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DETERMINATION OF BIOLOGICAL ACTIVITIES (ANTIBACTERIAL, ANTIOXIDANT AND ANTIPROLIFERATIVE) AND METABOLITE ANALYSIS OF SOME LICHEN SPECIES FROM TURKEY

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ABSTRACT
The aim of this study was to evaluate in vitro antibacterial, antioxidant and antiproliferative activities of 3 different lichens species (*Anaptychia ciliaris, Bryoria capillaris* and *Cetraria islandica*) and their chemical constituents. Lichen species were collected from different provinces of Bolu, Turkey, and extracted with methanol and acetone. Antibacterial activity was evaluated by disc diffusion method against 10 pathogenic bacteria. Antioxidant activity was determined by using DPPH method. Total phenol and flavonoid were determined by using Folin-Ciocaltaeu and aluminum chloride ($AlCl_3$) colorimetric method, respectively. Antiproliferative activity was evaluated against MCF-7 (Human breast adenocarcinoma) and HepG2/C3A (Human hepatocellular carcinoma) cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The chemical constituents of selected lichens were detected by High Performance Liquid Chromatographic Diode Array (HPLC-DAD) via chosen five standards (usnic acid, atranorin, stictic acid, evernic acid and fumarprotocetraric acid).The most effective antibacterial agent was acetone extract of *C. islandica* with inhibition values ranging from 20.3-28.0 mm against Gram-positive bacteria. *B. capillaris* exhibited broad spectrum of antibacterial activities against both Gram-positive and Gram-negative bacteria. *B. capillaris* had the highest free radical scavenging activity at 100 µg/ml concentration (67.4%). Moreover, consistent relationship was observed among total phenolic and flavonoid contents, and antioxidant effect of tested species. Strong antiproliferative activity was not observed with tested lichen species (IC_{50} value of >200 µg/ml). For HPLC analysis, acetone extracts had higher lichen acid content than methanol extracts. Aceton extract of *B. capillaris* was significant source of usnic acid and only *C. islandica* consisted of fumarprotocetraric acid. The present study showed that tested lichen species demonstrated an effective antibacterial and antioxidant activities. The results suggest that detected compounds can be possible agents for using pharmaceutical purposes.

KEYWORDS: *Anaptychia ciliaris*, antibacterial, antioxidant, antiproliferative, *Bryoria capillaris*, *Cetraria islandica*, DPPH, Folin-Ciocalteau, Aluminum chloride colorimetric, HPLC.

INTRODUCTION
Lichens are a symbiotic life form between a fungus (mycobiont) and an alga or a cyanobacterium (photobiont) with unique characteristics in plant kingdom.[1] It is estimated that there are approximately 25.000 species of lichens.[2] They are proven as the earliest colonizers of terrestrial habitats on the earth with a worldwide distribution from arctic to tropical regions and from the plains to the highest mountains.[3] These organisms have historically been used as food, dyes, in production of alcohol and perfume industry. They have also, for hundreds of years, been used in many countries as a cure for diseases of humans.[4]

Their specific, even extreme, range of habitats, slow growth, and long life are the reason for them being able to produce numerous protective secondary metabolites against different physical and biological influences.[5] They are mostly produced by the mycobiont, and accumulate in the cortex or in the medullary layer as extracellular tiny crystals on the outer surfaces of the hyphae.[6] Past and current studies show that lichen secondary metabolites exert a wide variety of biological activities that include antibiotic, antimycobacterial, antiviral, antiinflamatory, analgesic, antipyretic, plant growth inhibitory, enzyme inhibitory, antiproliferative and cytotoxic effects.[7]

The therapeutic potential of many lichen species and their metabolites has largely remained unexplored. Thus, the aim of this study is to evaluate the antioxidant capacity, antibacterial and antiproliferative activities of the lichens *Anaptychia ciliaris*, *Bryoria capillaris* and *Cetraria islandica* and to identify their chemical constituents by HPLC-DAD as possible therapeutic agents.

MATERIALS AND METHODS

Lichen Material and Extraction

Lichen materials [*Anaptychia ciliaris* (L.) Körb., *Bryoria capillaris* (Ach.) Brodo & D. Hawksw. and *Cetraria islandica* (L.) Ach.] were collected from different provinces (Lake Abant province, Aladağlar province and Lake Seben province) of Bolu, Turkey between 2014-2015 years. They were identified by Dr. Gulsah Ozyigitoglu and voucher specimens were deposited at the Abant Izzet Baysal University Herbarium, Bolu, Turkey.

