at 415 nm in spectrophotometer ("Janway" GBR) against blank samples. The total concentration of flavonoids in the extracts determined as microgram of rutin equivalent according to the formula that was obtained from standard rutin graph as

$$\text{Absorbance} = \frac{0.0144 \times \text{total flavonoid } [\mu\text{g rutin}] + 0.0556}{(R^2 = 0.9992)}$$

STATISTICAL ANALYSES

Statistical analyses were carried out with the EXCEL and SPSS software packages. ANOVA statistical method was used to determine the statistical significance of antioxidant activity. 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, Pearson's bivariate correlation test was used. All values are expressed as mean \pm SD of three measurements.

RESULTS

In this study were investigated DPPH radical scavenging, superoxide anion radical scavenging, reducing power, determination of total phenolic compounds and determination of total flavonoid content of the acetone, methanol and aqueous extracts of the lichen *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudovernia furfuraceae* and *Umbilicaria cylindrica*.

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 effect on DPPH radical. The scavenging activity of all lichen extracts were 32.68-94.70%. There was a statistically significant difference between extracts and control ($P < 0.05$). Extracts from lichen *Lecanora*

atra 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 94.70, 93.32 and 93.23% 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 *Pseudoevernia furfuraceae* (87.27% for the acetone, 57.88% for the methanol and 33.91% for the aqueous extracts). The remaining tested species showed a lower DPPH radical scavenging activity.

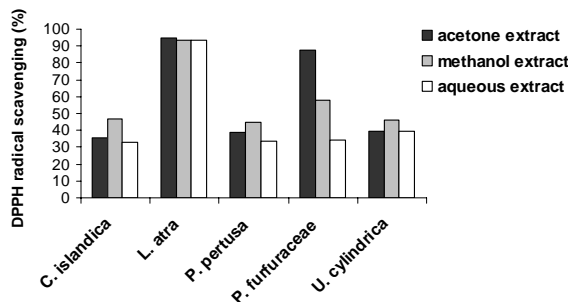


Fig. 1: DPPH radical scavenging of the acetone, methanol and aqueous extracts of the lichen *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudoevernia furfuraceae* and *Umbilicaria cylindrica*.

Reducing power capacity

The results of the reducing power assay of lichen extracts are summarized in fig. 2. High absorbance of extracts means high reducing power. Measured values of absorbance varied from 0.016 to 0.109. Among the tested lichen species, *Lecanora atra* gave highest reducing power, although the activity was lower than ascorbic acid, BHA and α -tocopherol. The reducing capacity of the tested extracts decreased in the following order: *L. atra* (acetone) > *L. atra* (methanol) > *L. atra* (aqueous) > *P. furfuraceae* (acetone) > *P. furfuraceae* (methanol) > *U. cylindrica* (methanol) > *C. islandica* (methanol) > *P. pertusa* (methanol) > *C. islandica* (acetone) > *U. cylindrica* (acetone) > *P. pertusa* (acetone) > *U. cylindrica* (aqueous) > *C. islandica* (aqueous) > *P. pertusa* (aqueous).

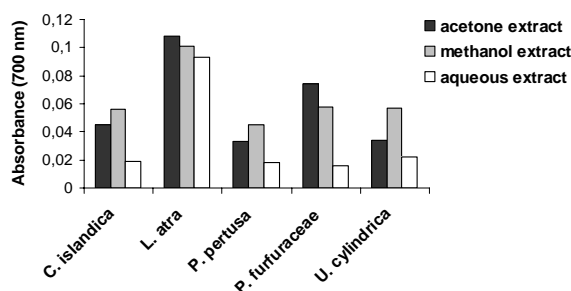


Fig. 2: Reducing power of the acetone, methanol and aqueous extracts of the lichen *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudoevernia furfuraceae* and *Umbilicaria cylindrica*.

Superoxide anion scavenging activity

Results of superoxide anion scavenging activities of tested extracts are shown in fig. 3. 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 7.31-84.51%. There was a statistically significant difference between extracts and control ($P < 0.05$). Maximum scavenging activity (84.51%) was in the acetone extracts of the lichen *Lecanora atra*. Aqueous extract of lichen *Parmelia pertusa* demonstrated weakest superoxide anion scavenging activity (7.31%).

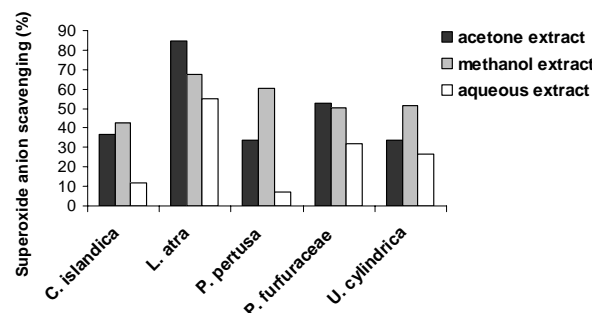


Fig. 3: Superoxide anion scavenging of the acetone, methanol and aqueous extracts of the lichen *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudoevernia furfuraceae* and *Umbilicaria cylindrica*.

Total phenolics and flavonoid content

Contents of total phenolic and flavonoid compounds of tested extracts are given in table 1.

The total phenolic content in the extract determined as microgram of pyrocatechol equivalent according to the formula that was obtained from standard pyrocatechol graph ($y = 0.0021 \times -0.0092$, $R^2 = 0.9934$). Results of the research showed that the phenolic compound of the tested extracts varied from 12 to 76.42 μ g of pyrocatechol equivalent. Highest phenolic compounds was identified in acetone extract of *Pseudoevernia furfuraceae* at a 76.42 μ g of pyrocatechol equivalent while *Parmelia pertusa* showed the lowest content at 12 μ g of pyrocatechol equivalent. High phenolic contents were also found in acetone, methanol and aqueous extract of *Lecanora atra* with 73.014, 71.048 and 69.77 μ g of pyrocatechol equivalent, respectively.

