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An insight into the variation of the antioxidative and antibacterial activity of extracts from populations of the subalpine and montane lichen *Cetraria islandica*

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ABSTRACT:
Lichens are supra-organismal symbiotic systems found in most environments. Environmental factors, such as temperature, altitude, precipitation, UV irradiation, or pathogens, significantly influence the physiology of lichens, and thus their secondary metabolism. The thalli of the same lichen species from different environments exhibit variation in the production of secondary metabolites and protective pigments. We selected two populations of the lichen *Cetraria islandica* from habitats differing in altitude, temperature, and precipitation. Then we compared their antioxidative and antibacterial activity. The lichen thalli were divided into two parts: the upper parts were exposed to light and the lower parts hidden from extensive radiation. The results show that the thalli from harsh alpine environments have higher 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity suggesting better tolerance to oxidative stress. On the other hand, the individuals from milder montane environments generally produce more secondary metabolites, leading to increased antibacterial activity of the extracts. The extracts of *C. islandica* containing fumarprotocetraric and paraconic acids exhibit inhibitory effects against gram-positive bacteria (e.g. *Staphylococcus aureus*) and some lower activity against gram-negative bacteria (e.g. *Escherichia coli*).

Keywords:
agar diffusion method, DPPH assay, montane zone, phenols, secondary metabolites, subalpine zone

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INTRODUCTION

Lichens are a group of fungi living in symbiosis with algae and/or cyanobacteria. These photosynthetic partners (photobionts) produce carbonaceous substances for fungi (mycobiont). Therefore, lichens are small self-sustaining ecosystems formed by the interaction between a fungus and one or more photosynthetic partners and other microscopic organisms, such as bacteria or yeast (Hawksworth & Grube 2020). Lichens can be found in most environments in the world, and in alpine, subpolar, or polar zones they present a major life form. They are subjected to various complex biotic and abiotic interactions and influences to form the lichen thallus' phenotype (Spribille *et al.* 2016).

One of the best examples of the uniqueness of lichens is the production of their secondary metabolites seldom found in other organisms, which can be present in high

amounts within the thallus (Varol 2019). Most of the lichen secondary metabolites are synthesised by mycobionts (Hager *et al.* 2008) and their production in a single lichen thallus is also influenced by the temperature, precipitation and UV irradiation which can be linked to habitat altitude. Lichens growing in alpine habitats are influenced by numerous environmental factors, several of which seem to be rather significant for their eco-physiological properties (Shukla *et al.* 2015) such as UV irradiation (Begora & Fahselt 2001), water availability, or temperature (Podterob 2008).

Thalli growing in habitats exposed to high UV radiation usually contain a higher amount of protective pigments, such as phenolic compounds: depsides or depsidones (Chowdhury *et al.* 2017). Stunted growth was also observed. The thalli of the same species growing in milder montane conditions have less protective allomelanins (Rassabina *et al.* 2020) and more robust growth (Beckett *et al.* 2019). The concentration of secondary metabolites in lichen thalli is expected to differ according to geographical location, altitude and changing seasons. These factors influence each metabolite differently. For example, the level of atranorin in the lichen *Parmotrema hypotropum* (Nyl.) Hale was positively correlated with exposure to light. In contrast, the amount of norstictic acid in the same thallus decreased (Armaleo *et al.* 2008). The phenolic compounds in *Cladonia* lichens [*C. mitis* Sandst., *C. rangiferina* (L.) F. H. Wigg., and *C. uncialis* (L.) F. H. Wigg.] showed a pattern of increasing concentration under UV-A light, but decreasing under UV-B light (Begora & Fahselt 2001). The lowest level of usnic acid was recorded in the *Cladonia mitis* species during spring and summer (Begora & Fahselt 2001). This may be the result of a cumulative effect of higher light exposure, drought, and heat stress affecting the metabolic activity of the thallus (Bjerke *et al.* 2005).