Collected lichens were dried in a room avoiding sun light and then ground into a powder. Each lichen was extracted by two different solvents (methanol and acetone). For methanol extracts (ME), 10 grams of each lichen species have been extracted with 200 ml methanol at 45 °C in a water bath for 12 hours. The extracts were evaporated under low pressure at a temperature not higher than 45 °C using rotary evaporator. In acetone extractions (AE), about 10 grams of each lichen sample were extracted by soxhlet with 200 mL of acetone at 50°C for 12 hours and then filtered. Filtrates were evaporated under vacuum using rotary evaporator to give the crude extracts. Lichen materials, locations and extraction yields are represented in Table 1.

Table 1: Information about tested lichen species.

Lichen Species	Location	Coordinate	Altitude	Collection number	Extract	Yield (%)
A. ciliaris	Lake Abant Province	40°36.589 N, 031°17.519 E	1340 m	isa1005	Methanol	5.3
					Acetone	2.3
B. capillaris	Aladağlar Province	40°36.014 N, 031°16.655 E	1353 m	isa1006	Methanol	6.7
					Acetone	7.4
C. islandica	Lake Seben Province	40°31.518 N, 031°36.880 E	1455 m	isa1009	Methanol	3.7
					Acetone	1.5

Antibacterial Bioassay

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibacterial activity.[8] The following bacteria strains were employed in the screening: Gram-positive *Streptococcus pyogenes* (ATCC® 19615), *Staphylococcus aureus* (ATCC® 25923) and *Staphylococcus epidermidis* (ATCC® 12228) and Gram-negative *Escherichia coli* (ATCC® 25922), *Pseudomonas aeruginosa* (ATCC® 27853), *Salmonella typhimurium* (ATCC® 14028), *Serratia marcescens* (ATCC® 8100), *Proteus vulgaris* (ATCC® 13315), *Enterobacter cloacae* (ATCC® 23355) and *Klebsiella pneumoniae* (ATCC® 13883).

Agar cultures of the test microorganisms were prepared as described by Turker et al.[8] For this purpose, the turbidity of each broth culture of bacteria was adjusted with saline to obtain turbidity visually comparable to that of a 0.5 McFarland standard and then Mueller Hinton agar plates for each bacterium were inoculated by using cotton swabs.

All extracts were dissolved in Dimethyl sulfoxide (DMSO) to the final concentration of 100 mg/mL and sterilized by filtration through a 0.22 µm sterilizing Millipore express filter (Millex®). Sterile, 6-mm diameter filter paper discs (Glass microfibre filters, Whatman®) were impregnated with 13 µl of extract. Positive controls consisted of five different antimicrobial susceptibility test discs (Bioanalyse®): Erythromycin (15 µg) (E-15), Ampicillin (10 µg) (AM-10), Carbenicillin (100µg) (CB-100), Tetracycline (30 µg) (TE-30) and Chloramphenicol (30 µg) (C-30). DMSO was used as a negative control. Paper discs containing lichen extracts and antibiotic discs were placed onto Mueller Hinton agar. Inoculated plates with discs were placed in a 37°C incubator. After 16 to 18 hours of incubation, inhibition zone diameter (mm) was measured. All experiments were repeated three times.

Antioxidant Assay

Free radical scavenging activity

Free radical scavenging activity of the methanol extracts of lichen species was determined spectrophotometrically by monitoring the disappearance of 2,2-diphenyl-1-picrylhydrazil (DPPH•, Sigma-Aldrich Chemie, Steinheim, Germany) at 517 nm, according to the method described by Brand-Williams et al. [9] Briefly, 0.15 mM solution of DPPH in ethanol was prepared. Then 1 mL of this solution was added to 3 mL of the extracts at different concentrations (25, 50, 100 and 200 µg/ml). These solutions were vortexed thoroughly and incubated in the dark. Thirty minutes later, the decrease in the absorbance of these solutions was measured at 517 nm with Hitachi U-1900, UV-VIS Spectrophotometer 200V against blank samples. All analyses were made in

triplicate. The DPPH• scavenging capacity of the extracts was calculated using the following equation:
DPPH· Scavenging Effect (% inhibition) = [(A0−A1/A0) x 100][10] where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of tested extracts.

