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ANTIOXIDANT ACTIVITY OF LICHEN CETRARIA ACULEATA

KORESPONDENT

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SUMMARY

The aim of the present study is to investigate the antioxidant properties of the lichen Cetraria aculeata. Antioxidant activity of the methanol and ethyl acetate extracts of lichen was tested by different methods including determination of total phenolics content, determination of total antioxidant capacity, DPPH free radical scavenging activity, inhibitory activity towards lipid peroxidation, ferrous ion chelating ability and hydroxyl radical scavenging activity. The extracts of the lichen C. aculeata showed significant antioxidant activity. The methanol extract showed higher values for total phenolics and total antioxidant capacity compared to the ethyl acetate extract, while the ethyl acetate extract demonstrated better results for DPPH radical scavenging, inhibitory activity towards lipid peroxidation, chelating ability and hydroxyl radical scavenging than the methanol extract. This is the first report of the antioxidant properties of Cetraria aculeata growing in Serbia. The results of antioxidant activity indicate the application of this lichen as source of natural antioxidants that could be used as a possible food supplement, in the pharmaceutical industry and in the treatment of various diseases.

Keywords: ROS, antioxidant activity, lichen, Cetraria aculeata.

INTRODUCTION

Active oxygen exists in different forms, such as superoxide anion radicals ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O2$). These forms of oxygen are highly reactive intermediates, and have a collective name of reactive oxygen species (ROS) [1,2,3,4,5]. ROS are free radicals very important for living organisms, in which are formed in different ways. In normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most oxidants produced by cells. However, despite the fact that ROS are necessary for cell function, in high concentrations leads to oxidative stress and to the development a large number of diseases such as arthritis, carcinogenesis, aging. In addition to endogenous sources of free radicals, a major contribution of the accumulation of free radicals in cells are provided by exogenous sources such as ionizing radiation, tobacco smoke, certain pollutants, organic solvents and pesticides [4, 6, 7, 8, 9, 10]. The above mentioned diseases and accelerated aging are the consequence of oxidative tissue damage by free radicals because of unbalanced mechanisms of antioxidant protection under the influence of endogenous and exogenous factors [11]. ROS attack unsaturated fatty acids in membrane proteins, causing lipid peroxidation, and the result is damage to membrane proteins [12]. This leads to reduced permeability of membranes, receptors and enzyme activity, and reduced activation of cells. Free radicals attack the DNA, while leading to DNA damage, resulting in mutations that cause cancer. Therefore, the prevention of many diseases are important antioxidant defense systems, including food, drugs and antioxidant enzymes [13,14].

Antioxidants are compounds of natural and synthetic origin, which have the ability to inhibit or delay the process of oxidation caused by free radicals. They prevent the initiation of oxidizing chain reactions. In this way, protect the body from oxidative stress caused by free radicals [15,16,17]. However, despite widespread use of synthetic antioxidants, at the moment it is limited because of suspicions that they manifest toxic and carcinogenic effects such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertbutylhydroquinone (TBHQ) and propyl gallate (PG). Because of this there is a great interest for finding natural antioxidants, which do not cause adverse effects [18, 19]. Therefore, attention will be focused to the lichen as a natural source of antioxidants due to insufficient research of their antioxidant properties.

Lichens are complex associations composed of fungi ("micobiont") and one or more algae or cianobacteria ("photobionts") living in symbiosis [20]. So far more than 20.000 known species of lichens have been determined and more than 1000 primary and secondary metabolites

of lichens have been identified [21,22]. Secondary metabolites of lichens represent different classes of chemical compounds (dibenzofurans, depsides, depsidones, depsones, lactones, quinones, etc.), which contain in their structure a phenolic groups that have the ability to scavenge toxic free radicals. Because these metabolites exhibit strong antioxidant activity, about which are reported [23,24,25]. It has been found that depsidones are more efficient antioxidants than depsides [26].

