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PLATISMATIA GLAUCA AND PSEUDEVERNIA FURFURACEA LICHENS AS SOURCES OF ANTIOXIDANT, ANTIMICROBIAL AND ANTIBIOFILM AGENTS

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ABSTRACT

The antioxidative, antimicrobial and antibiofilm potentials of acetone, ethyl acetate and methanol extracts of lichen species *Platismatia glauca* and *Pseudevernia furfuracea* were evaluated. The phytochemical analysis by GC, GC/MS and NMR revealed caperatic acid, atraric acid, atranorin and chloroatranorin as the predominant compounds in *Platismatia glauca*. Atraric acid, olivetoric acid, atranorin and chloroatranorin were the major constituents in *Pseudevernia furfuracea*. The strong antioxidant capacities of the *Platismatia glauca* and *Pseudevernia furfuracea* extracts were assessed by their total phenolic and flavonoid contents and DPPH scavenging activities. The methanol extracts of both species exhibited the strongest antioxidant activities with the highest IC_{50} value for *Pseudevernia furfuracea* (95.33 µg/mL). The lichen extracts demonstrated important antibacterial activities against 11 bacterial strains with detectable MIC values from 0.08 mg/mL to 2.5 mg/mL for *Platismatia glauca* and from 0.005 mg/mL to 2.5 mg/mL for *Pseudevernia furfuracea*. While the antibacterial activities of *Pseudevernia furfuracea* were solvent–independent, the acetone and ethyl acetate extracts of *Platismatia glauca* showed higher antibacterial activities compared to its methanol extract. The methanol extracts of both species demonstrated significant antifungal activities against 9 fungal strains with detectable MIC values from 0.04 mg/mL to 2.5 mg/mL. The best antifungal activities were determined against *Candida* species in *Pseudevernia furfuracea* extracts with remarkable MIC values which were lower than the MIC values of the positive contol fluconazole. The acetone and ethyl acetate extracts of *Platismatia glauca* showed better antibiofilm activities on *Staphylococcus aureus* and *Proteus mirabilis* with BIC value at 0.63 mg/mL then its methanol extract. On the other hand, the methanol extract of *Pseudevernia furfuracea* was more potent with BIC value at 1.25 mg/mL on *Staphylococcus aureus* and 0.63 mg/mL on *Proteus mirabilis* compared to other types of extracts. Our study indicates a possible use of lichens *Platismatia glauca and Pseudevernia furfuracea* as natural antioxidants and preservatives in food, pharmaceutical and cosmetic industry.

INTRODUCTION

Lichens are a large group of lower plants with unique features. They represent symbiotic organisms consisting of the mycobiont (a fungus) and the photobiont (an alga or cyanobacterium). Approximately 25,000 lichen species inhabit all terrestrial ecosystems ranging from arctic to tropical regions and from plains to the highest mountains (Muggia et al., 2009). A list of 599 lichen species has been reported in Serbian flora (Bilovitz and Mayrhofer, 2008). Harsh living conditions, slow growth and long lifetime (up to several thousand years) have resulted in protective metabolites against different physical and biological influences. Lichens produce unique, small but complex organic compounds known as secondary metabolites or lichen substances. Structures for 1059 different lichen substances have been elucidated (Molnar and Farkas, 2010). Most of them have been derived from the acetyl-polymalonyl pathway (secondary aliphatic acids, esters and related derivates and polyketide derived aromatic compounds), while others have originated from the mevalonic acid pathway (terpenes and steroids) and the shikimic acid pathway (terphenyl-quinones and pulvinic acid derivates). A structurally diverse group of polyketide secondary metabolites comprises monocyclic phenols (orsellinic acid and β-orsellinic acid), bicyclic or tricyclic phenols joined by ester bond (depsides, tridepsides and benzyl esters), both ester and ether bonds (depsidones, depsones and diphenyl ethers) or a furan heterocycle (dibenzofurans, usnic acids and derivates), antraquinones, chromones, naphtaquinones and xanthones.

Various aliphatic, cycloaliphatic, aromatic and/or terpenic lichen compounds have demonstrated antiviral, antibacterial, antifungal, antioxidant, antitumor, antimutagenic, antiprotozoal, antiherbivore, antiulcerogenic, antinociceptive, antipyretic, anti-inflammatory, anti-prion, enzyme inhibiting, etc. effects. Since ancient times these effects have been recognized and exploited in traditional remedies for treatment of external wounds, burns, gastritis, cold, asthma, tuberculosis, etc. in humans and animals. For instance, the medicinal use of the lichen *Pseudevernia furfuracea* was well documented in an old tonic for intestinal weakness, a foreign drug imported to Egypt from Europe, a popular 15th century drug "Lichen quercinus virdes" (a cocktail of *Evernia prunastri, Pseudevernia furfuracea* and *Hypogimnia physodes*) in Europe, a decoction for respiratory complaints in Andalucia (Spain), an antiasthmatic, anticatarrhal and hypotensive drug in the region of Pallars (Spain) and a healing cream with clay for wounds, eczema and hemorrhoids in Kutahya Province (Turkey) (Bilgin et al., 2012; Guvenc et al., 2012). Besides, many cultures have utilized lichens as preservatives, embalming agents, perfumes, cosmetics, dyes, in food and alcohol production (Guvenc et al., 2012). Archeological remains dated back to ancient Egypt revealed the exploitation of the preservative and/or aromatic properties of the lichen *Pseudevernia furfuracea* for embalming and its nourishing potential for bread making. Throughout history vast amounts of *Pseudevernia furfuracea* have been processed and are still being processed for fragrances and fixatives in perfumes, soaps, cosmetics (Joulain and Tabacchi, 2009). Traditionally, *Platismatia glauca* is well-known as a source of a chamois-colored dye for wool in Europe.

Lichens as valuable sources of natural antioxidants and antimicrobial agents have been the focus of many studies (Gulluce et al., 2006; Guvenc et al., 2012; Turk et al., 2006). Also, the property of lichen extracts and their isolated constituents to inhibit bio-

film formation has been reported (Nomura et al., 2013). Strong antioxidant, antimicrobial and antiproliferative activities have been demonstrated for some lichen species from Serbia (Ranković et al., 2012; Kosanić et al, 2013; Kosanić and Ranković 2011; Mitrović et al., 2011; Stojanović et al., 2010).