When the ecologically optimal requirements of a lichen thallus are met, the lifespan of thalli can reach extreme old age spanning hundreds or even thousands of years (Grube & Hawksworth 2007). This longevity requires efficient protective mechanisms against oxidative damage and pathogen infection. The distribution of the secondary metabolites in the lichen thallus is not random and it is characteristic for each species (Le Pogam *et al.* 2016). The secondary metabolites stored extracellularly on mycobiont hyphae contribute to protection against invasion by pathogenic bacteria. Usnic acid, one of the most common secondary metabolites in the cortex, protects the lichen against gram-positive bacteria (Grube *et al.* 2009). The antioxidative and antibacterial activity of lichens depends on their phenolic compound content (Kosanić *et al.* 2011). However, several studies suggest antagonistic or synergistic interactions between phenolics and other organic compounds in lichen extracts, which explains the lower antioxidative activity for some isolated phenols (Lohézic-Le Dévéhat *et al.* 2007; Lopes *et al.* 2008). The antibacterial activity of lichen extracts was studied for the first time in the 1940s (Burkholder & Evans 1945; Vartia 1949), but did not receive a great deal of attention in research until recent years when they were proven effective against a wide range of bacterial species (Araújo *et al.* 2015; Basnet *et al.* 2018; Aoussar *et al.* 2020; Goga *et al.* 2021). However, many questions remain unanswered.

Cetraria islandica (L.) Ach has an upright branched fruticose thallus, which is 5–10 cm tall. The distribution of the metabolites in the thallus varies with predominantly protolichesterinic and roccellaric acids in the tips and fumarprotocetraric acid and quinone-like substances in the base (Boustie *et al.* 2011), which manifests as red colouring at the base. The species usually develops extensive carpets of brownish cushions, particularly over acidic soils in wind-swept habitats in montane zones, where the thalli are exposed to rapid climatic changes (Boustie *et al.* 2011), but it can also be found along a wide altitudinal gradient from lowlands to alpine zones (Wirth *et al.* 2013). This causes significantly higher exposure of the thalli tips to sunlight and other environmental factors.

With the altitude gradient, many environmental factors change. These factors also affect the production of secondary metabolites in lichens. Variance in sunlight, temperature or the duration of different factors all result in variation in lichen metabolism leading to differences among populations from distinct areas. The production of melanin by mycobionts reduces the transmittance of UV irradiance and affects the levels of photosynthetically active radiation reaching the photobiont cells (Daminova *et al.* 2022).

A widely distributed lichen species, *C. islandica*, has been used in traditional medicine for centuries (Crawford 2019). Even today, the extract of *C. islandica* is used to treat colds as it contains the highly effective polysaccharide lichenan. Other secondary metabolites present in *C. islandica* include paraconic acids (protocetraric acid, protolichesterinic acid and roccellaric acid), fumarprotocetraric acid, and quinone-like compounds (Stepanenko *et al.* 1997; Horhant *et al.* 2007; Anonymous 2014; Sánchez *et al.* 2022). Several studies (Kristinsson 1969; Xu *et al.* 2018) have reported two chemotypes within this species, fumarprotocetraric acid-producing and fumarprotocetraric acid-deficient. However, the distribution of these chemotypes shows no clear patterns and so far it remains unknown whether it is correlated with lichen morphology and/or habitat type. Other studies have reported the antioxidative and antibacterial activity of *C. islandica* extracts (Gülçin *et al.* 2002; Kosanić *et al.* 2011; Grujičić *et al.* 2014). Protolichesterinic and fumarprotocetraric acids showed inhibition against *Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus,* or *Escherichia coli* (Ingólfsdóttir 2000; Türk *et al.* 2003; Ranković & Mišić 2008).

In this research, we compared two ecotypes of *C. islandica*. We focused on potential biochemical differences, namely selected secondary metabolite contents, influenced by several factors linked with altitudinal gradient, such as temperature, precipitation, and UV irradiation. We hypothesised that the secondary metabolites extracted from lichens from higher altitudes would be more promising in the test of antibacterial and antioxidative activity. With the aim of determining the chemotypes, the presence of fumarprotocetraric acid (depsidone) in both populations of *C. islandica* was evaluated using HPLC analyses.

