STEREOCAULON PASCHALE LICHEN AS ANTIOXIDANT, ANTIMICROBIAL AND ANTICANCER AGENT

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Abstract

In this study, antioxidant, antimicrobial and anticancer activities of the methanol extract of the lichen *Stereocaulon paschale* were determined by: free radical and superoxide anion scavenging activity, reducing power, determination of total phenolic compounds and determination of total flavonoid content, the minimal inhibitory concentration by the broth microdilution method against five species of bacteria and five species of fungi and the microculture tetrazolium test on FemX (human melanoma) and LS174 (human colon carcinoma) cell lines. As a result of the study methanol extract of *Stereocaulon paschale* had moderate free radical scavenging activity with IC_{50} values 879.56 µg/mL. Moreover, the tested extract had effective reducing power and superoxide anion radical scavenging. Values of minimum inhibitory concentration against the tested microorganisms ranged from 0.625 to 10 mg/mL. In addition, the tested extract had a strong anticancer activity towards both cell lines with IC_{50} values of 46.67 and 71.71 µg/mL.

Rezumat

În acest studiu au fost evaluate acțiunile antioxidantă, antimicrobiană și antitumorală a unui extract metanolic obținut din lichenul *Stereocaulon paschale*. S-au efectuat următoarele determinări: capacitatea de neutralizare a radicalilor liberi, puterea reducătoare, conținutul total de fenoli și flavonoide, concentrația minimă inhibitorie prin metoda microdiluției asupra a 5 specii de bacterii și 5 specii de ciuperci. De asemenea pe 2 tipuri de linii celulare tumorale umane s-a efectuat testul cu tetrazoliu (MTT). Rezultatele obținute sugerează că lichenul *Stereocaulon paschale*. poate fi un agent antioxidant, antimicrobian, genotoxic și antitumoral, natural.

Keywords: *Stereocaulon paschale*, biological activity, methanol extract.

Introduction

Lichens are complex symbiotic associations between a fungus (mycobiont) and photobiont which can be either an alga or cyanobacteria [1]. They are proven as the earliest colonizers of terrestrial habitats on the earth with a worldwide distribution from artic to tropical regions and from the plains to the highest mountains. Their specific, even extreme, conditions

of existence, slow growth and long life are the reason for producing numerous protective secondary metabolites against different physical and biological influences [2].

Lichens exert a wide variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and anticancer effects [3, 4]. Due to a relatively recent resurgence in lichen bioactivity, therapeutic potential of many classes of lichens in medicine has largely remained unexplored. For this reason, in this study, significant attention was focused on the lichen *Stereocaulon paschale.*

Stereocaulon paschale is a large, fruticose species with erect stalks (pseudopodetia); cephalodia abundant, blue-greenish to dark brownish, scabrid, usually forming blackish, spiky clusters, containing *Stigonema*. Stalks to 6 cm tall, naked below with white to rosy tomentum above; phyllocladia white or grey white, occasionally rose-tinged, warty, coralloid, or rarely somewhat squamulose; and apothecia uncommon, to 3 mm diameter [5]. It grows on soil, humus or soil over rock. *Stereocaulon paschale* have been fully investigated for their taxonomy, but very little data on its biological activity. Because of this, in the present study, there were examined *in vitro* the antioxidant, antimicrobial and anticancer activities of the methanol extract of the lichen *Stereocaulon paschale.*

Materials and Methods

Lichen Sample

Lichen sample of *Stereocaulon paschale* (L.) Hoffm., was collected from Kopaonik, Serbia, in September 2011. Voucher samples are preserved in the Department of Biology and Ecology of Kragujevac, Faculty of Science. Determination of the investigated lichen was accomplished using standard methods.

Extraction

Finely dry ground thalli of the examined lichen (100 g) were extracted using methanol 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 in the tests. The extract was dissolved in 5% dimethyl sulfoxide (DMSO) for the experiments. DMSO was dissolved in sterile distilled water to the desired concentration.

Antioxidant activity

Scavenging DPPH radicals

The free radical scavenging activity of extract was measured using 1,1-diphenyl-2-picryl-hydrazil (DPPH) method. The method used is similar

to Dorman *et al*. [6] but was modified in details. The stock solution of the extract was prepared in 5% DMSO to achieve the concentration of 1000 μg/mL. Further, two-fold dilutions were made to obtain concentrations of 500, 250, 125, 62.5 μg/mL. Diluted solutions of extract (1 mL each) were mixed with 2 mL of methanol solution of DPPH radical (0.05 mg/mL) 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 in a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK) against methanol as blank. Ascorbic acid was used as standard. The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A0 - A1)/A0] \times 100,$$

where A0 is the absorbance of the negative control (2 mL of methanol solution of DPPH radical + 1 mL of 5% DMSO) and A1 is the absorbance of reaction mixture or standard.