The aim of this study is the evaluation of antioxidant, antimicrobial and antibiofilm capacities of the two Parmeliaceae species - *Platismatia glauca* and *Pseudevernia furfuracea*. This is the first report of detailed chemical composition, antioxidant capacity and antibiofilm activity of Platismatia glauca.

MATERIALS AND METHODS

Chemicals

The organic solvents and the sodium hydrogen carbonate were purchased from Zorka Pharma, Šabac, Serbia. The gallic acid, the rutin hydrate, the chlorogenic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. The Folin-Ciocalteu phenol reagent, 3-tert-butyl-4-hydroxy-anisole (BHA) and the aluminium chloride hexahydrate (AlCl$_3$·6H$_2$O) were purchased from Fluka Chemie AG, Buchs, Switzerland. The nutrient liquid mediums for microorganisms, the Mueller–Hinton broth and the Sabouraud dextrose broth, were obtained from Torlak, Belgrade, Serbia. The antibiotic doxycycline was purchased from Galenika A.D., Belgrade, Serbia and the antimycotic fluconazole from Pfizer Inc., USA. All other solvents and chemicals were of analytical grade.

Lichen samples

The lichen samples of *Platismatia glauca* (L) W.L. Culb and C.F. Culb and *Pseudevernia furfuracea* (L) Zopf. were collected from the mountain Tara (900 m altitude) in western Serbia during summer 2012. The voucher specimens of the lichens were determined and deposited in the licheno-logical herbarium of the Department of Biol-ogy and Ecology, Faculty of Sciences and Mathematics, University of Niš, Serbia.

Preparation of lichen extracts

The air-dried lichen thalli (10 g) were powdered and transferred to dark-coloured flasks. The extractions were performed with 250 ml of solvent (methanol, acetone and ethyl acetate) at room temperature for a peri-od of 24 h. The infusions were filtered using Whatman No.1 filter paper and the residue was re-extracted with an equal volume of solvents. After 48 h, the process was repeated. The combined supernatants were evaporated to dryness under vacuum at 40 °C using a rotary evaporator. The obtain-ed extracts were kept in sterile sample tubes and stored at 4 °C.

Methylation of extracts

An excess of diazomethane in diethyl ether was added to the solutions of the ex-tracts (10 mg) in 1 ml of diethyl ether, the mixture was left for 24 h in the refrigerator and then analyzed (GC and GC/MS).

Gas chromatography and gas chromatography-mass spectrometry (GC and GC/MS) analyses

The chemical composition of the ace-tone, methanol and ethyl acetate extracts was investigated by GC and GC/MS (three repe-titions), which were carried out using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5 % phenylmethylsiloxane, 30 m x 0.25 mm; film thickness, 0.25 mm; Agilent Technologies, USA) and coupled with a 5975B mass-selective detector from the same company. The injector and interface were operated at 250 and 300 °C, respective-ly. Oven temperature was raised from 70 to 315 °C at a heating rate of 5 °C/min, and the program ended with an isothermal period of 10 min. Helium was used as a carrier gas (1.0 ml/min). The samples, 1 μl of the ex-tract solutions (10 mg/ml), were injected in a pulsed-split mode (the flow was 1.5 ml/min for the first 0.5 min and then set to

1.0 ml/min throughout the remainder of the analysis; split ratio, 40:1). MS conditions were as follows: ionization voltage, 70 eV; acquisition mass range, 35–650; scan time, 0.32 s; *m/z* (rel. int. [%]). The percentage composition of the extracts was computed from the GC peak areas without any corrections. The constituents were identified based on their linear retention indices matching with literature values (relative to C_{14}–C_{34} alkanes on the DB-5MS column) and through the comparison of the mass spectra with those from a number of commercial and in-house MS libraries (NIST11, Wiley06, MassFinder and Adams) by the application of AMDIS software (automated Mass Spectral Deconvolution and Identification System, Ver. 2.1, DTRA/NIST, 2002).

Nuclear magnetic resonance (NMR) analyses

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II spectrometer (Bruker, Germany) operating at 400 and 100.6 MHz, respectively. All NMR spectra were measured at 25 °C in CD_3SOCD_3 with tetramethylsilane as internal standard.

Total phenolic content, total flavonoid content and antioxidant activity

Determination of total phenolic content

The total phenolic content of the lichen extracts was determined spectrophotometrically by the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 0.5 ml of methanol extract solution (1 mg/ml) and 2.5 ml of 1:10 Folin-Ciocalteu reagent were mixed and then 2 ml of sodium carbonate (75 g/l) were added. After 15 min of incubation at 45 °C, the absorbance at 765 nm was measured. The total phenolic concentration was calculated from gallic acid (GA) calibration curve. The data were expressed as the gallic acid equivalents (GAE)/g of the extract averaged from 3 measurements.

Determination of total flavonoid content

The total flavonoid content was evaluated using aluminum chloride (Quettier et al., 2000). The sample for determination was prepared by mixing 1 ml of methanol extract solution (1 mg/ml) and 1 ml of aluminum chloride (20 g/l). After 1 h of incubation at room temperature, the absorbance at 415 nm was measured. The total flavonoid concentration in the lichen extract was calculated from rutin (Ru) calibration curve and expressed as the rutin equivalents (RuE)/g of the dry extract. The measurements were done in triplicates.

Determination of free radical scavenging activity

The antioxidant activity of the lichen extract was evaluated according to the scavenging activity of stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical (Kumarasamy et al., 2007; Tekao et al., 1994). Serial dilutions of the extract were made from 1000 µg/ml to 0.97 µg/ml. 1 ml of each dilution was mixed with 80 µg/ml DPPH. After 30 min of incubation in darkness at room temperature, the absorbance was measured at 517 nm. The control sample contained all the reagents except the extract. The percentage of inhibition was calculated using the following equation:

$$\% \ inhibition = \left(\frac{A \ control - A \ sample}{A \ control} \right) x \ 100$$

[1]

where A control was the absorbance of the control sample and A sample is the absorbance of the extract.