MATERIALS AND METHODS

Lichen material sampling. The thalli of *Cetraria islandica* were collected in September 2020 from the Seetaler Alpen Mts. in Austria and the Slovenské Rudohorie Mts. In Slovakia (Table 1). The Austrian location (subalpine zone) was characterised by an altitude of 2000 m at Schlosserkogel Mt., Seetaler Alpen, Austria (N 47.0828947°, E 14.5637200°, WGS84). The bedrock at this location is siliceous phyllite, and the vegetation at this altitude consists of *Vaccinium* spp. shrubs and *Cladonia arbuscula* (Wallr.) Rabenh., *Cladonia rangiferina, Cladonia uncialis* and *Alectoria ochroleuca* (Hoffm.) A. Massal. lichen communities. The site is open spaced, without any shade or phanerophytes, rocky, and with a slightly western exposition. The Slovak location (montane zone) was characterised by an altitude of 1246 m at Kojšovská hoľa Mt., the Slovenské Rudohorie Mts., Slovakia (N 48.7821819°, E 20.9879181°, WGS84). Geologically, the bedrock consists of siliceous phyllite. The vegetation here comprises *Vaccinium* spp. shrubs with *Cladonia coniocraea* (Flörke) Sprengel, *Cladonia arbuscula* subsp. *mitis* (Sandst.) Ruoss and *Flavocetraria cucullata* (Bellardi) Kärnefelt & A. Thell, with occasional *Picea abies* (L.) H. Karst. trees. The collection site is at the summit, open-spaced, without shade, and rocky. At both locations, *C. islandica* forms extensive carpets. Thalli with distinctly coloured bases were harvested from each of the 5 sublocations at each site. The air-dried thalli were stored in paper bags in a refrigerator at 4°C prior to analyses.

The thalli from the two locations were visually distinctly different (Fig. 1A). The montane zone thalli were larger, greener and the branches were overgrown and more difficult to separate than the smaller and browner subalpine zone samples. The bases of the montane zone lichen samples were more yellowish in contrast to the subalpine zone reddish bases. For further analysis, the thalli were divided into two parts – the tips and base. Since there is no clear line between the tips and the basis of a single thallus, we divided the thalli approximately in the middle. For all analyses, methanol extracts were prepared: (WT) extract from the whole thallus, (UP) only the upper part, and (LP) only the lower part of the thallus. The thallus of *C. islandica* has an uneven distribution of tissue density, where the middle part has the highest, ergo the highest weight, while the senescing base of the thallus has the lowest tissue density. In order to achieve the dry weight (DW) required for analyses, we needed to combine the pieces of several thalli in one sample. The voucher specimens are deposited in the Herbarium of the Botanical Garden of Pavol J. Šafárik University in Košice, Slovakia (acronym KO) as KO35863 and KO35864.

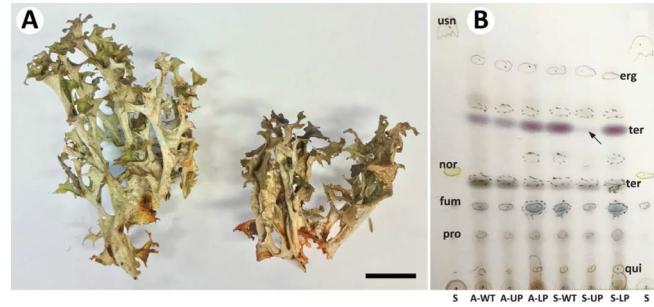

Fig. 1 A. The habitus of the *Cetraria islandica* thalli sampled in the montane zone (left) and those sampled in the subalpine zone (right); B. Part of the developed TLC plate in solvent B after spraying with 10% H_2SO_4 and heating. The arrow marks the missing substance in the montane lower part of the thallus extract. S – standard, A-WT – subalpine whole thallus, A-UP – subalpine upper part, A-LP – subalpine lower part sample (resp. S-WT, S-UP, S-LP for the montane samples), usn – usnic acid, erg – ergosterol, ter – unidentified terpenoids, nor – norstictic acid, fum – fumarprotocetraric acid, pro – protocetraric acid, qui – quinone-like compounds. Scale = 1 cm.