The total flavonoid content in the extract determined as microgram of rutin equivalent according to the formula that was obtained from standard rutin graph ($y = 0.0144x + 0.0556$, $R^2 = 0.9992$). As a shown in the Table 1, excellent flavonoid content was found in extracts of the lichen *Lecanora atra* (54.77 μ g of rutin equivalent for the acetone, 53.71 μ g for the methanol and 52.60 μ g for the aqueous extract). Good flavonoid content was also found in the acetone extract of lichen *Pseudoevernia*

furfuraceae (37.6 µg of rutin equivalent). Other lichen extracts showed lower flavonoid content.

Correlations between antioxidant activity and total phenolic content

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 ($r = 0.966$), with reducing power ($r = 0.944$) and with superoxide anion radical scavenging ($r = 0.823$). 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 ($r = 0.971$, $r = 0.972$ and $r = 0.831$).

DISCUSSION

Free radical scavenging action is one of the numerous mechanisms for antioxidation (Sini and Devi, 2004). Antiradical activity of lichen extracts was studied by screening its possibility to bleach the stable DPPH radical. This method is based on the formation of non-radical form DPPH-H in the presence of alcoholic DPPH solution and hydrogen donating antioxidant (AH) by the reaction $\text{DPPH} + \text{AH} \rightarrow \text{DPPH-H} + \text{A}$ (Koleva *et al.*, 2002; Anandjiwala *et al.*, 2008).

The reducing power of a component may indicate their potential antioxidant activity. The reducing features are mainly related with the presence of reductones. Gordan *et al.*, (1990) found that the antioxidant effect of reductones is based on the destruction of the free radical chain by donating a hydrogen atom. The reduction of ferrous ion

(Fe^{3+}) to ferric ion (Fe^{2+}) is measured by the strength of the green-blue color of 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 way as reductones react with free radicals to turn them into more stable products and abort free radical chain reactions (Sasikumar *et al.*, 2010).

Various biological reactions produce superoxide radicals they are a highly toxic species. Superoxide anion radicals cannot directly initiate lipid oxidation, but they can be a potential precursors of damaging oxygen species and therefore the research of the scavenging of these radicals is significantly (Jayasri *et al.*, 2009). In the PMS/NADH-NBT system, superoxide anion is produced using a reaction of phenazine methosulphate in the presence of NADH and molecular oxygen (Robak and Gryglewski, 1998). When absorbance at 560 nm decreases with antioxidants it means that superoxide anion in the reaction mixture disappear (Gulcin *et al.*, 2004). The superoxide radical scavenging activity of extracts was estimated based on their ability to destroy the superoxide radical produced from the PMS/NADH reaction.

Phenolic components are potential antioxidants (Shahidi and Wanasundara, 1992). Phenolic compounds can donate hydrogen to free radicals and this way to stop the chain reaction of lipid oxidation at the initial stage. This ability of phenolic compounds to scavenge radicals comes due to the presence of their phenolic hydroxyl groups (Sawa *et al.*, 1999). Flavonoids are widely group of natural compounds and also the most important natural phenolics. These compounds have a large number of biological and chemical activities including radical scavenging

Table 1: Total phenolics and flavonoid content of different extracts of *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudoevernia furfuraceae* and *Umbilicaria cylindrica*.

Lichen species	Extracts	Phenolics content µg of pyrocatechol equivalent	Flavonoid content µg of rutin equivalent
<i>C. islandica</i>	A ^a	25 ± 1.09	7.714 ± 1.012
	B	38.083 ± 1.031	25.809 ± 1.097
	C	18.19 ± 1.018	1.375 ± 1.078
<i>L. atra</i>	A	73.019 ± 1.275	54.77 ± 1.231
	B	71.048 ± 1.269	53.714 ± 1.128
	C	69.77 ± 1.612	52.597 ± 1.195
<i>P. pertusa</i>	A	18.197 ± 1.341	6.625 ± 1.092
	B	34.611 ± 1.208	18.201 ± 1.213
	C	12 ± 1.075	5.34 ± 1.106
<i>P. furfuraceae</i>	A	76.417 ± 1.206	37.6 ± 1.121
	B	37 ± 1.019	21.048 ± 1.099
	C	18.666 ± 1.027	1.764 ± 1.014
<i>U. cylindrica</i>	A	19.143 ± 1.318	12.953 ± 1.091
	B	42.528 ± 1.211	19.143 ± 1.105
	C	19.145 ± 1.018	11.139 ± 1.097

^aA-acetone extract; B-methanol extract; C-aqueous extract.

properties (Ghafar *et al.*, 2010). In this study, we found that the tested extracts exhibited the highest radical scavenging activity, ferric reducing power and superoxide anion radical scavenging with the greatest amount of phenolic content. Previous researches also showed a high correlations between antioxidative activities and phenolic content (Odabasoglu 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.

On the basis of the results, it can be concluded that tested lichen extracts show a strong antioxidant activity *in vitro*. The intensity of antioxidant activity depended on the tested lichen species and the solvent that used for extraction. Different antioxidant activity of different solvents depends on 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 not very soluble in water (Kinoshita *et al.*, 1994).

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

In the end, it may be concluded that the tested lichen extracts exhibited potent antioxidant activities. These results indicate that lichen can be good and safe natural sources of antioxidants. Further studies should be done to isolation compound from lichen that exhibit strong antioxidative activities.

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