Determination of total phenolic content
The phenolic contents of acetone and methanol extracts of lichen species were determined according to the procedure described by Slinkard and Singleton[11] with the slight modification of using a Folin-Ciocalteu phenolic reagent. Gallic acid were used as a standard phenolic compound. Briefly, 2mL of distilled water was added to 0.01 g of extracts (5mg/ml) of lichens. Prepared stock solution was then diluted to 1mg/mL. To prepare a calibration curve; 0, 50, 100, 150, 200, 250 and 500 mg/L gallic acid was prepared. 20 µL from each calibration solution, sample, or blank was placed into separate cuvettes. Then, 1.58 mL of water and 100 µL of Folin-Ciocalteu reagent (Sigma®) was added to each, and then mixed well. After 2 minutes, 300 µL of Na_2CO_3 solution was added and was shaken very well. The solutions were incubated at 20 ^{0}C for 2 hours and measured the absorbance of each solutions at 765 nm against the blank (the "0 mL" solution) using the spectrophotometer.

The amounts of total phenolic compounds in lichen extracts were determined as micrograms of gallic acid equivalent, using an equation that was obtained from a standard gallic acid graph (R^2: 0.9966). All analyses were made in triplicate.

Determination of total flavonoid
The amount of total flavonoids in the acetone and methanol extracts was measured by aluminum chloride ($AlCl_3$) colorimetric assay. Catechol was used as a reference flavonoid. 1250 mg/mL and 500 mg/mL concentrations of the extracts were prepared in ethanol. Different concentrations of catechol (20, 40, 60, 80 and 100 mg/mL) were prepared in order to obtain standard calibration curve of catechol. Briefly, 500 µL of extract solution or standard solution of catechol was added to a 10-mL test tube containing 2 mL of distilled water. Then, 150 µL of 5% $NaNO_2$ was added to the test tubes. After 5 min, 150 µL of 10 % $AlCl_3$ was added. At 6 min, 1000 µL of 1M NaOH was added to the mixture. Immediately, the reaction tube was diluted to volume of 5 mL with the addition of 1200 µL of distilled water and thoroughly mixed.

Absorbance of the mixture was determined at 510 nm versus a blank. Samples were analyzed in three replications. [12] The total flavonoid contents of lichen extracts were expressed as mg catechol equivalents (CE)/100g dried weight of plant.

Human cancer cell lines and culture conditions
Human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were grown in Dulbecco's minimum essential medium (DMEM) with Earle's salts (Mediatech Cellgro, Herndon, USA). Culture medium was supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA), a solution of vitamins, sodium pyruvate and non-essential amino acids (all at 1:100 v/v dilution of supplied solutions), penicillin (100 I.U./ml) and streptomycin (100 µg/ ml) (Mediatech Cellgro, VA). Cells were cultured at 37 °C in a humidified environment containing 5% CO_2.

Cell viability assay
Exponentially growing cells were plated in 96-well microplates (Costar, Corning Inc.) at a density of 10×10^3 cells per well in 100 µL of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of each extract in DMSO (Sigma-Aldrich) were then added (100 µL per well) and the cells were incubated for 24 h. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a yellow tetrazole (MTT) assay on an automated 96-well Multiskan FC microplate photometer reader (Thermo Fisher Scientific Inc.) at 570 nm. The proliferation test is based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. The culture medium was removed and replaced with 90 µl of fresh culture medium. Ten microlitres of sterile filtered MTT solution (5 mg/ml) in phosphate buffered saline (PBS, pH 7.4) were added to each well, reaching a final concentration of 0.5 mg MTT/ ml which was then incubated at 37 ˚C in 5% CO_2 for 4 h. After 4 h, 100 µl/well of DMSO were added to all samples for dissolving of formazan that is the final product of MTT reaction and were allowed to incubate at 37 °C, in a 5% CO_2 humidified incubator for a night. After incubation, absorbance of formazan was measured spectrophotometrically in a Multiskan FC microplate photometer reader at 570 nm. Each experiment was carried out three times in triplicate. The relative cell viability (%) related to control wells containing cell culture medium without samples was calculated as:

100 × A570 nm (sample) / A570 nm (control).

High Performance Liquid Chromatography (HPLC) analysis
Methanol and acetone extracts were analyzed using a HPLC system (VWR-Hitachi LaChrom Elite®) equipped with a Hitachi L-2455 Diode-Array Detector (DAD), Hitachi L-2130 Pump, Hitachi L-2200 Autosampler. Chromatographic separation was achieved using Hitachi column oven L-2300 and Venusil XBP C18 column (Bonna-Agela Technologies, particle size 5 µm, 4.6 x 250 mm). Flow rate was 1 ml/min with 25 ºC oven and injection volume was 20 µl. All solvents were HPLC

grade (Merck) and mobile phased was composed of methanol/water/phosphoric acid (90/10/0.9 v/v/v).[13] Mobile phases and ultrapure water (SG Labostar) were filtered through a 0.45 µm hydrophilic polypropylene membrane filter (47 mm) (Pall Corporation) prior to HPLC injection. Spectra data were recorded from to 200 to 400 nm during the entire run. The chromatograms were obtained at 254 nm.