For a climatological comparison of the two locations, see Table 1 (Anonymous 2021a, b). The subalpine location has a slightly more oceanic-type climate. Both locations are considered pollution-free, away from heavy industry or dense population. In Central Europe, a high mountain landscape is where the altitude exceeds 1600–1700 m (Troll 1973).

Total extraction yield of metabolites. For an approximate comparison of the total content of metabolites in the thalli of each sample, we prepared acetone extracts. The lichen extracts were prepared by mixing 1 g DW of each sample in 10 mL of acetone for 24 h. The extracts were filtered using Whatman® qualitative filter paper, Grade 1, reduced to dry extract, and weighed.

Thin Layer Chromatography (TLC). The small pieces of thalli (20 mg DW) were extracted with acetone and another set with methanol for 24 hours at laboratory temperature. For the thin layer chromatography, 20 × 20 cm Silica gel 60 F254, 0.25 mm thick glass plates (Sigma Aldrich, Germany) were used. The solvents used were:

Table 1. An overview of the climate conditions at both locations (Anonymous 2021a, b). Avg. = average.

	Subalpine location	Montane location
Altitude	2000 m	1246 m
Avg. annual air temperature	0-2°C	2-4°C
Avg. July air temperature	3-10°C	12-14°C
Avg. January air temperature	-6 to -8°C	-5 to -6°C
Avg. annual global solar radiation	800-1000 kWh.m^{-2}	1050-1100 kWh.m^{-2}
Avg. annual precipitation	1800-2100 mm	900-1000 mm
Avg. precipitation in:	Summer: 500-600 mm	July: 100-120 mm
Avg. number of days with snow cover	180-210	100-120

A (toulene : dioxane : acetic acid, 180:45:5), B (hexane : diethyl ether : formic acid, 130:80:20), and C (toulene : acetic acid, 170:30) (Orange *et al.* 2001), which are ideal for the identification of lichen substances such as depsides or depsidones. The developed plates were visualized under UV irradiance (254 and 365 nm). The plates were sprayed with 10% H_2SO_4 and heated to 100–110°C. The retardation factor (R_f) values were recorded, and the substances were identified according to the Elix (2014) and LIAS metabolite online database (Elix *et al.* 2012). *Cladonia foliacea* (Huds.) Willd. f. *foliacea* (usnic acid, fumarprotocetraric acid, protocetraric acid) and *Cladonia symphycarpa* (Flörke) Fr. (norstictic acid, atranorin) were used as the standard.

Determination of fumarprotocetraric acid using HPLC. The cleaned lichen specimens (50–60 mg) were placed in Eppendorf (Safe-Lock, 2.0 mL) tubes and extracted in 1.5 mL of cool acetone for 60 min (Feige *et al.* 1993). Extraction was repeated at least three times. The acetone extracts were collected and evaporated, and the residues were dissolved with 1.5 mL of fresh acetone.

The filtered acetone extracts were analysed using high performance liquid chromatography (Dionex Ultimate 3000, Thermo Scientific) by gradient HPLC (Feige *et al.* 1993; Lumbsch 2002), under the following conditions: column Hypersil GOLD™ C18 (250 mm × 4 mm, particle size 5 µm; Thermo Fisher Scientific), flow rate 0.7 mL min^{-1}. For the mobile phase, A=H_2O and B=90% acetonitrile. The gradient program was 0 min, 25% B; 5 min, 50% B; 20 min, 100% B; 25 min, 25% B. Detection was performed at a wavelength of 245 nm (detector Dionex Ultimate 3000, DAD, Thermo Scientific). Three replicates were used for each time and variant of experiment. Fumarprotocetraric acid used as the standard was isolated from lichen *Cladonia rangiferina* as previously described by Kosanić *et al.* (2014). The lichens used for the standard were collected randomly in November 2022 in the village of Špania dolina, 810 m a. s. l. (Slovak Republic).