The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity. A lower IC_{50} meant better radical scavenging activity.

Reducing power

The reducing power of the extract was determined according to the method of Oyaizu [7]. The stock solution of the extract was prepared in 5% DMSO to achieve the concentration of 1000 μg/mL. Further, two-fold dilutions were made to obtain concentrations of 500, 250, 125, 62.5 μg/mL. One millilitre of each extract was 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 ^{0}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 ferric chloride (0.5 mL; 0.1%). The absorbance of the solution was measured at 700 nm. 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 was used as positive control.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of extract was detected according to the method of Nishimiki *et al.* [8]. The stock solution of the extract was prepared in 5% DMSO to achieve the concentration of 1000 μg/mL. Further, two-fold dilutions were made to obtain concentrations of 500, 250, 125, 62.5 μg/mL. Briefly, 0.1 mL of each extract was mixed with 1 mL nitroblue tetrazolium (NBT) solution (156 μM in 0.1 M phosphate buffer, pH 7.4) and 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 μM in 0.1 M phosphate buffer, pH 7.4). The reaction

was started by adding 100 μL of phenazine methosulfate (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 against blank sample (phosphate buffer). Decreased absorbance indicated increased superoxide anion radical scavenging activity. Ascorbic acid was used as standard. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging activity (\%)} = [(A0 - A1)/A0] \times 100,$$

where A0 is the absorbance of the negative control (consisting of all the reaction agents except the extract) and A1 is the absorbance of reaction mixture or standard.

The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity. A lower IC_{50} meant better radical scavenging activity.

Determination of total phenolic compounds

Total soluble phenolic compounds in the extract were determined using Folin-Ciocâlteu reagent according to the method of Slinkard and Singleton [9] using pyrocatechol as a standard phenolic compound. Briefly, 1 mL of the extract (1 mg/mL) in a volumetric flask diluted with distilled water (46 mL). One millilitre of Folin-Ciocâlteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of sodium carbonate (2%) were added and then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm against blank consisting of all the reaction agents except the extract. The total concentration of phenolic compounds in the extract was determined as microgram of pyrocatechol equivalent (PE) per milligram of dry extract by using an equation that was obtained from a standard pyrocatechol graph as follows:

$$\text{Absorbance} = 0.0057 \times \text{total phenols [μg PE/mg of dry extract]} - 0.1646$$
$$(R^2 = 0.9203)$$

Total flavonoid content

The total flavonoid content was determined using the Dowd method [10]. Two millilitres of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (1 mg/mL). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nmA negative control, without extract was used as the blank. The total flavonoid content was determined as microgram of rutin equivalent (RE) per milligram of dry extract by using an equation that was obtained from a standard rutin graph as follows:

$$\text{Absorbance} = 0.0296 \times \text{total flavonoid [μg RE/mg of dry extract]} + 0.0204$$
$$(R^2 = 0.9595)$$

Antimicrobial activity

Microorganisms and media

The following bacteria were used as test organisms in this study: *Staphilococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 10987), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC 29906). All the bacteria used were obtained from the American Type Culture Collection (ATCC). Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). The fungi used as test organisms were: *Aspergillus flavus* (DBFS 267), *Candida albicans* (DBFS 316), *Fusarium oxysporum* (DBFS 292), *Penicillium purpurescens* (DBFS 418) and *Trichoderma harsianum* (DBFS 379). All fungi were from the mycological collection maintained at the Mycological Laboratory within the Department of Biology of Kragujevac University's Faculty of Science (DBFS). Fungal cultures were maintained on potato dextrose (PD) agar except *Candida albicans* that was maintained on Sabourad dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4°C and subcultured every 15 days.

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 spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS [11].

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method with using 96-well microtiter plates [12]. A series of dilutions with concentrations ranging from 40 to 0.156 mg/mL for extract was used in the experiment against every microorganism tested. The starting solutions of extract were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of extracts were prepared in Müller-Hinton broth for bacterial cultures and 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 changing colour of resazurin was defined as the MIC for the tested microorganism at the given concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria,

ketoconazole in the case of fungi. A DMSO solution was used as a negative control for the influence of the solvents.