Sample Preparation for HPLC Analysis

Methanol and acetone extracts of all lichen samples (10 mg) were dissolved in 10 ml methanol (1 mg/ml) in volumetric flasks. Five different lichen substances were used as reference standards which consist of usnic acid (Sigma®), evernic acid (Sigma®), stictic acid (Chromadex®), atranorin (Chromadex®) and fumarprotocetraric acid (BOC sciences®). All standards were separately prepared at 1 mg/ml in MeOH (stictic acid, evernic acid and usnic acid) or acetone (atranorin and fumarprotocetraric acid) and diluted five different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/ml) to calibrate the standard curve. All extracts and standards were filtered through a 0.2 µm GHP Acrodisc (25 mm) (Pall Corporation) into 2 ml HPLC vials. Procedures were repeated 3 times for each sample tested.

RESULT AND DISCUSSION

Screening of antibacterial antioxidant and antiproliferative capacities of 3 different lichen species was conducted (Table 1,2,3,4 and 5). Six crude extracts were prepared using 2 different solvents (acetone and methanol) for the experiments (Table 1). Some lichen acids were also analyzed (Table 7).

Best antibacterial activity was obtained with acetone extract of *C. islandica* against Gram-positive bacteria (*S. aureus*, *S. epidermidis* and *S. pyogenes*) with the inhibition zones between 20.3-28 mm. Tested 3 lichens exhibited strong antibacterial activity against Gram-positive bacteria. Vulnerability of Gram-positive bacteria may originate from cell wall structure containing single layer. On the other hand, Gram-negative cell wall contains multi-layered and complex structure. Methanol and acetone extracts of *A. ciliaris*, and methanol extract of *C. islandica* did not exhibit antibacterial activity against tested Gram-negative bacteria.

Generally, acetone extracts of tested lichens exhibited more effective antibacterial activity than methanol extract. Acetone extracts of *B. capillaris* exhibited a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. Acetone extract of *B. capillaris* had antibacterial activity against 8 bacterial strains with the inhibition zones between 8.0-19.0 mm. Only five bacteria were inhibited by methanolic extract of *B. capillaris*. The acetone extract of *B. capillaris* showed higher inhibition zones (14.8 mm and 9.5 mm, respectively) than reference antibiotic Erythromycin in the case of *P. vulgaris* and *S. marcescens* with the inhibition zones of 13.6 and 11 mm, respectively. Cobanoglu et al.[14] reported that *B. capillaris* did not exhibit antibacterial activity against *S. aureus* and *P. aeruginosa*. However, these bacteria were vulnerable to all extracts of *B.capillaris* in our study (Table 2). In consistent with our study, *E. coli* was not sensitive to acetone extract of *B. capillaris*.[14] On the other hand, Sariozlu et al. [15] reported that methanol and acetone extracts of *B. capillaris* displayed antibacterial activity against *E. coli*. Similar to our result, *S. aureus* and *P. vulgaris* were vulnerable to methanol and acetone extracts of *B. capillaris*. [15] Antibacterial activities of *B. capillaris* against *S. epidermidis*, *S. marcescens*, *S. pyogenes*, *S. typhimurium* and *E. cloaca* were presented for the first time with this study (Table 2). Antibacterial results of *B. capillaris* supported the traditional uses of *B. capillaris* in the remedy of diarrhea, vaginal discharge and skin abrasions.[16]

K. pneumonia and *E. coli* did not show sensitivity against to the tested lichen extracts. In addition, only acetone extract of *B. capillaris* inhibited *S. marcescens* and *S. typhimurium* bacteria (Table 2). Positive controls (antibiotics) generally showed antibacterial activity to our test organisms. Since final concentrations of all lichen extracts were adjusted with DMSO, it was used as a negative control and no inhibition was observed with it (Table 2).