DPPH assay. For the estimation of the antioxidative properties the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay was used. The lichen extracts were prepared by mixing 50 mg DW of each sample in 1 mL of methanol for 2 hours. All the measurements were done in triplicate for each sublocation. The free radical scavenging activity was measured according to Dorman *et al.* (2003) and modified as in Goga *et al.* (2021). For the antioxidative analysis, 100 µL of the methanol lichen extract was diluted with 900 µL of methanol, and 2 ml of 0.1 mM DPPH in methanol was added. The samples were incubated at laboratory temperature in the dark for 30 min. Absorbance was registered at 517 nm on a Synergy HT microplate reader (Biotek, USA), using methanol as the blank control and ascorbic acid as the standard. The DPPH radical concentration was calculated in % according to Dorman *et al.* (2003).

Total phenol content. For estimating total phenols, the lichen extracts were prepared by mixing 50 mg DW of each sample in 1 mL of methanol for 2 hours. All the measurements were done in triplicate for each sublocation. The total phenol content in the methanol lichen extracts was measured as in Ghatak *et al.* (2014). 200 µL of methanol lichen extracts were mixed with 500 µL of Folin-Ciocalteau's reagent and 2 mL of aqueous 20% Na_2CO_3 solution. The mixture was incubated at laboratory temperature for 15 minutes. Absorbance was registered at 650 nm on a Synergy HT microplate reader (Biotek, USA). Methanol was used as the blank control and gallic acid as the standard for estimating the calibration curve. The total phenolic content was calculated as the equivalent of gallic acid (in µg) by applying the equation (1):

$$\text{Total phenols} = (\text{absorbance} - 0.1059)/0.0525 \quad (1)$$

which was obtained by the calibration standard of gallic acid (correlation value $R^2 = 0.9647$) as used in Goga *et al.* (2021).

Antibacterial activity. The thalli of *C. islandica* were dried at laboratory temperature, cleaned from debris, and homogenised in a mortar. Around 3.5 g DW of thalli for each sample was used for extraction with acetone

(100 mL) at room temperature for 24 h. The extracts were filtered using Whatman® qualitative filter paper, Grade 1, and reduced to dry extract.

For the antibacterial activity of the samples, the agar diffusion method with a slight modification was used (Rojas *et al.* 2006). The gram-negative bacteria (*Escherichia coli* CCM 3988) and gram-positive bacteria (*Staphylococcus aureus* CCM 4223) were acquired from the Czech collection of microorganisms (CCM).

The bacteria were cultured aerobically at 37°C in nutrient broth (Oxoid, United Kingdom) with agitation. The stock of frozen cultures was stored at -20°C. The cultures were then transferred to liquid media and incubated for 24 h and then sub-cultured in liquid media, again incubated for 24 h and used for the experiments. The agar was autoclaved and cooled down to 42°C. The bacterial cultures were inoculated to a cell density of 5×10^5 cfu mL^{-1} and left overnight. 20 mL of the inoculated agar was then pipetted into Petri dishes (90 mm in diameter). After solidification of the agar, wells of 5 mm in diameter were cut out and the holes were filled with 50 µL of the samples. All the samples were prepared by extraction. 10 mg of the extract sample was dissolved in 1 mL of 5% DMSO. A 10 mM concentration of gentamicin sulphate (Sigma Aldrich, USA) was used as the positive control and 5% DMSO as the negative control. The plates were incubated for 24 h at 37°C. After 24 h, the plates were photographed for the calculation of the inhibition zones by means of ImageJ software (National Institute of Mental Health, USA). The calculations were from 3 replicates for each sample. The antibacterial activity was calculated by applying the formula (3):

% RIZD = [(IZD sample – IZD negative control)/IZD gentamicin]*100 (3)

RIZD is the percentage of the relative inhibition zone diameter and IZD is the inhibition zone diameter in mm.

Statistical analysis. The statistical analyses were done using MINITAB 18 software (Pennsylvania State University, USA). Significant differences were determined by one-way ANOVA and Tukey's pairwise comparison of means. The values are given as average mean ± standard deviation. Pearson's correlation coefficient (r) was used to assess the relationships between different parameters.