Cytotoxic Activity

Cell Lines

The human melanoma FemX and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (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, L-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), and 25 mM HEPES and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Treatment of Cell Lines

Stock solution (100 mg/mL) of test sample, made in dimethyl-sulfoxide (DMSO), was dissolved in the corresponding medium to the required working concentrations. Neoplastic FemX cells (5000 cells per well) and neoplastic LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates, and 24 h later, after the cell adherence, five different, double diluted, concentrations of the investigated sample, were added to the wells. The final concentrations applied to target cells were 200, 100, 50, 25 and 12.5 μg/mL, except for the control wells, where only nutrient medium was added to the cells. The nutrient medium was RPMI 1640 medium, supplemented with L-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), 10% heat inactivated (56 °C) fetal bovine serum (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 test sample on cancer cell survival was determined by MTT test (microculture tetrazolium test), according to Mosmann [13] with modification by Ohno and Abe [14], 72 h upon addition of the test sample, as described earlier. Briefly, 20 μL of MTT solution (5 mg/mL PBS) was added to each well. Samples were incubated for a further 4 h at 37 C in 5% CO_2 and a humidified air atmosphere. Then, 100 μL of 10% SDS was added to the extract of 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 the cell survival (%), A of a sample with cells grown in the presence of various concentrations of the investigated test sample was divided by the 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. 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*-diamminedichloro-platinum (*cis*-DDP) was used. All experiments were performed in triplicate.

Statistical analyses

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

Results and Discussion

The tested extract had antioxidant activity against various oxidative systems *in vitro* as a shown in Table I and Table II.

Table I

DPPH radical scavenging activity and superoxide anion scavenging activity of methanol extract of *Stereocaulon paschale*

	DPPH radical scavenging activity IC_{50} (µg/mL)	Superoxide anion scavenging activity IC_{50} (µg/mL)
Stereocaulon paschale	879.56	973.71
Ascorbic acid	6.42	115.61

Table II

Reducing power of methanol extract of *Stereocaulon paschale*

Absorbance (700 nm)					
	1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL
S. paschale	0.6108	0.4978	0.3165	0.2176	0.1221
Ascorbic acid	2.1131	1.6543	0.9572	0.4784	0.2472

DPPH radical scavenging and superoxide anion radical scavenging of the studied extract are summarised in Table I, while the reducing power is shown in Table II. The tested extract revealed lower antioxidant activities than ascorbic acid. The IC_{50} values were 879.56 and 973.71 µg/mL for DPPH radicals and superoxide anion radicals scavenging activity, respectively. Measured values of absorbance for reducing power in the tested extract varied from 0.1221 to 0.6108. As shown in Table II, the reducing power was concentration dependant (high concentration exhibited high reducing power).

Total phenolic and flavonoid constituents of tested extract are given in Table III. The amount of total phenolics and flavonoids in the extract were 52.67 µg PE/mg and 24.86 µg RE/mg, respectively.

Table III

Total phenols and flavonoid content of methanol extract of *Stereocaulon paschale*

Phenols content (µg PE/mg of extract)	Flavonoid content (µg RE/mg of extract)
52.67	24.86

The antimicrobial activity of the tested extract against the tested microorganisms was shown in the Table IV.

Table IV

Minimum inhibitory concentration of methanol extract of *Stereocaulon paschale*

Microorganisms	Methanol extract	S	K
Staphylococcus aureus	1.25	31.25	-
Bacillus subtilis	0.625	15.62	-
Bacillus cereus	0.625	15.62	-
Escherichia coli	2.5	62.5	-
Proteus mirabilis	1.25	62.5	-
Aspergillus flavus	10	-	7.81
Candida albicans	1.25	-	3.9
Fusarium oxysporum	5	-	3.9
Penicillium purpurescens	10	-	15.62
Trichoderma harsianum	5	-	7.81

Values given as mg/mL for extract and as µg/mL for antibiotics. Values are the mean of three replicates. Antibiotics: K - ketoconazole, S – streptomycin.

The methanol extract of the tested lichen showed relatively similar antibacterial and antifungal activity. The MIC for extract related to the tested bacteria and fungi were 0.625 - 10 mg/mL. The lowest measured MIC value (0.625 mg/mL) was related to the *Bacillus subtilis* and *Bacillus subtilis* species.

The antimicrobial activity was compared to streptomycin (standard antibiotic) and ketoconazole (standard antimicotic). The results showed that streptomycin and ketoconazole had a stronger activity than the tested extract as shown in Table IV. In the negative control, DMSO had no inhibitory effect on the tested organisms.

The anticancer activity of the tested lichen extract against the tested cell lines is shown in Table V. The IC_{50} against FemX and LS174 cell lines was 23.52 and 40.22 µg/mL, respectively. As shown in table, positive control (Cis-DDP) had slightly better anticancer activity than tested sample.