Until now, the extracts of Cetraria aculeata have been explored for antimicrobial activity [27] and genotoxic/antigenotoxic and cytotoxic activities [28].

STUDY OBJECTIVE

The aim of the present study was to investigate the antioxidant properties of Cetraria aculeata in order to find an easily accessible source of natural antioxidants that could be used as a possible food supplement, in the pharmaceutical industry and in the treatment of various diseases.

MATHERIAL AND METHODS

LICHEN MATERIAL

The lichen Cetraria aculeata (Schreb.), family Parmeliaceae, was collected from Kopaonik (Ploce) in Serbia during the april 2011. Voucher specimens (9064, HMN) were deposited in the herbarium of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis.

PREPARATION OF THE LICHEN EXTRACTS

The extraction of the lichen Cetraria aculeata was performed by macerating lichen sample with separately methanol and ethyl acetate. The lichen material was dried one week at room temperature (26°C), after which it was ground to a uniform powder. Then, 500g dry powdered lichen material was soaked in 2000 mL of an appropriate solvent (methanol and ethyl acetate) at room temperature for three days. After which extracts were filtered through a Whatman no. 42 (125 mm) filter paper and concentrated in a rotary evaporator. In this way, both extracts has been prepared.

DETERMINATION OF THE TOTAL PHENOLICS

Determination of total phenolics content was performed using the Folin-Ciocalteu method [29]. The lichen extract was diluted to the concentration of 1mg/mL, and aliquots of 0.5mL were mixed with 2.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of NaHCO3 (7.5%). The resulting mixture was staying 15 min at the 45°C, after which absorbance was measured at 765nm on spectrophotometer against blank sample. Total phenolic content in the extracts were expressed in the form of gallic acid equivalents (mg GA/g extract). The values are presented as means of triplicate analyses.

DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY

The total antioxidant activity of the lichen extracts was determined using the phosphomolybdenum method [30]. This test is based on the reduction of Mo (VI)-Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 mL of sample extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes with reaction solution were incubated at 95 °C for 90 min. After which the absorbance of the solution was measured at 695 nm using spectrophotometer versus blank after cooling to room temperature. Methanol in the place of extract was used as the blank. As standard was used ascorbic acid (AA). The total antioxidant capacity was determined as milligrams of ascorbic acid per gram of the dry extract (mg AA/g extract).

DETERMINATION OF DPPH FREE RADICAL SCAVENGING ACTIVITY

The free radical scavenging activity of extracts was measured using the stable radical DPPH (1,1-diphenyl-2-picryl-hydrazil) according to method [31] was adopted with suitable modifications from [32]. DPPH (8 mg) was dissolved in 100 mL methanol to obtain a concentration of 80 μg/ mL. Then serial dilutions were carried out with the stock solution (1mg/mL) of the extract. The resulting solutions (2mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517nm. As reference standards were used ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) and dissolved in methanol were used to make the stock solution with the same concentration (1mg/ mL). Control sample was prepared containing the same volume without test compounds or reference antioxidants. Methanol 95% was used as blank. Inhibition DPPH free radical scavenging activity (%) of lichen extract was calculated using the following equation:

$$\% \text{ inhibition} = [(Ac - As) / Ac] \times 100$$

(1) where Ac was the absorbance of the control (containing DPPH of the stock solution and methanol), and As was the absorbance of the sample (containing sample extract solution or standard solution without DPPH of the stock solution).

Results are presented as the IC50 values (minimum concentration of the each tested sample that reduces 50% of the DPPH radical, was calculated as μg/ mL through sigmoidal dose-response curve).