Both acetone and methanol extracts of *C. islandica* exhibited strong activity against Gram-positive bacteria; *S. aureus*, *S. epidermidis* and *S. pyogenes*. Especially acetone extract showed best antibacterial activity with the inhibition zone of 20.3, 25.5 and 28.0 mm, respectively. Acetone extract of *C. islandica* also showed antibacterial activity against some Gram-negative bacteria; *P. aeruginosa* and *P. vulgaris* (10.0 and 8.3 mm, respectively). This was the best antibacterial effect against *P. aeruginosa* as compared to other tested lichen extracts and also better than some reference antibiotics. Dulger et al.[17] reported that acetone and ethanol extract of *C. islandica* did not show antibacterial activity against *S. aureus*, *S. epidermidis* and *E. coli*. Similarly, *E. coli* was not vulnerable to acetone and methanol extract of *C. islandica*. But, *S. aureus* and *S. epidermidis* were very sensitive to both extracts in our study (Table 2). Grujicic et al.[18] found that methanol extract of *C. islandica* showed antibacterial activity against *E. coli* and *S. auerus*. However, *E. coli* was not susceptible to methanol extract of *C. islandica* and moderate antibacterial activity (11 mm) was observed against *S. auerus* in our study (Table 2). Antibacterial activities of acetone extract of *C. islandica* against *S. epidermidis*, *S. pyogenes*, *P. aeruginosa* and *P. vulgaris* were recorded for the first time with our study.

Methanol and acetone extracts of *A. ciliaris* exhibited moderate antibacterial activity against only Gram-positive bacteria with the inhibition zones between 8.3-14.8 mm. Karagoz et al.[19] showed that ethanol extracts of *A. ciliaris* exhibited no inhibition against S. *aureus*, *S.*

epidermidis, *P. aeruginosa*, *E. coli* and *K. pneumonia*, and aqueous extract of *A. ciliaris* displayed moderate inhibition against *E. coli* and *S. aureus*. Rankovic et al.[20] reported that *S. aureus*, *P. aeruginosa* and *K. pneumonia* were susceptible to methanol extract of *A. ciliaris* and *E. coli* was not vulnerable to this extract. On the other hand, methanolic extract of *A. ciliaris* showed moderate inhibition against *S. epidermidis* and *S. pyogenes*, and no activity against *S. aureus*, *P. aeruginosa*, *K. pneumonia* and *E. coli* in our study (Table 2). Antibacterial activity of *C. islandica* against *S. aureus*, *S. epidermidis*, *S. progenes*, *P. aeruginosa* and *P. vulgaris* may explain why it uses in traditional medicine to treat wounds, edemas, uterine cysts, nephritis and some respiratory diseases such as tuberculosis, asthma and whooping cough.[16]

Table 2: Antibacterial activity of tested lichen species. Data presented as zone of inhibition of bacterial growth in mm. Means with the same letter within columns are not significantly different at *P*>0.05. AE: Acetone, ME: Methanol

Treatment		Zone of inhibition (mm ± SE)									
		S. auerus	*S. epidermidis*	*S.pyogenes*	*S. marcescens*	*S.typhimurium*	*P.aeruginosa*	*P. vulgaris*	*K. pneumonia*	*E. cloacae*	*E.coli*
A. ciliaris	AE	9.5 ± 0.3^{i}	8.3 ± 0.3^{h}	14.8 ± 0.3^{f}	-	-	-	-	-	-	-
	ME	-	10.8 ± 0.5^{g}	12.3 ± 0.6^{f}	-	-	-	-	-	-	-
B. capillaris	AE	16.0 ± 1.1^{g}	19.0 ± 0.4^{e}	14.8 ± 0.5^{f}	9.5 ± 0.3^{f}	9.8 ± 0.5^{c}	$8.0 \pm 0.4^{b,c}$	14.8 ± 0.3^{c}	-	8.5 ± 0.3^{e}	-
	ME	8.5 ± 0.3^{j}	11.5 ± 0.5^{g}	12.8 ± 0.8^{f}	-	-	-	9.8 ± 0.3^{d}	-	8.0 ± 0.4^{e}	-
C. islandica	AE	20.3 ± 0.6^{f}	25.5 ± 0.3^{d}	28.0 ± 1.2^{d}	-	-	10.0 ± 0.4^{b}	8.3 ± 0.3^{d}	-	-	-
	ME	11.0 ± 0.0^{h}	16.5 ± 0.3^{f}	$24.0 \pm 1.2^{d,e}$	-	-	-	-	-	-	-
Ampicillin (10 mg)		39.8 ± 0.8^{b}	31.0 ± 0.0^{c}	48.6 ± 2.5^{a}	14.6 ± 0.3^{d}	27.4 ± 0.3^{a}	-	27.0 ± 1.4^{b}	8.4 ± 1.1^{c}	27.0 ± 0.0^{c}	21.2 ± 0.5^{c}
Carbenicillin (100mg)		43.8 ± 0.8^{a}	40.0 ± 1.4^{a}	42.8 ± 3.3^{b}	28.0 ± 0.0^{a}	25.2 ± 2.2^{b}	10.0 ± 0.0^{b}	36.8 ± 1.9^{a}	8.0 ± 1.4^{c}	32.0 ± 1.4^{a}	24.4 ± 0.3^{b}
Chloramphenicol (30 mg)		25.0 ± 0.0^{e}	32.6 ± 1.7^{b}	$32.0 \pm 1.4^{c,d}$	26.8 ± 0.8^{b}	28.8 ± 0.8^{a}	9.0 ± 0.0^{b}	27.0 ± 1.4^{b}	29.2 ± 0.8^{a}	28.8 ± 0.5^{b}	28.2 ± 0.5^{a}
Erythromycin (15 mg)		30.0 ± 0.0^{d}	40.0 ± 0.0^{a}	35.2 ± 3.6^{c}	11.0 ± 0.0^{e}	10.8 ± 0.5^{c}	-	13.6 ± 0.3^{c}	13.0 ± 0.0^{b}	9.6 ± 0.3^{d}	12.0 ± 0.0^{d}
Tetracycline (30 mg)		32.0 ± 0.0^{c}	8.0 ± 0.0^{h}	36.2 ± 1.2^{c}	23.8 ± 0.8^{c}	25.2 ± 0.5^{b}	16.0 ± 0.0^{a}	37.6 ± 1.6^{a}	29.8 ± 0.5^{a}	30.6 ± 0.3^{a}	28.8 ± 0.8^{a}
DMSO		-	-	-	-	-	-	-	-	-	-