IC_{50} values (concentration of the extract in the reaction mixture which decreases the initial DPPH concentration to 50 %) were estimated from % inhibition versus the concentration sigmoidal curve using the non-linear regression analysis. The data were presented as mean values ± standard deviation (n = 3). Chlorogenic acid was used as a standard (IC_{50} value 11.65 ± 0.52).

In vitro antimicrobial assays

Test microorganisms

The antimicrobial activity of the lichen extracts was tested against 20 microorganisms including 11 strains of bacteria (6 strains of Gram-positive bacteria: *Bacillus pumilus* NCTC 8241, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Sarcina lutea*, *Enterococcus faecalis*; 5 strains of Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhimurium*); 5 species of filamentous fungi (*Aspergillus flavus*, *Botrytis cinerea*, *Penicillium italicum*, *Penicillium digitatum*, *Penicillium verrucosum*) and 4 yeast species (*Candida albicans* ATCC 10231, *Candida albicans*, *Rhodotorula sp.* and *Saccharomyces boulardii*). All clinical isolates of the bacteria were a generous gift from the Institute of Public Health, Kragujevac, while the fungi and the ATCC strains were provided from a collection held by the Laboratory of Microbiology, Faculty of Science, University of Kragujevac.

The antibiofilm activity was tested against *Staphylococcus aureus* ATCC 25923 and *Proteus mirabilis* ATCC 12453 held by the Laboratory of Microbiology, Faculty of Science, University of Kragujevac.

Suspension preparation

The bacterial suspensions and the yeast suspension were prepared by the direct colony method (Andrews, 2005). The colonies were taken directly from the plate and were suspended in 5 ml of sterile 0.85 % saline. The turbidity of the initial suspension was adjusted using 0.5 McFarland densitometer (BioSan, Latvia). 1:100 dilutions of the initial suspension were additionally prepared in the sterile 0.85 % saline.

The suspensions of the fungal spores were prepared by a gentle stripping of the spore from the agar slants with growing fungi. The resulting suspensions were 1:1000 diluted in the sterile 0.85 % saline.

Microdilution method

The antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC) using the microdilution method with resazurin (Sarker et al., 2007). The twofold serial dilutions of the lichen extracts were prepared with the Mueller-Hinton broth (Torlak, Serbia) for the bacteria and the Sabouraud dextrose broth (Torlak, Serbia) for the fungi at a volume of 0.1 ml per well in sterile 96-well flat-bottom microtitre plates. The final concentrations ranged from 0.02 mg/ml to 2.5 mg/ml. The microtitre plates were inoculated with the suspensions to give a final concentration of 5 x 10^5 CFU/ ml for the bacteria and 5 x 10^3 CFU/ml for the fungi. The inoculated microtitre plates were incubated at 37 °C for 24 h for the bacteria, at 28 °C for 48 h for the yeasts and at 28 °C for 72 h for the filamentous fungi. 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 inoculated plates were incubated at 37 °C for 24 h for the bacteria, at 28 °C for 48 h for the yeast and at 28 °C for 72 h for filamentous fungi. The MIC was defined as the lowest concentration of the tested substance that prevented resazurin color change from blue to pink. For the filamentous fungi, the MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelia growth. To determine the MMC, 10 μl of the samples from the wells above the MIC was inoculated on a nutrient agar medium. At the end of the incubation period the lowest concentration with no growth was defined as the minimum microbicidal concentration (MMC). Doxycycline (Galenika A.D., Belgrade) and fluconazole (Pfizer Inc., USA), dissolved in a nutrient liquid medium, were used as positive controls. Stock solutions of crude extracts were obtained by dissolving in DMSO then diluted into a nutrient liquid medium to achieve a concentra-

tion of 10 % DMSO. It was observed that 10 % DMSO did not inhibit the growth of microorganism. Each test included growth control and sterility control. All tests were performed in duplicate and the MICs were constant.

Biofilm susceptibility assay

The biofilm inhibitory concentration (BIC) and the minimum inhibitory concentration (MIC) were determined using a modified procedure with a microtiter plate with a peg lid (Nalgene Nunc International, Rochester, N.Y.) described by Moskowitz et al. (2004). The solutions of the extracts and the concentration range were made as described above. The BIC values were defined as the lowest concentration without growth. The MICs were obtained using the MBEC assay system (Ceri et al., 2001), parallel with the biofilm testing.

Statistical analysis

All data were analyzed using the one-way analysis of variance (ANOVA) and the Student's *t*-test. In all cases *p* values <0.05 were considered statistically significant. All statistical analyses were performed using the SPSS package (SPSS for Windows, ver. 17, 2008) (Chicago, IL, USA).

RESULTS AND DISCUSSION

Chemical profile of lichen extracts

A preliminary GC-MS run of the *Platismatia glauca* extracts resulted in complex chromatograms containing a number of interconnected and/or tailing peaks with mass spectra suggesting a presence of highly related aliphatic compounds. We assumed that these might form during the GC-MS analyses (i.e. that they might be artifacts of the analytical procedure being applied) and decided to try to suppress this by conversion of the originally present lichen metabolites into their methyl esters/ethers (Culberson, 1972). The methylation, accomplished by a treat-

ment with diazomethane, was eventually rewarded by a significant simplification of the GC profile. Almost all non-Gaussian peaks had disappeared and were replaced by a single highly abundant peak corresponding to dimethyl caperate, an ester of a lichen metabolite (caperatic acid) known to occur in the genus *Platismatia* and this particular species (Nash et al., 2001). In order to further confirm the presence of caperatic acid in the extracts, an NMR analysis of the crude acetone extract was performed. In addition to the signals belonging to the caperatic acid (Huneck and Yoshimura, 1996), the ^{1}H NMR spectrum of this extract appeared to contain a second set of predominant signals. After a literature search and a thorough comparison (Huneck and Yoshimura, 1996), these signals were in fact assigned to two closely related lichen compounds atranorin and chloroatranorin. The two depsidones were known to be constituents of this particular lichen species (Nash et al., 2001). According to a subsequent quantitative NMR (qNMR) experiment, the content of caperatic acid, atranorin and chloroatranorin was estimated to be 91.7 %, 3.7 % and 4.6 %, respectively, of the original crude solvent extracts. Taking into account the yield of the solvent extracts and these quantitative results one can conclude that *Platismatia glauca* is a prolific source of caperatic acid.