DETERMINATION OF THE INHIBITORY ACTIVITY TOWARD LIPID PEROXIDATION

The antioxidant activity of extracts was determined using the thiocyanate method [33]. Serial dilutions were carried out with the stock solution (1mg/mL) of the extracts, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0,2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50mL 40mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5mL with 40 mM phosphate buffer, pH 7.0. After incubation at 37°C in the

dark for 72 hours, a 0.1mL aliquot of the reaction solution was mixed with 4.7mL of ethanol (75%), 0.1 mL $FeCl_2$ (20 mM) and 0.1 mL ammonium thiocyanate (30%). The absorbance of this mixture was measured at 500 nm, after it was stirred for 3 min. As reference compounds were used ascorbic acid, gallic acid, α-tocopherol and BHT. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition of linoleic acid peroxidation (%) was calculated using following formula:

$$\% \text{ inhibition} = [(Ac - As) / Ac] \times 100$$

(2) where Ac was the absorbance of the control and As was the absorbance of the sample.

The results of inhibitory activity towards lipid peroxidation are presented as the IC50 values.

MEASUREMENT OF FERROUS ION CHELATING ABILITY

Based by decrease in absorbance at 562 nm of the iron (II)- ferrozine complex was measured by ferrous ion chelating ability [34, 35]. One milliliter of 0.125 mM $FeSO_4$ was added to 1.0 mL sample (with different dilutions), followed by 1.0 mL of 0.3125 mM ferrozine. Before measuring the absorbance, mixture was allowed to equilibrate for 10 min. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

$$\text{Chelating effect } (\%) = [(Ac - As) / Ac] \times 100$$

(3) where Ac was the absorbance of the control and As was the absorbance of the sample.

The results of ferrous ion chelating ability are presented as the IC50 values.

DETERMINATION OF HYDROXYL RADICAL SCAVENGING ACTIVITY

The ability of lichen Cetraria aculeata to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according method described by [36]. The reaction mixture contained 100 µL of extract dissolved in water, 500 µL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4-NaOH buffer (50 mM, pH 7.4), 200 µL of premixed 100 µM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100 µL of 1.0 mM H_2O_2 and 100 µL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50°C for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50°C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (Ac) and of the sample (As), using following formula:

$$\% \text{ inhibition} = [(Ac - As) / Ac] \times 100$$

(4) where the controls contained all the reaction reagents except the extract or positive control substance.

The results of hydroxyl radical scavenging activity are presented as the IC50 values.

STATISTICAL ANALYSIS

All computations were made by employing the statistical software (SPSS, version 11.0). Experimental results are presented as mean±standard deviations of three measurements. Statistical analyses were performed using Student's t-test and one way analysis of variance while the probability value of 0.05 was considered significant.

The obtained results of antioxidant activity for methanol extract of C. aculeata were compared to the published results of the antioxidant analysis of methanol extract of T.candida [40], using the Student's t-test.

RESULTS

Antioxidant activity

Table 1 shows the results of the determination of the total phenols and antioxidant capacity of the examined C. aculeata extracts. Total phenolic contents, expressed as gallic acid equivalents were amounted to 80.8±0.79 mg GA/g and 64.12±0.58 mg GA/g, for the methanol and ethyl acetate extracts, respectively. Results for total antioxidant capacity were amounted to 91.52±0.34 µg AA/g and 71.5±0.29 µg AA/g, for methanol and ethyl acetate extracts, respectively.

In Table 2 are given the results of DPPH scavenging activity for the examined C. aculeata extracts. For the methanol extract IC50 value was 51.65±1.38 µg/mL, while this value for the ethyl acetate extract was 41.4±0.94 µg/mL.

The results of inhibitory activity towards lipid peroxidation (Table 2) of the tested extracts of C. aculeata were amounted to 45.55±0.99 µg/mL and 38.55±0.76 µg/mL for methanolic and ethyl acetate extracts, respectively.

In Table 2 are shown IC50 values for the metal chelating activity for the methanol and ethyl acetate extract. These values were amounted of 50.43±0.98 µg/mL and 40.55±0.93 µg/mL, respectively.

The hydroxyl radical scavenging activity of the examined extracts are given in Table 2. For the methanol extract IC50 value was 90.1±0.47 µg/mL while for the ethyl acetate extract this value was 79.4±0.65 µg/mL.