DPPH antioxidant assay is used to assess the free radical scavenging effectiveness of various antioxidant substances. Scavenging activity of antioxidant substances is evaluated using DPPH radical as a substrate. The reduction capability on the DPPH radical is determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical. Free radical scavenging action is one of the important mechanisms for antioxidation. [21] In the present study, antioxidant activity of methanol extracts of tested lichens was evaluated (Table 3). Scavenging activity of the extracts was studied by screening the ability to bleach the stable DPPH radical. *B. capillaris* and *C. islandica* exhibited better DPPH radical scavenging activity than *A. ciliaris*. *B. capillaris* and *C. islandica* showed a concentration dependent free radical scavenging activity (%) by scavenging DPPH radical. Free radical scavenging activity raised when concentrations increased from 5 to 200 µg/ml. *A. ciliaris* extract has little tendency to scavenge DPPH radicals (Table 3). Similar to our result, Rankovic et al. [20] revealed a weak antioxidant activity of methanol extract of *A. ciliaris*. Although *B. capillaris* exhibited the highest DPPH radical scavenging activity (67.4%) at 100 µg/ml concentration, *C. islandica* showed the highest activity (65.6%) at 200 µg/ml concentration (Table 3). Grujicic et al.[18] and Fernández-Moriano et al.[22] reported that IC_{50} value of methanol extract of *C. islandica* was 678.38 µg/ml and 1183.55 µg/ml, respectively for DPPH radicals scavenging activity. *C. islandica* in our study showed better radical scavenging activity having IC_{50} value of around 75 µg/ml (Table 3). Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups. There is a correlation between total phenol and DPPH radicals scavenging activity of the tested lichen extracts. In consistent with DPPH radicals scavenging activity results, among the methanol extracts of tested lichens, the highest phenolic content was obtained with *B. capillaris* and the lowest phenolic content was observed with *A. ciliaris* (Table 3 and 4). This was the first report for antioxidant activity of *B. capillaris*.

Table 3: % inhibition of DPPH by methanolic lichen extracts.

Treaatments	% Inhibition of DPPH Concentrations				
	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
Ascorbic acid	92.83	97.08	97.49	98.10	98.12
A. ciliaris	1.4	0.1	0.7	19.3	28.9
B. capillaris	38.9	45.8	54.3	67.4	60.4
C. islandica	17.5	27.4	41.5	58.1	65.6