RESULTS

Secondary metabolites. The total extraction yield of metabolites (Table 2) was significantly higher for the upper part extracts. The TLC analysis showed a similar composition of secondary metabolites in the extracts between locations but different amounts of metabolites within the thallus parts. Fumarprotocetraric acid, protocetraric acid, protolichesterinic acid, and ergosterol were identified (Fig. 1B). Several quinone-like compounds, triterpenoids, and sterols were separated on the plates, but these need further research to be identified. One of the triterpenoids was found in high levels in the lower part extract of the montane sample but only as a trace in the tips (indicated by the arrow in Fig. 1B). In the subalpine samples, the same substance showed a more or less even distribution throughout the thallus. Fumarprotocetraric acid was found mainly in the bases of the thalli from both locations. The montane upper part sample contained an unidentified red pigment.

Table 2. The total content of secondary metabolites in the lichen *Cetraria islandica* extracts in mg g^{-1} DW. DW = dry weight.

Cetraria islandica accessions		**Yield of secondary metabolites (mg.g^{-1} DW)**
Whole thallus	**Subalpine**	3.62
	Montane	7.95
Upper part	**Subalpine**	3.48
	Montane	7.44
Lower part	**Subalpine**	2.20
	Montane	2.44

The HPLC analyses confirmed the presence of fumarprotocetraric acid in both populations of *C. islandica* (Table 3). However, its content in the subalpine populations of the lichen was significantly higher when compared to the montane populations. The lower parts of the subalpine lichens demonstrated a significantly higher content of fumarprotocetraric acid than the upper parts. In the montane zone samples the content of fumarprotocetraric acid in the upper parts was low and even undetectable using the conventional HPLC protocol for the determination of secondary metabolites in lichens.

Antioxidative activity. The antioxidative activity of lichens is strongly dependent on the content of their phenolic compounds. The total phenol content differed significantly between locations in the extracts from the whole thallus (subalpine 0.024 mg.g^{-1} DW, resp., montane 0.013 mg.g^{-1} DW). DPPH radical scavenging showed higher scavenging capacity in the subalpine samples than the montane samples (10.2%, resp., 1.9%). This correlates with the amount of total phenolics.

The data indicate that the antioxidant activity of the metabolites extracted from *Cetraria islandica* is based mainly on the activity of phenolics and the most potent antioxidants are stored in the base of the thallus (Table 4). The subalpine lichen samples possess more phenolic compounds thus resulting in higher antioxidative activity.

Antibacterial activity. The antibacterial activity of the extracts against the tested bacteria is shown in Table 5. In all cases the effects of the extracts obtained from the montane samples were higher than from the subalpine

Table 3. The content of fumarprotocetraric acid in the tested accession of *Cetraria islandica* (mean ± SD). %FPCA = fumarprotocetraric acid % DW thalli (w/w), DW = dry weight, n/d = undetectable. The values in the same row followed by the same letter do not differ significantly at $P < 0.05$ by Tukey's pairwise comparison. N = 3.

Cetraria islandica	Whole thallus		Upper part		Lower part	
	Subalpine	Montane	Subalpine	Montane	Subalpine	Montane
%FPCA (DW)	0.10 ± 0.03A	0.02 ± 0.01C	0.07 ± 0.02AB	n/d	0.12 ± 0.04A	0.03 ± 0.01BC

Table 4. The antioxidant activity of all the *Cetraria islandica* thallus extracts (mean ± SD, N = 5) represented in total phenol content and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity. N = 5. DW = dry weight.

Cetraria islandica accessions	Whole thallus	
	Subalpine	Montane
Phenols (mg g^{-1} DW)	0.024 ± 0.001A	0.013 ± 0.001B
DPPH scavenging (%)	10.2 ± 2.26A	1.9 ± 0.59B

samples. The highest antibacterial activity was present in the montane whole thallus sample (77.32 ± 11.51%) against *S. aureus* CCM 4223. Overall, we found higher antibacterial activity against gram-positive bacteria *S. aureus* CCM 4223 than gram-negative *E. coli* CCM 3988 (Table 5), while the subalpine samples exhibited almost no antibacterial activity. None of the tested extracts exhibited activity higher than the standard antibiotic gentamicine, which was used as the positive control.