DISCUSSION

Until now, many researchers investigated the antioxidant properties of many species of lichens and some of them have very good antioxidant activity [37, 38, 39, 40]. Secondary metabolites that have been identified from various species of lichen extracts manifested high antioxidant activity [23, 24, 25].

Some metabolites of lichens in their structure contain phenolic groups which are considered to be a key element for the antioxidative efficiency [41]. Protolichesterinic acid (aliphatic α- methylene-γ-lactone) is the active substance which has been identified and isolated from lichens C. islandica and C. aculeata. It was shown that this substance exhibits antimicrobial activity against E. coli, B. subtilis, P. aeruginosa, L. monocytogenes [27] and antiproliferative activity towards three human cancer lines (MCF-7, HeLa and HCT-116), while did not exhibit free radical scavenging activity [42]. How are tested extracts of C. aculeata showed significant antioxidant activity which may be the result of high phenolic

Table 1. Total phenolic and total antioxidant capacity of the examined Cetraria aculeata extracts

Lichen species	Extracts	Total phenolic (mg GA/g)	Total antioxidant capacity (μg AA/g)
Cetraria aculeata	Methanol	80.8±0.79	91.52±0.34
	Ethyl acetate	64.12±0.58	71.5±0.29

Table 2. The antioxidant activity of the examined Cetraria aculeata extracts

C. aculeata extracts/ standards	[a]IC_{50} (μg/mL)			
	DPPH scavenging activity	Inhibitory activity against lipid peroxidation	Metal chelating activity	Hydroxyl radical scavenging activity
Methanol	51.65±1.38	45.55±0.99	50.43±0.98	90.1±0.47
Ethyl acetate	41.4±0.94	38.55±0.76	40.55±0.93	79.4±0.65
Gallic acid	3.79±0.69	255.43±11.68	–	59.14±1.10
Ascorbic acid	6.05±0.34	> 1000	–	160.55±2.31
BHT	15.61±1.26	1.00±0.23	–	33.92±0.79
α-Tocopherol	–	0.48±0.05	–	–

[a]*IC50 values were determined by nonlinear regression analysis. Results are mean values±SD from three experiments.*

Table 3. IC_{50} values (means ± SD) of methanol extract of C. aculeata compared with methanol extract of T. candida, using the Student's t-test

IC_{50} (μg/mL)	*Cetraria aculeata* methanol extract	*Toninia candida* methanol extract [40]	t-test
DPPH scavenging activity	51.65±1.38	51.45±1.78	n.s.
Inhibitory activity against lipid peroxidation	45.55±0.99	46.46±1.68	n.s.
Metal chelating activity	50.43±0.98	41.91±0.88	*
Hydroxyl radical scavenging activity	90.1±0.47	67.11±0.23	*

Data were analysed by Student's t-test. *(* $p<0.05$; n.s. not significant)*

content. Future research of lichen C. aculeata can be focused to the identification and isolation of compounds on which depends the antioxidant activity of the tested extracts.

Previous studies have reported the antioxidant properties of aqueous extracts of C. islandica [43], but this is the first time to study the antioxidant activity of extracts C. aculeata.