In order to identify additional minor constituents present in the extracts of *Platismatia glauca*, we continued with the GC-MS analyses of the methylated extracts of this lichen species. On the other hand, the native extracts of *Pseudevernia furfuracea*, showing no noticeable degradation during the GC analyses, were further analyzed non-derivatized in order to avoid an ambiguous situation concerning the origin of a number of methyl esters detected in the extracts of this taxon. A total of 22 constituents were identified in this way in both taxa and these are presented in Table 1.

Table 1: Chemical composition of *Platismatia glauca* and *Pseudevernia furfuracea* extracts

RI[a]	Constituents	*Platismatia glauca*			*Pseudevernia furfuracea*		
		A[b]	E[b]	M[b]	A[b]	E[b]	M[b]
1419	2,5-Dimethyl-1,3-Benzenediol (syn. β-orcinol)	-[c]	-	-	-	tr[d]	-
1494	3-Chloro-2,6-dihydroxy-4-methyl-benzaldehyde (syn. chloroatranol)	1.8	2.7	-	9.1	14.8	tr
1549	2,6-Dihydroxy-4-methylbenzaldehyde (syn. atranol)	2.5	2.6	-	12.7	8.7	tr
1659	Methyl 2,4-dihydroxy-6-methyl-benzoate (syn. methyl orsellinate)	tr	tr	0.3	0.4	0.6	0.4
1666	Methyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (syn. methyl haematommate)	-	-	1.5	0.2	0.1	9.5
1706	Methyl 2,4-dihydroxy-3,6-dimethylbenzoate (syn. methyl β-orcinolcarboxylate; atraric acid)	14.3	12.1	15.9	47.7	31.5	46.6
1746	Ethyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (syn. ethyl haematommate)	-	0.1	-	tr	1.0	-
1752	5-Pentyl-1,3-benzenediol (syn. olivetol)	1.1	0.4	0.2	12.9	17.2	21.7
1765	Methyl 2,4-dihydroxy-3,5,6-trimethylbenzoate	-	-	-	0.2	0.2	tr
1784	Isopropyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (syn. isopropyl haematommate)	-	-	-	-	0.1	-
1824	5,7-Dihydroxy-6-methylphtalide	0.4	0.7	0.3	-	-	-
1882	Methyl 5-chloro-3-formyl-2,4-dihydroxy-6-methylbenzoate (syn. methyl chlorohaematommate)	tr	-	0.1	-	-	2.0
1921	Methyl hexadecanoate (syn. methyl palmitate)*	tr	tr	0.1	-	-	tr
1957	Ethyl 5-chloro-3-formyl-2,4-dihydroxy-6-methylbenzoate (syn. ethyl chlorohaematommate)	-	tr	-	-	0.3	-
2076	Methyl (Z,Z)-9,12-octadecadienoic acid (syn. methyl linoleate)*	-	-	-	-	-	0.2
2094	Methyl (Z)-9-octadecenoate (syn. methyl oleate)*	-	-	tr	-	-	-
2118	5-(2-Oxoheptyl)-resorcinol	0.2	tr	-	1.6	2.1	2.3
2338	Olivetonide	0.3	-	-	14.6	22.8	16.9
2400	Tetracosane	-	tr	-	-	-	-
1500	Pentacosane	-	tr	-	-	-	-
2724	Trimethyl 2-hydroxyheptadecane-1,2,3-tricarboxylate (syn. dimethyl caperate)*	78.1	80.1	80.0	-	-	-
3378	30-Nor-21α-hopan-22-one (syn. isoadiantone)	0.6	0.4	0.7	-	-	-
	Total amount (%)	99.3	99.1	99.1	99.4	99.4	99.6

[a]Experimental retention indices (DB-5MS column); [b]**A** = acetone, **E** = ethyl acetate extracts, **M** = methanol; [c]- =not detected; [d]tr = trace amount (<0.05%); *Formed after methylation of the extracts

The GC and GC-MS analyses showed that two compounds dominated in the derivatized (methylated) extracts of *Platismatia glauca*: atraric acid (12.1-15.9 %, present in the essentially unaltered amount in the total ion current chromatogram (TIC) of the non-

derivatized samples) and trimethyl 2-hydroxyheptadecane-1,2,3-tricarboxylate (dimethyl caperate; 75.1-79.6 %). These GC percentages give an underestimate of the true amount of the caperatic acid present in the extracts. This is understandable having in mind that the MS detector is not equally sensitive to aliphatic (caperatic acid) and aromatic compounds (atraric acid). A previous study (Hveding-Bergseth et al., 1983) concerning the composition of an extract (hexane) of *Platismatia glauca* has reported depsides, phenols and alkanes as the dominant constituents. The authors have also identified a nor-triterpene ketone (30-nor-21α-hopan-22-one) that has also been detected in our extracts of *Platismatia glauca* but in a relatively low amount (0.4-0.7 %). There are two hypotheses about sterols and triterpenes as lichens metabolites. First, it is believed that the triterpenes detected in lichen extracts are lichen metabolites as it is known that lichens can biosynthesize triterpenes and steroids, even when growing in the absence of a host tree, e.g. on rocks (Joulain and Tabacchi, 2009). Other hypothesis has classified those constituents as secondary metabolites of the host tree that have migrated to the lichen (Stojanović et al., 2011). However, both hypotheses need additional work with all available lichen species and host trees in order to be accepted.

Finally, a recent thin layer chromatography (TLC) analysis of *Platismatia glauca* by Obermayer and Randlane (2012) has revealed jackinic acid and pseudoplacodiolic acid along with the usual marker molecules for this specie caperatic acid and atranorin.