Table V

Growth inhibitory effects of methanol extract of *Stereocaulon paschale* on FemX and LS 174 cell lines

	FemX	LS 174
Test sample	IC_{50} (µg/mL)	
Stereocaulon paschale	23.52± 1.08	40.22 ± 0.08
Cis-DDP	0.94 ± 0.35	2.3 ± 0.31

The tested lichen extract had a strong antioxidant activity against various oxidative systems *in vitro*.

Free radical scavenging action is one of the numerous mechanisms of antioxidation. Antiradical activity of extract 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

$$DPPH + AH \rightarrow DPPH\text{-}H + A$$ [15].

The reducing power of methanol extract of *Stereocaulon paschale* also may indicate its potential antioxidant activity. The reducing features are mainly related with the presence of reductones. Gordan [16] 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 ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was measured by the strength of the green-blue colour of solution which absorbs at 700 nm. The results obtained indicated that the marked ferric reducing power activity of extract was due to the 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 [17].

The superoxide radical scavenging activity of methanol extract of *Stereocaulon paschale* was estimated based on its ability to destroy the superoxide radical produced from the PMS/NADH reaction. The decrease in absorbance at 560 nm indicates that superoxide anion in the reaction mixture disappeared [18].

Antioxidative nature of methanol extract of *Stereocaulon paschale* might depend on its phenolic content. Phenolic components are potential antioxidants. Phenolic compounds can donate hydrogen to free radicals and this way they 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 [19]. Flavonoids are a wide group of natural compounds and also the most important natural phenols. In

most lichens, phenols are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals [20]. Numerous researches found a high correlation between antioxidative activities of lichens and phenolic content [4, 21].

In the literature no data on antioxidant activity of *Stereocaulon paschale,* but antioxidant activity of some other lichens were studied by other researchers. For example, Kosanić *et al*. [3] found antioxidant activity for the acetone extracts of the lichen *Evernia prunastri* and *Pseudoevernia furfuraceae.* Manojlović *et al*. [22] explored antioxidant properties of *Umbilicaria cylindrica*. Compared with their research, the results of this study suggest that the methanol extract of *Stereocaulon paschale* showed a relatively powerful antioxidant activity.

Numerous lichens, such as *Parmelia reticulata, Toninia candida, Usnea barbata* were screened for antimicrobial activity in search of the new antimicrobial agents [4, 23], but in this study, for the first time, the antimicrobial activity of the methanol extract of *Stereocaulon paschale* was investigated. In correlation with results obtained in experiments with other lichens, we noticed that the methanol extract of *Stereocaulon paschale* showed relatively strong antimicrobial activity.

In these experiments, the examined extract in the same concentrations showed a slightly stronger antibacterial than antifungal activity. These results could be expected due to the fact that numerous tests proved that bacteria are more sensitive to the antibiotic compared to fungi [24]. The reason for different sensitivity between the fungi and bacteria can be found in different transparency of the cell wall. The cell wall of the gram-positive bacteria consists of peptidoglycan (murein) and teichoic acids, while the cell wall of the gram-negative bacteria consists of lipopolysaccharides and lipopoliproteins [25] whereas; the cell wall of fungi consists of polysaccharides such as hitchin and glucan [26].

In the present study, the results clearly demonstrate that methanol extract of the studied lichen induced significant anticancer effect on the tested cancer cell lines. Until now, only few researches proved that lichen extracts have anticancer activity. Kosanić *et al*. [3] reported significant anticancer effect for *Evernia prunastri* and *Pseudoevernia furfuraceae*. Manojlović *et al*. [27] explored anticancer properties of *Thamnolia vermicularis*. Trigiani *et al*. [28] found strong anticancer activity for *Xanthoria parietina.*

The importance of lichens as anticancer agents was confirmed in recent years, which suggests that lichens can be used as biological agents in the treatment of cancer. The mechanism of action of the tested extracts and

their compounds is yet to be tested. Further research will be necessary for fractionation in order to identify compounds responsible for the observed anticancer effect, and to establish the opportunities reinforcement activities and to improve the selectivity.

Conclusions

In conclusion, it can be stated that the tested extract had a certain level of antioxidant, antimicrobial and anticancer activities *in vitro*, which suggest that *Stereocaulon paschale* could be a natural antioxidant, antimicrobial, genotoxic and anticancer agent. Further studies should be done on the isolation and characterization of pure compounds from this lichen, which are responsible for its antioxidant, antimicrobial and anticancer activities.

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