DISCUSSION

In the present study, we demonstrated the quantitative and qualitative variance of secondary metabolites within two accessions of lichen *C. islandica* from two environmentally different locations. Due to different temperatures, wind conditions, and humidity, the extracts of the same species differ slightly in terms of the composition of the same metabolites. Presuming that sunlight exposure differs with changing altitude, this is also a factor influencing the secondary metabolite composition. It has already been confirmed that the presence of minor secondary metabolites in lichens may vary (Stocker-Wörgötter *et al.* 2004). These environmental conditions directly influence polyketide synthase transcription in lichens, which is the major family of enzymes involved in the synthesis of lichen secondary metabolites via the malonate pathway (Deduke *et al.* 2012). The majority of secondary metabolites in *C. islandica,* e.g. fumarprotocetraric, protocetraric, protolichesterinic, and roccellaric acids, are phenolic compounds which are synthesised via the malonate pathway (Goga *et al.* 2018). Each lichen species holds a unique mixture of secondary metabolites. Lichen *Hypogymnia physodes* (L.) Nyl. contains atranorin, chloratranorin, physodalic acid, physodic acid, 3-hydroxyphysodic acid, 2′-O-methylphysodic acid, and protocetraric acid (Studzińska-Sroka & Zarabska-Bożjewicz 2019), while *Cladonia foliacea* contains usnic acid and fumarprotocetraric acid (Farkas *et al.* 2020). Therefore, the antioxidative and antibacterial activity of the extracts is the result of the interaction of these metabolites, but the amount and production seem also to be affected by the environment.

Several secondary metabolites identified by TLC were shown to be present in higher amounts in the bases of the thalli even if the total yield of the extract was higher from the tips. This is not surprising, since the secondary metabolites prevalent in the tips of the thalli, namely protolichesterinic and roccellaric acids, are hard to detect and evaluate by the TLC technique (Horhant *et al.* 2007; Elix 2014).

The crude extracts showed high differences in the total yield of metabolites both within the different locations and thallus parts. The basal parts of the thalli in both locations showed a significantly lower content of extractible secondary metabolites suggesting the metabolites' accumulation in the metabolically more active parts. These results contradict the radical scavenging activity data where the lower part extracts of the montane samples exhibited the highest scavenging activity. This could be explained by the higher content of carotenoids, anthocyanins, and terpenoids, known to be effective as free radical scavengers (Orak 2007; Young & Lowe 2018; Wang *et al.* 2019).

The accumulation of protective secondary metabolites in the thallus is considered an adaptive response to harsh environmental conditions (Solhaug *et al.* 2010). However, a 40% decrease in the content of phenolics in *C. islandica* exposed to UV-B irradiation was observed (Boustie *et al.* 2011), which could explain the lower yield in the tested subalpine samples.

Extreme conditions in high mountain or alpine environments lead to the production of reactive oxygen species. Protective mechanisms are necessary for lichens, which grow in such environments. The whole thallus extracts indicate a clear gap between the locations, where the alpine samples were more efficient in free radical scavenging along a higher phenolic content.

The total phenolic content was measured, as this is a group of metabolites known as potential antioxidants (Férnandez-Moriano *et al.* 2016). Phenolic compounds are able to donate hydrogen to peroxy radicals

Table 5. The percentage of the relative inhibition zone diameter (%RIZD) values of the *Cetraria islandica* extracts tested against *Escherichia coli* CCM 3988 and *Staphylococcus aureus* CCM 4223. Gentamicine was used as the standard with %RIZD = 100, representing group A. The values in the same row followed by the same letter do not differ significantly at $P < 0.05$ by Tukey's pairwise comparison. N = 5.