In a similar way, GC-MS analyses of the extract of *Pseudevernia furfuracea* led to the identification of atraric acid (47.7, 46.6 and 31.5 %), olivetonide (14.6, 16.9 and 22.8 %) and olivetol (12.9, 21.7 and 17.2 %) as the major constituents of the acetone, methanol and ethyl acetate extracts, respectively. Our results (Table 1) are in agreement with the previously published work on volatile constituents in *Pseudevernia furfuracea* (Joulain and Tabacchi, 2009). However, some of the

identified constituents could be regarded as artifacts generated during the analysis of the extracts in either of the following ways: a consequence of the extraction process (Bats et al., 1990), hydrolytic pre-treatment (Joulain and Tabacchi, 2009) or heating in the GC inlet. For example, 5-(2-oxoheptyl)-resorcinol is probably the product of thermal decarboxylation of olivetoric acid (Joulain and Tabacchi, 2009). Thus, the relative amounts of such compounds should be taken with some reserve.

The chemical composition of *Pseudevernia furfuracea* has been investigated on several previous occasions. Kosanić et al. (2013) used high performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV) for the identification and quantification of the following lichen compounds: 3-hydroxyphysodalic acid, physodalic acid, physodic acid, atranorin and chloroatranorin as major metabolites in an acetone extract of *Pseudevernia furfuracea*. Previously, Guvenc and associates (2012) applied the TLC technique for the detection of atraric acid and a mixture of methyl hematommate and methyl chlorohematommate from the butanol, dichloromethane, ethyl acetate and methanol extracts of *P. furfuracea*. Moreover, the same method (TLC) was used for the differentiation of the two chemical races of *Pseudevernia furfuracea* (var. *furfuracea* and var. *ceratea*). The phenomenon of chemical variation among *Pseudevernia furfuracea* was first described by Halvorsen and Bendiksen (1982) for lichens in Norway. Turk et al. (2006) identified physodic acid, atranorin and chloroatranorin as the main chemical constituents of the ethanol, chloroform, diethyl ether and acetone extracts of *Pseudevernia furfuracea* var. *furfuracea*, whereas olivetoric acid, atranorin and chloroatranorin were found in *Pseudevernia furfuracea* var. *ceratea*. Based on these facts we could conclude that our specimen of *Pseudevernia furfuracea* represents var. *ceratea*.

The acetone and ethyl acetate extracts from both of these lichen species *Platismatia glauca* and *Pseudevernia furfuracea* growing

in Serbia were chemically similar. The methanol extracts contained a much lower amount of atranol and chloroatranol accompanied with a greater amount of methyl haematommate and methyl chlorohaemammatomate. This is understandable as the latter probably formed as the result of methanolysis of the parent depsidones that occurred during the extraction.

Total phenolic content, total flavonoid content and antioxidant activity

The antioxidant potential of acetone, methanol and ethyl acetate extracts from *Platismatia glauca* and *Pseudevernia furfuracea* was evaluated by determing their total phenolic and flavonoid contents and their ability for a free radical scavenging. The results are summarized in Table 2.

The total phenolic content of the studied lichen extract was estimated by the Folin-Ciocalteu method (Singleton et al., 1999). The amount of phenolic compounds was species- and solvent-dependent and varied from 39.75 to 131.82 mg GA/g extract. Generally, *Pseudevernia furfuracea* was 2.07-, 2,47- and 3.10-fold richer in phenols than *Platismatia gluca* depending on the solvent used for extraction (acetone, ethyl acetate and methanol, respectively). The best yield of the extracted phenols was observed with acetone as a solvent and the worst result was obtained with methanol in both species.

The total flavonoid content was evaluated using aluminum chloride (Quettier et al., 2000). The amount of flavonoid compounds ranged from 22.93 to 51.84 mg Ru/g of the lichen extract. Naturally, species- and solvent-dependence of the yield of the extracted flavonoids was observed. *Pseudevernia furfuracea* was a 1.88-fold and 2.26-fold richer source of flavonoids than *Platismatia glauca* when the extraction with acetone and ethyl acetate was performed. The extraction with methanol in both species gave a similar yield of flavonoids. Within a specie, the highest flavonoid content was obtained by methanol extraction in *Platismatia glauca* and ethyl acetate extraction in *Pseudevernia furfuracea*.

The DPHH radical scavenging capacities of the studied lichens were assessed by a modified method of Tekao et al. (1994). The IC50 value, i.e. the concentration of the extract decreasing the initial DPHH concentration to 50 %, varied from 95.33 to 2151.28 µg/mL. The scavenging effects of all lichen extracts were significantly higher for *Pseudevernia furfuracea* than for *Platismatia glauca*. Methanol extracts gave the best scavenging effect in both species. The methanol extract of *Pseudevernia furfuracea* showed IC50 value 95.33 µg/mL, while the methanol extract of *Platismatia glauca* showed IC50 value 656.98 µg/mL. These results were consistent with the determined 3.10-fold higher amount of phenols for the methanol extract of *Pseudevernia furfuracea*.

Table 2: The comparison of the total phenolic content, the total flavonoid content and the antioxidant activity of the lichen extracts

	Platismatia glauca			*Pseudevernia furfuracea*		
	Acetone	**Ethyl acetate extract**	**Methanol**	**Acetone**	**Ethyl acetate extract**	**Methanol**
Total phenolic content[a**]	**63.69 ± 0.47**	51.18 ± 0.63	39.75 ± 0.52	**131.82 ± 0.84**	126.43 ± 0.58	123.09 ± 0.90
Total flavonoid content[b**]	26.90 ± 0.43	22.93 ± 0.93	**37.58 ± 0.76**	50.61 ± 0.35	**51.84 ± 0.78**	37.83 ± 0.21
Antioxidant activity[c**]	2151.28 ± 1.33	1100.57 ± 1.19	**656.98 ± 1.08**	140.31 ± 0.83	183.43 ± 1.58	**95.33 ± 0.94**

** Each value is the average of three analyses ± standard deviation.
[a] Total phenolic content in the extracts of investigated lichen species expressed in terms of gallic acid equivalent, GAE (mg GA/g of extract)
[b] Flavonoid content in the extracts of investigated lichen species expressed in terms of rutin equivalent, RuE (mg Ru/g of extract)
[c] Antioxidant activity of the extracts of investigated lichen species expressed in terms of IC_{50} values (µg/mL)

Also we should mention a higher content of olivetol, methyl haematommate, methyl chlorohaemammatomate and 5-(2-oxoheptyl)-resorcinol for methanol extracts. Having in mind that the methanol extracts of both species possessed equal amounts of flavonoids and the lowest amounts of phenols compared to the other types of the extraction preformed in our study, we concluded that the antioxidant activity could be partly attributed to some non-polar and non-phenol components.