These results showed that the methanol extract has a higher total phenolic content and total antioxidant capacity than the ethyl acetate extract. The high total phenolic content explains the strong antioxidant activity of C. aculeata assessed by the different systems. Recent study have been proved positive correlation between phenolic composition and antioxidant activity [44, 45]. Results of DPPH scavenging activity of ethyl acetate extract (41.4±0.94 μg/mL) shows a higher activity than methanol extract (51.65±1.38 μg/mL). Our study have shown that ethyl acetate extract of C. aculeata displayed a higher scavenging activity than the activity of methanol, chloroform and petrol ether extracts of Toninia candida [40]. Both tested extracts of C. aculeata showed higher scavenging activity compared to the methanol extracts of Parmelia sulcata, Flavoparmelia caperata, Evernia prunastri and Cladonia foliacea, while the methanol extract of C. aculeata showed the lower scavenging activity than the methanol extract of Hypogymnia physodes [46]. Significant antioxidant activity of extract of lichen Hypogymnia physodes arises from his lichen compounds (depsides, depsidones and usnic acid), which demonstrated strong antioxidant effects [24]. Phenol compounds are very important plant constituents because their hydroxyl groups contributed to their scavenging ability [47]. The results of inhibitory activity towards lipid peroxidation demonstrated that both tested extracts exhibited significant inhibitory activity (45.55±0.99 μg/mL and 38.55±0.76 μg/mL for methanolic and ethyl acetate extracts), respectively. The metal chelating activity of ethyl acetate extract with an IC50 value of 40.55±0.93 μg/mL displayed a higher chelating activity than methanol extract (IC50 values of 50.43±0.98 μg/mL).

The ethyl acetate extract of C. aculeata showed stronger hydroxyl radical scavenging activity than the methanol extract of this lichen. While both tested extracts of C. aculeata showed similar hydroxyl radical scavenging activity with the examined extracts of Umbilicaria cylindrica [48] and lower scavenging activity than the examined extracts of Toninia candida [40]. The results of hydroxyl radical scavenging activity are significant and suggest that the methanol and ethyl acetate extracts of C. aculeata acting as primary antioxidants. The tested extracts of C. aculeata showed stronger antioxidant activity than the many other species of lichens [38].

Table 3 showed the IC50 values (means±SD) of the methanol extract of C. aculeata compared with the methanol extract of Toninia candida [40]. Statistical analysis IC50 values of the antioxidant potential of the methanol extracts of C. aculeata and T. candida [40] showed the existence of statistical significance in the metal chelating and hydroxyl radical scavenging activities.

CONCLUSION

In conclusion, the results of our study showed that the tested extracts of C. aculeata demonstrated antioxidant activity. These results indicate the application of this lichen as source of natural antioxidants that could be

used as a possible food supplement, in the pharmaceutical industry and in the treatment of various diseases. The obtained results represent a good basis for a more detailed phytochemical examination of C. aculeata. Future research can be focused on the identification and isolation of the active components from C. aculeata and examination of their biological activities.

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SRPSKI

ANTIOKSIDATIVNA AKTIVNOST LIŠAJA CETRARIA ACULEATA

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SAŽETAK
Cilj ovog istraživanja je da se odrede antioksidantna svojstva lišaja Cetraria aculeata koji raste u Srbiji. Antioksidativna aktivnost metanolnog i etilacetatnog ekstrakta lišaja je testirana različitim metodama: određivanje ukupnog fenolnog sadržaja, određivanje ukupnog antioksidativnog kapaciteta, aktivnosti slobodnih radikala DPPH, inhibitorna aktivnost prema lipidnoj peroksidaciji, sposobnost vezivanja fero jona i aktivnost hidroksil radikala. Ekstrakti lišaja C. aculeata pokazali su značajnu antioksidativnu aktivnost. Metanolni ekstrakt je pokazao veće vrednosti za ukupne fenole i ukupni antioksidativni kapacitet u odnosu na etilacetatni ekstrakt, dok je etilacetatni ekstrakt pokazao bolje rezultate za aktivnosti DPPH radikala, inhibitornu aktivnost prema lipidnoj peroksidaciji, sposobnost i aktivnosti hidroksil radikala nego metanolni ekstrakt. Ovo je prvi prikaz antioksidativnih svojstava vrste Cetraria aculeata. Rezultati antioksidativne aktivnosti ukazuju na primenu ovog lišaja kao izvora prirodnih antioksidanata koji se mogu koristiti kao mogući dodatak ishrani, u farmaceutskoj industriji i u lečenju različitih bolesti.
Ključne reči: ROS, antioksidativna aktivnost, lišaj, Cetraria aculeata.