	E. coli		*S. aureus*	
***Cetraria islandica* accessions**	**Subalpine**	**Montane**	**Subalpine**	**Montane**
Whole thallus	5.92 ± 4.82CD	22.65 ± 9.87B	15.15 ± 0.29BC	77.32 ± 11.51AB
Upper part	4.33 ± 3.79D	19.95 ± 4.46BC	34.38 ± 15.84C	59.3 ± 30.5ABC
Lower part	5.72 ± 4.67CD	27.82 ± 4.73B	15.82 ± 1.21C	41.4 ± 31.2BC

converting them to hydroperoxides and can accept uncoupled electrons from free radicals (Goga *et al.* 2018). Indeed, the fumarprotocetraric, protocetraric and protolichesterinic acids identified in the samples of *C. islandica* belong to phenolic compounds. The antioxidative activity correlated with the total phenolic content in all the tested extracts (r = 0.8876).

The antibacterial activity of the lichen extracts correlated with the content of secondary metabolites in the extracts in the case of *S. aureus* (r = 0.8593) but not for *E. coli* (r = 0.4227). We found that the *C. islandica* extracts showed antibacterial activity against gram-positive bacteria *S. aureus* but almost no activity against gram-negative bacteria *E. coli*. The antibacterial activity of fumarprotocetraric and protolichesterinic acid has already been reported (Yilmaz *et al.* 2004; Ranković & Mišić 2008; Celenza *et al.* 2013). Several authors have examined *C. islandica* extracts for their antibacterial activity and reported no antibacterial activity against gram-negative bacteria, such as *E. coli* (Dülger & Gücin 1998; Grujičić *et al.* 2014). In our experiment, antibacterial activity of 27% for the montane sample and 5% for the subalpine sample were achieved. The whole thallus extract from the montane sample showed the highest inhibitory activity of 77%. Fumarprotocetraric acid is present mainly in the base of the thallus and protolichesterinic acid in the tips of the thallus. Several studies (Türk *et al.* 2003; Bellio *et al.* 2017) have proved protolichesterinic acid activity against gram-negative *E. coli, B. subtilis,* or *P. aeruginosa*.

CONCLUSIONS

The lichen thalli collected from the subalpine zone showed higher antioxidative activity than the montane zone thalli due to the higher phenolic compound content. On the other hand, antibacterial activity was strongly correlated with the total amount of secondary metabolites in the thallus, and was higher for the thalli from the montane zone. In conclusion, while *C. islandica* from the subalpine environment seems to be more adapted to higher oxidative damage due to its secondary metabolites production, it lacks significant antibacterial activity.

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REZIME

Botanica SERBICA

Uvid u variranje antioksidativne i antibakterijske aktivnosti ekstrakata iz populacija subalpskih i planinskih lišajeva *Cetraria islandica*

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Lišajevi su supra-organski simbiotski sistemi koji su prisutni u većini okruženja. Faktori sredine, kao što su temperatura, nadmorska visina, padavine, UV zračenje ili patogeni, značajno utiču na fiziologiju lišajeva, a time i njihov sekundarni metabolizam. Talusi iste vrste lišajeva koji dolaze iz različitih sredina pokazuju varijacije u proizvodnji sekundarnih metabolita i zaštitnih pigmenata. Za istraživanja su izabrane dve populacije lišaja *Cetraria islandica* sa staništa koji se razlikuju po nadmorskoj visini, temperaturi i količini padavina. Nakon toga su upoređivani njihova antioksidativna i antibakterijska aktivnost. Lišajski talusi su podeljeni na dva dela: gornji deo izložen svetlosti, i donji deo sakriven od intenzivnog zračenja. Rezultati pokazuju da talusi iz surovog alpijskog okruženja pokazuju jaču 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging aktivnost, sugerišući bolju podršku oksidativnom stresu. Sa druge strane, jedinke iz umereno planinske oblasti produkuju generalno više sekundarnih metabolite, koji izazivaju veću antibakterijsku aktivnost ekstrakata. Ekstrakti *C. islandica* koji sadrže fumarprotocetransku i parakonsku kiselinu pokazuju inhibitorni efekat protiv gram-pozitivnih bakterija (npr. *Staphilococcus aureus*) i nešto nižu aktivnost protiv gram-negativnih bakterija (npr. *Escherichia coli*).

Ključne reči: metoda difuzije agara, DPPH esej, planinska zona, fenoli, sekundarni metaboliti, subalpijska zona