According to most studies, lichen phenolic substances, including depsides, depsidones and dibenzofuranes, are superior antioxidants due to their capacity to inactivate and stabilize free radicals through their hydroxyl groups and their redox properties to act as reducing agents, hydrogen and electron donors and singlet oxygen quenchers. Flavonoids, being the most important natural phenols, are proven antioxidants as well. Previous studies of various lichens in Serbian flora have confirmed a strong correlation of their total phenolic and flavonoid contents and their antioxidant capacities (Ranković et al, 2012; Kosanić et al., 2013, Mitrović et al., 2011). Our findings with *Platismatia glauca* and *Pseudevernia furfuracea* are similar to a report from Odabasoglu and colleagues (2004) with contrary findings, i.e. the absence of correlation between the total phenolic content and the antioxidant activity. This team suggested that the antioxidant activity of lichens might be dependent on other, non-polar and non-phenol components.

Our study represents the first report of the evident antioxidant activity of lichen species *Platismatia glauca*. Previously, the antioxidant activity of *Platismatia glauca* and the other lichen species from Artvin province in Turkey has not been detected by Gulluce and colleagues (2006). The methanol extract of *Platismatia glauca, Parmelia saxatilis, Ramalina pollinaria, Ramalina polymorpha* did not exert any antioxidant activity by the scavenging of free radical DPPH and the inhibition of linoleic acid oxidation, whereas the methanol extract of *Umbilicaria nylanderiana* provided a significant antioxidant activity in both assays. The observed activity could be related to the amount of polar phenols in the tested species *Platismatia glauca, Parmelia saxatilis, Ramalina pollinaria, Ramalina polymorpha* and *Umbilicaria nylanderiana* (1.1, 1.0, 1.0, 0.8 and 3.0 %, respectively).

The antioxidant activity of the second lichen specie *Pseudevernia furfuracea* has been investigated earlier usually from crude extracts, rarely from pure compounds. Guvenc et al. (2012) investigated butanol, dichloromethane, ethyl acetate and methanol extracts and isolated compounds (atraric acid and a mixture of methyl hematommate and methyl chlorohematommate) of *Pseudevernia furfuracea* growing in Kutahya province in Turkey. The scavenging activity was the lowest in the methanol extracts, higher in the ethyl acetate extracts and the highest in the dichloromethane extracts, while it was not detected in the butanol extract. A mixture of methyl hematommate and methyl chlorohematommate was more active than atraric acid in DPPH test.

Bilgin et al. (2012) reported a strong antioxidant activity of the acetone extracts of *Pseudevernia furfuracea* collected from the Back Sea region of Turkey and gave a detailed analysis of the previous studies with this specie.

Kosanić and Ranković (2011) have explored the antioxidant potential of acetone, methanol and aqueous extracts of the lichen *Pseudevernia furfuracea, Cetraria islandica, Lecanora atra, Parmelia pertusa* and *Umbilicaria cylindrica* from the mountain Kopaonik in Serbia. The highest intensity of DPPH scavenging activity was delineated for the acetone extracts of *Lecanora atra* and *Pseudevernia furfuracea* according to the highest phenolic and flavonoid contents determined. The aqueous extracts of the tested lichens showed the weakest antioxidant activity probably because the active components were not soluble in water.

In the following study Kosanić and colleagues (2013) estimated the antioxidant activity of the crude acetone extract and isolated pure compounds of *Pseudevernia furfuracea* and *Evernia prunastri* (physodic acid and evernic acid, respectively) from the same location. Physodic acid from *Pseudevernia furfuracea* demonstrated the strongest DPPH radical scavenging activity.

Antimicrobial and antibiofilm activity

The antibacterial, antifungal and antibiofilm activity of methanol, acetone and ethyl acetate extracts from *Platismatia glauca* and *Pseudevernia furfuracea* were tested in vitro against a panel of microorganisms including human pathogenic bacteria, yeasts and moulds in order to evaluate a broad-spectrum of antimicrobial activity. The results are presented in Tables 3-5. The intensity of antimicrobial activity varied depending on the species of microorganisms. The detectable MIC values for *Platismatia glauca* were in the range from 0.08 mg/mL to 2.5 mg/mL and for *Pseudevernia furfuracea* were in the range from 0.005 mg/mL to 2.5 mg/mL. For both lichen species, a statistically significant difference in the activity among methanol, acetone and ethyl acetate extracts on all tested microorganisms was not noticed ($p > 0.05$). Regarding the bacteria, a statistically significant difference in the sensitivity between Gram-positive and Gram-negative bacteria was observed for *Platismatia glauca* and *Pseudevernia furfuracea* ($p < 0.05$).

The extracts from *Platismatia glauca* exhibited a strong effect against Gram-positive bacteria: *Bacillus* species, *Staphylococcus aureus* and *Sarcina lutea* with the inhibitory and bactericidal concentrations varying from 0.08 mg/mL to 1.25 mg/mL (Table 3). On the other side, the tested extracts showed a moderate effect against Gram-negative bacteria with the MIC values at 1.25 mg/mL and 2.5 mg/mL. The acetone and ethyl acetate extracts of *Platismatia glauca* showed slightly higher antibacterial activities compared to its methanol extract. Previous investigations

of the antimicrobial activity of the lichen species *Platismatia glauca* are limited. Gulluce and colleagues (2006) have tested a methanol extract of *Platismatia glauca* against 35 tested bacterial strains and inhibited the growth of 4 Gram-positive bacterial strains (two strains of *Bacillus subtilis*, *Bacillus macerans* and *Clavibacter michiganense*). However, Cobanoglu et al. (2010) have determined the activity of acetone and chloroform extracts of *Platismatia glauca* against Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Acinobacter sp.*). The differences in sensitivity between Gram-positive and Gram-negative bacteria are mainly due to their different cell wall structures and permeability. The cell wall of Gram-positive bacteria consists of a single layer of peptidoglucanes and teichoic acids, while the cell wall of Gram-negative bacteria is a multilayered structure bounded by an outer cell membrane and made of peptidoglucanes, lipopolysacharides and lipoproteins. Bacteria are generally more sensitive to antimicrobial agents than fungi since the cell wall of fungi is poorly permeable and made of polysaccharides chitin and glucan (Kosanić et al., 2013).