Phenolics and flavonoids are significantly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals. They prevent reactive oxygen formation, chelate trace elements found in free-radical production, scavenge reactive species and protect antioxidant defense system in the body. [23] Methanol extract of *B. capillaris* had the highest total phenolic content (163.3 mg Gallic acid (GA) equivalent (E)/g dried extract). Acetone extract of *B. capillaris* also contained high flavonoid content (154.5 mg Catechol (C) E/g dried extract). Acetone extract of *C. islandica* included the highest flavonoid amount (158.2 mg CE/g dried extract) (Table 4). Grujicic et al. [18] reported that the total phenolics and flavonoids in methanol extract of *C. islandica* were 38.08 µg pyrocatechol equivalent/mg and 25.81 µg rutin equivalent/mg, respectively. In another report, Fernández-Moriano et al. [22] found that total phenolic amount of methanol extract of *C. islandica* was 57.34 µg GAE/mg. *C. islandica* in our study contained higher total phenolic and flavonoid contents having 78.3 mg GAE/g and 55.6 mg CE/g, respectively (Table 4). Rankovic et al. [20] reported that methanol extract of *A. ciliaris* included 27.6 mg GAE/g. In consistent with their results [20], *A. ciliaris* in our study contained similar amount having 29.2 mg GAE/g. Total phenolic and flavonoid contents of *B. capillaris* were presented for the first time with this study.

Table 4: Total phenolic and flavonoid content of tested lichen species. AE: Acetone, ME: Methanol

Lichen species	Treatments	Total Phenolics mg GAE/ g dried mass	Total Flavonoids mg CE/ g dried mass
A. ciliaris	ME	29.2 ± 0.00	93.3 ± 0.00
	AE	34.2 ± 0.00	53.4 ± 0.00
B. capillaris	ME	163.3 ± 0.00	154.5 ± 0.001
	AE	160.1± 0.00	157.1 ± 0.001
C. islandica	ME	78.3 ± 0.00	55.6 ± 0.00
	AE	45.3± 0.00	158.2 ± 0.00

The cytotoxic effects of acetone extracts at various concentrations were evaluated with in vitro cytotoxicity assay against MCF-7 (human breast cancer) and HepG2/C3A (human hepatocellular carcinoma) cell lines (Table 5). Strong antiproliferative activity was not observed with tested lichen species (IC_{50} value of >200 µg/ml) (Table 5). Grujicic et al. [18] showed significant anticancer effects of methanolic extract of *C. islandica*, against Femx (human melanoma) and LS174 (human colon carcinoma) cell lines with IC_{50} values of 22.68 µg/ml and 33.74 µg/ml, respectively. Fernández-Moriano et al. [22] reported the cytotoxic activity of methanolic extract of *C. islandica* against HepG2 (human hepatocellular carcinoma) and MCF-7 (breastadenocarcinoma) cell lines having IC_{50} values of 181.05 µg/ml and 19.51 µg/ml, respectively. However, acetone extract of *C. islandica* did not show any antiproliferative activity against to MCF-7 and HepG2/C3A cell lines at all used concentrations in our study.

Table 5: Antiproliferative activities of acetone extracts of tested lichen species.

Lichen Extracts	IC_{50} (µg/mL)	
	MCF-7	HepG2/C3A
A. ciliaris	> 200	> 200
B. capillaris	> 200	> 200
C. islandica	> 200	> 200

HPLC with diode array detector (DAD) system provides the identification of substances (qualitative analyzes) by comparison of retention time data and UV spectra of peaks, and determination the amount of substances (quantitative analyzes). Quantification of the chosen five lichen acid standards (usnic acid, atranorin, stictic acid, evernic acid and fumarprotocetraric acid) in three different lichen species was performed. The values of retention time and absorbance maxima of the used standards were given in Table 6. The chromatogram of the standards used in the experiment was shown in Figure 1. Quantitative analysis of the lichen acids in tested lichen species were exhibited in Table 7. HPLC analysis showed that acetone was the most efficient solvent for obtaining lichen compounds. Acetone extract of *B. capillaris* was detected as usnic acid source significantly (101.43 mg/g dry extract). *A. ciliaris* and acetone extract of *C. islandica* didn't possess any amount of usnic acid. The dibenzofuran usnic acid has anti-inflamatory, antimicrobial, anticancer, anti-viral, gastroprotective, antipyretic and non-genotoxic anticancer activites [1,24,25]. Fumarprotocetraric acid was only found in *C. islandica*. Acetone extract had higher amount than methanol (233.18 mg/g and 1515.64 mg/g, respectively). Fumarprotocetraric acid has strong antimicrobial, antioxidant and anticancer activities. [13,26,27] We observed that any of tested lichen species included other standard compounds; atranorin, evernic acid and stictic acid by HPLC analysis. Gudjonsdottir and Ingolfsdottir [28] reported that acetone extract of *C. islandica* contained fumarprotocetraric acid and protocetraric acid. Fernández-Moriano et al. [22] reported that methanol extract of *C. islandica* contained the depsidone fumarprotocetraric acid as its main constituent and trace amount of related depsidone protocetraric acid. Similarly, acetone and methanol extracts of *C. islandica* contained high amount of fumarprotocetraric acid in our study (Table 7). Noticeable antibacterial activities of acetone extracts of *C. islandica* against Gram-positive bacteria may be attributed to the high content of fumarprotocetraric acid in *C. islandica*. Furthermore, broad spectrum of antibacterial activity of acetone extract of *B. capillaris* may be explained by high content of usnic acid in *B. capillaris* in our study (Table 2 and 7).