The antibacterial activity of *Pseudevernia furfuracea* was more prominent and almost solvent–independent compared to the same activity of *Platismatia glauca*. *Pseudevernia furfuracea* showed a strong activity against *Bacillus* species and *Staphylococcus aureus* (MIC and MMC values between 0.005 mg/mL and 0.08 mg/mL), a moderate activity against *Sarcina lutea*, *Enterococcus faecalis*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (MICs at 0.31 mg/mL and 0.63 mg/mL) and a weak activity against *Escherichia coli* and *Salmonella typhimurium* (MICs at 1.25 mg/mL and 2.5 mg/mL) (Table 3). A similar antibacterial profile was established in studies described by other researchers. Kosanić et al. (2013) have tested the antimicrobial activity of the acetone extract of *Pseudevernia furfuracea* and determined a moderate sensitivity of the bacteria as follows *Bacillus mycoides > Bacillus sub-*

tilis > Staphylococcus aureus > Escherichia coli. The strongest antimicrobial activity was found for the isolated physodic acid. Guvenc et al. (2012) have observed significant antimicrobial activities of the dichloromethane and acetone extracts and the isolated mixture of methyl hematommate and methyl chlorohematommate from *Pseudevernia furfuracea* against Gram-positive bacteria and *Candida* species. While the crude extracts of *Pseudevernia furfuracea* were inactive against Gram-negative bacteria, the mixture of methyl hematommate and methyl chlorohematommate showed a good activity on *Proteus mirabilis*. Finally, Turk at al. (2006) examined the ethanol, chloroform, diethyl ether and acetone extracts and the isolated compounds (physodic acid, chloroatranorin, atranorin and olivetoric acid) of the chemical races of *Pseudevernia furfuracea* (var. *furfuracea* and var. *ceratea*). All extracts of both chemical races were active on Gram-positive bacteria and fungi but inactive on Gram-negative bacteria. An activity of the extracts was not detected against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae, Salmonella typhimurium, Alternaria citri, Alternaria tenuissima* and *Gaeumannomyces graminis* Chloroatrorin and olivetoric acid were active against filamentous fungi. Atranorin showed no activity against filamentous fungi, either.

Table 3: Antibacterial activity of *Platismatia glauca* and *Pseudevernia furfuracea* (mg/mL)

Species	Acetone		Ethyl acetate extract		Methanol		**Doxycicline**	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
Platismatia glauca								
B. pumilus NCTC 8241	0.08	0.08	0.16	0.31	0.31	0.31	0.0001	0.008
B. subtilis	0.08	0.08	0.16	0.16	0.31	0.31	0.0001	0.002
B. cereus	0.16	0.16	0.16	0.16	0.31	0.31	0.001	0.008
E. faecalis	2.5	> 2.5	2.5	> 2.5	2.5	2.5	0.008	0.063
E. coli ATCC 25922	2.5	> 2.5	2.5	> 2.5	2.5	> 2.5	0.016	0.031
E. coli	2.5	> 2.5	2.5	> 2.5	> 2.5	> 2.5	0.008	0.016
P. aeruginosa	1.25	2.5	1.25	2.5	1.25	> 2.5	0.25	> 0.25
P. mirabilis	1.25	1.25	1.25	2.5	2.5	> 2.5	0.25	> 0.25
S. aureus	0.08	0.31	0.08	0.63	0.63	1.25	0.0004	0.008
S. lutea	0.08	0.08	0.08	0.08	0.08	0.31	<0.0004	0.004
S. typhimurium	2.5	> 2.5	> 2.5	> 2.5	> 2.5	> 2.5	0.016	0.125
Pseudevernia furfuracea								
B. pumilus NCTC 8241	0.005	0.02	0.005	0.02	0.005	0.04	0.0001	0.008
B. subtilis	0.01	0.02	0.01	0.02	0.01	0.02	0.0001	0.002
B. cereus	0.04	0.04	0.04	0.04	0.04	0.08	0.001	0.008
E. faecalis	0.63	0.63	0.63	0.63	1.25	1.25	0.008	0.063
E. coli ATCC 25922	0.63	0.63	0.63	0.63	0.63	1.25	0.016	0.031
E. coli	2.5	> 2.5	1.25	2.5	2.5	> 2.5	0.008	0.016
P. aeruginosa	0.31	1.25	0.63	1.25	1.25	> 2.5	0.25	> 0.25
P. mirabilis	0.63	1.25	0.63	1.25	0.63	1.25	0.25	> 0.25
S. aureus	0.04	0.04	0.02	0.04	0.04	0.08	0.0004	0.008
S. lutea	0.63	0.63	0.63	0.63	0.63	0.63	<0.0004	0.004
S. typhimurium	1.25	2.5	1.25	2.5	2.5	> 2.5	0.016	0.125

The antifungal activity of the lichen extracts of *Platismatia glauca* and *Pseudevernia furfuracea* was investigated on 9 fungal strains, including yeasts and molds (Table 4). The detectable MIC values were in the range from 0.04 mg/mL to 2.5 mg/mL. Among the tested extracts, the methanol extract of *Platismatia glauca* and *Pseudevernia furfuracea* demonstrated a significant activity againts all yeast and filamentous fungi. In addition, all tested extracts inhibited the growth of food-spoilage fungi *Penicillium italicum, Penicillium digitatum, Penicillium verrucosum* and *Botrytis cinerea*. The most sensitive was the plant pathogenic fungus *Botrytis cinerea* on the acetone extract of *Platismatia glauca*. Gulluce et al. (2006) have also noticed an antifungal effect of the methanol extract on the plant pathogenic fungi, *Sclorotinia sclerotiorum* and *Trichophyton rubrum*. These findings suggest that lichen *Platismatia glauca* could be a potential natural pesticide.

Concerning the antifungal properties of *Pseudevernia furfuracea,* we observed stronger effects than with *Platismatia glauca.* All tested extracts of *Pseudevernia furfuracea* showed the best antifungal activities against *Candida* species with MICs below the 0.04 mg/mL. In some cases, the MIC values of the *Pseudevernia furfuracea* extracts were lower than the MIC values of the positive control, fluconazole.

The literature data also suggest that *Pseudevernia furfuracea* extracts possess a marked antifungal activity (Guvenc et al., 2012; Kosanić et al., 2013; Turk et al., 2006).

Table 4: Antifungal activity of *Platismatia glauca* and *Pseudevernia furfuracea* (mg/mL)

Species	Acetone		Ethyl acetate extract		Methanol		Fluconazole	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
Platismatia glauca								
A. flavus	> 2.5	> 2.5	> 2.5	> 2.5	0.63	0.63	1	1
B. cinerea	0.04	0.08	1.25	> 2.5	0.63	0.63	0.031	0.5
C. albicans ATCC 10231	> 2.5	> 2.5	> 2.5	> 2.5	0.31	0.63	0.031	1
C. albicans	1.25	2.5	1.25	2.5	1.25	1.25	0.063	1
P. italicum	0.63	2.5	1.25	2.5	0.16	0.16	1	1
P. digitatum	0.63	0.63	0.63	0.63	0.63	0.63	0.031	0.031
P. verrucosum	0.63	1.25	0.63	1.25	0.63	1.25	n.d.[**]	n.d.[**]
Rhodotorula sp.	> 2.5	> 2.5	> 2.5	> 2.5	0.16	1.25	0.063	1
S. boulardii	0.63	2.5	0.63	2.5	0.63	1.25	0.031	1
Pseudevernia furfuracea								
A. flavus	0.31	>2.5	1.25	>2.5	0.04	0.63	1	1
B. cinerea	0.63	1.25	2.5	>2.5	0.08	0.16	0.031	0.5
C. albicans ATCC 10231	< 0.04	0.08	<0.04	0.08	< 0.04	0.31	0.031	1
C. albicans	< 0.04	0.08	<0.04	0.08	< 0.04	0.31	0.063	1
P. italicum	0.31	0.31	<0.04	0.63	0.31	0.31	1	1
P. digitatum	< 0.04	0.63	0.31	0.31	0.16	0.63	0.031	0.031
P. verrucosum	0.63	1.25	0.63	1.25	0.31	0.63	n.d.[**]	n.d.[**]
Rhodotorula sp.	>2.5	>2.5	>2.5	>2.5	0.63	0.63	0.063	1
S. boulardii	0.63	0.63	1.25	1.25	0.31	0.31	0.031	1

[**] n.d. = not determined

The assemblage of bacteria on extracellular polymer matrices of biotic or abiotic surfaces known as a biofilm confer higher resistance to variable environmental conditions and biocides (antibiotics and disinfectants) compared to the planktonic form of bacteria (Nomura et al., 2013). Therefore, we investigated the effect of *Platismatia glauca* and *Pseudevernia furfuracea extracts* on the biofilm formation of Gram-positive *Staphylococcus aureus* ATCC 25923 and Gram-negative *Proteus mirabilis* ATCC 12453. The acetone and ethyl acetate extracts of *Platismatia glauca* showed better antibiofilm activities (BIC at 0.63 mg/mL) than their methanol extract (Table 5). The MIC values were higher than the BIC values due to the capability of the extracts to prevent biofilm formation better than to affect bacterial presence, giving a solid starting point for further investigation.

Concerning the antibiofilm activity of *Pseudevernia furfuracea,* a methanol extract of this specie was the most active with the BIC value at 0.63 mg/mL on *Proteus mirabilis* ATCC 12453 (Table 5). This was also the only case in which the BIC was lower than the MIC, showing an interesting nature of this extract in terms of preventing the biofilm resistance. The BICs were constant for the acetone and ethyl acetate of *Pseudevernia furfuracea* for both bacterial species.

The strong antimicrobial and antibiofilm activity of *Pseudevernia furfuracea* extracts could be attributed to specific lichen metabolites with antimicrobial actions. In general, the lichen compounds possess different mechanisms of antimicrobial activity. They affect specific targets on a microbial cell, including the cell wall, cell membrane, metabolic enzymes, protein synthesis, and genetic systems causing inhibition or inactivation of microorganisms. The major metabolites of *Pseudevernia furfuracea* are compounds with a proven antimicrobial activity such as: 3-hydroxyphysodalic acid, physodalic acid, physodic acid, olivetoric acid, atranorin, chloroatranorin, orselinic acid and methyl orselinate (Gulluce et al., 2006; Kosanić et al., 2013). Our study provides data for supporting the use of *Pseudevernia furfuracea* extracts as natural antimicrobial agents and confirms that these extracts represent a significant source of antimicrobial compounds.

CONCLUSION

The antioxidant, antimicrobial and antibiofilm activities of the two lichen species from Familia Parmeliacea (*Platismatia glauca* and *Pseudevernia furfuracea*) have been demonstrated. The antioxidant properties of

Table 5: Antibiofilm activity of *Platismatia glauca* and *Pseudevernia furfuracea* (mg/mL)

Type of extract		*Staphylococcus aureus* ATCC 25923		*Proteus mirabilis* ATCC 12453	
		MIC	BIC	MIC	BIC
Platismatia glauca	Acetone	0.63	0.63	2.5	0.63
	Ethyl acetate extract	1.25	0.63	2.5	0.63
	Methanol	2.5	2.5	>2.5	2.5
Pseudevernia furfuracea	Acetone	0.31	2.5	2.5	2.5
	Ethyl acetate extract	0.31	2.5	2.5	2.5
	Methanol	0.31	1.25	2.5	0.63

Pseudevernia furfuracea were significantly stronger than the antioxidant properties of *Platismatia glauca. Pseudevernia furfuracea* demonstrated more potent antimicrobial and antibiofilm activities than *Platismatia glauca.* Antioxidant, antimicrobial and antibiofilm agents detected in the investigated lichens extracts could be valuable for the prevention of pathogens in food industry, medicine and phytopharmacy. Our future study will focus on the isolation and evaluation of pure active components in these species which could be used as preservatives.

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