Table 6: Retention time of tested lichen standards and their absorbance maxima (nm).

Peaks No	Compound	Substance Class	Retention time (min)	Absorbance maxima UV spectrum (nm)
1	Stictic acid	Depsidone	3.47	212, 306
2	Fumarprotocetraric acid	Depsidone	5.42	212, 240, 318
3	Evernic acid	Depside	6.77	212, 270, 304
4	Usnic acid	Dibenzofuran	13.70	233, 282
5	Atranorin	Depside	14.87	210, 251, 320

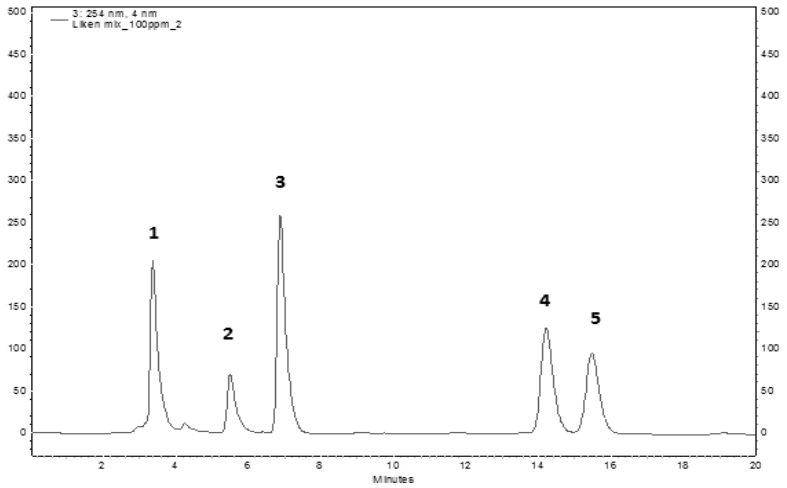

Figure 1: HPLC Chromatogram of standards used for identification. (1:Stictic acid, 2:Fumarprotocetraric acid, 3:Evernic acid, 4:Usnic acid, 5:Atranorin)

Table 7: Quantitative analysis of tested lichen species by HPLC. AE: Acetone, ME: Methanol

Lichens Species		Standard Compounds (mg/g dry extract)				
		Atranorin	Evernic acid	Fumarprotocetraric acid	Stictic acid	Usnic acid
A. ciliaris	AE	-	-	-	-	-
	ME	-	-	-	-	-
B. capillaris	AE	-	-	-	-	101.43 ± 0.15
	ME	-	-	-	-	84.94 ± 0.78
C. islandica	AE	-	-	233.18 ± 0.93	-	4.30 ± 0.01
	ME	-	-	151.64 ± 2.24	-	-

CONCLUSION

Taken together, it was determined that acetone extract of *C. islandica* exhibited strong antibacterial activities against Gram-positive bacteria and acetone extract of *B. capillaris* displayed broad spectrum of antibacterial activities. Antibacterial activity of *B. capillaris* against *S. epidermidis, S. macescens, S. pyogenes, S. typhimurium* and *E. cloaca*, and antibacterial activity of *C. islandica* against *S. epidermidis, S. pyogenes, P. aeruginosa* and *P. vulgaris* have been presented for the first time. Highest antioxidant activity (DPPH radicals scavenging activity, determination of total phenolic and flavonoid amount) was demonstrated by *B. capillaris*. This is the first report for the antioxidant activity of *B. capillaris*. Moreover, we detected that *B. capillaris* was a source of usnic acid and *C. islandica* was the significant source of fumarprotocetraric acid. *A. ciliaris* had the weakest biological activity and poorest content. As a result, the tested lichen species had a certain level of antioxidant and antibacterial activities. They represent a significant source of phenolic compounds. It is possible to use them for the treatment of infections caused by Gram-positive and Gram-negative bacteria and also can be a source of food additives as antioxidant due to preventing the liposomal oxidation and prevention the cancer in diet.

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