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ORIGINAL RESEARCH

Lasallia pustulata lichen as possible natural antigenotoxic, antioxidant, antimicrobial and anticancer agent

Marijana Kosanić · Branislav Ranković · Tatjana Stanojković · Ivana Stošić · Darko Grujičić · Olivera Milošević-Djordjević

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Abstract The methanol extract of the lichen *Lasallia pustulata* was tested for genotoxic, antioxidant, antimicrobial and anticancer activities. We did this using a cytokinesis block micronucleus (MN) assay on peripheral blood lymphocytes, by measuring free radical and superoxide anion scavenging activity, reducing power, determining of total phenolic compounds and determining the total flavonoid content, measuring the minimal inhibitory concentration by the broth microdilution method against five species of bacteria and five species of fungi and by using the microculture tetrazolium test on FemX (human melanoma) and LS174 (human colon carcinoma) cell lines. As a result of this study, we found that the methanol extract of *L. pustulata* did not modify the frequency of the MN and nuclear division index in comparison to untreated cells ($p > 0.05$). These results revealed that the methanol extract had moderate free radical scavenging activity with IC_{50} values of 395.56 μg/mL. Moreover, the extract tested had effective reducing power and superoxide anion radical scavenging. The values of the minimum inhibitory concentration against the tested microorganisms ranged from 0.625 to 20 mg/mL. In addition, the extract tested had strong anticancer activity against both cell lines with IC_{50} values of 46.67 and 71.71 μg/mL.

Keywords *Lasallia pustulata* · Methanol extract · Genotoxic · Antioxidant · Antimicrobial · Anticancer activities · In vitro

M. Kosanić (✉) · B. Ranković · I. Stošić · D. Grujičić · O. Milošević-Djordjević
Department of Biology, Faculty of Science, University of Kragujevac, 34000 Kragujevac, Serbia
e-mail: marijanakosanic@yahoo.com

T. Stanojković
Institute of Oncology and Radiology of Serbia, 11000 Belgrade, Serbia

O. Milošević-Djordjević
Faculty of Medical Sciences, University of Kragujevac, 34000 Kragujevac, Serbia

Introduction

Lichens are complex symbiotic associations between a fungus (mycobiont) and photobiont which can be either an alga or cyanobacteria (Bates et al. 2011). They have been proven to be the earliest colonizers of terrestrial habitats on the earth with a worldwide distribution from artic to tropical regions and from the plains to the highest mountains. Their specific, even extreme, range of habitats, slow growth and long life are the reason for their being able to produce numerous protective secondary metabolites against different physical and biological influences (Mitrović et al. 2011).

Lichens exert a wide variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and anticancer effects (Kosanić et al. 2013; Ranković

et al. 2012). Due to a relatively recent resurgence in lichen bioactivity, the therapeutic potential of many classes of lichen in medicine has largely remained unexplored. For this reason, in this study, significant attention was focused on the lichen *Lasallia pustulata.*

Lasallia pustulata is a large, foliose species, with rounded thalli attached centrally, and an upper surface with conspicuous, convex, scabrid-pruinose pustules. It is brown to whitish grey when dry, brownish to yellowish green when wet, with margins with dense tufts of black isidia and rare apothecia. It grows on rocks in upland and mountainous areas (Purvis et al. 1992; Dobson 2000). *Lasallia pustulata* have been fully investigated for their taxonomy, but there is very little data on its biological activity.

Because of this, this study was designed to evaluate several biological activities (genotoxic, anti-oxidant, antimicrobial and anticancer) of the methanol extract obtained from the lichen *Lasallia pustulata*.

Materials and methods

Chemicals and reagents

Media for microorganisms (Müller-Hinton agar, Müller-Hinton broth, Sabouraud dextrose agar and Sabouraud dextrose broth) were purchased from Torlak (Belgrade, Serbia). The microorganisms, human melanoma FemX and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Methanol was obtained from Kefo (Belgrade, Serbia). Giemsa was from Alfapanon (Novi Sad, Serbia). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Lichen sample

Lichen samples of *Lasallia pustulata* (L.) Merat, were collected from Kopaonik, Serbia, in September 2011. The samples were preserved in the facilities of the Department of Biology and Ecology of Kragujevac (Faculty of Science). Determination of the lichen species investigated was accomplished using standard methods.

Extraction

Finely dry ground thalli from the lichen (100 g) were extracted using methanol in a Soxhlet extractor (IKA, Werke, Staufen, Germany). The extract was filtered then concentrated under reduced pressure in a rotary evaporator (IKA, RV 10, Werke, Staufen, Germany). The dry extract was stored at −18 °C until it was used in the tests. The extract was dissolved in 5 % dimethyl sulphoxide (DMSO) for the experiments. The DMSO was dissolved in sterile distilled water to the desired concentration.

In vitro cytokinesis-block micronucleus test (CBMN test)

Peripheral blood samples were taken from three healthy, non-smoking female donors aged between 30 and 34 years, who had not been exposed to known mutagens. The experiments conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki) and informed consent was obtained from all of the donors.

In this study, a cytokinesis block micronucleus assay (CBMN) was carried out using the procedure described by Fenech (2000). Briefly, whole heparinized blood (0.5 mL) was added to 5 mL of the complete medium for lymphocyte culture PBMax Karyotyping (Invitrogen, CA, USA). All of the cultures were carried out in duplicate and incubated at 37 °C for 72 h. After a 24 h incubation period, the concentrations of lichens extract tested (12.5, 25, 50, 100 and 200 μg/mL) were added to the cultures. Untreated cultures served as negative controls. Cytochalasin B was added 44 h after initiation of incubation at a final concentration of 4 μg/mL. At the end of the incubation period the cultures were harvested. The cells were collected by centrifugation, treated with cold (+4 °C) hypotonic solution (0.56 % KCl) and fixed with methanol: glacial acetic acid in a 3:1 solution. The cell suspensions were re-suspended in a small volume of fixative and dropped onto clean slides, air-dried and stained with 2 % Giemsa.

The scoring was performed using a light microscope (Nikon E50i, Otawara, Tochigi, Japan) at 400× magnification. The MN frequencies were scored in one thousand binucleated cells (BN) from each donor (3000 BN cells per concentration) following the criteria for MN scoring as described by Fenech (2007). The nuclear division index (NDI) was calculated using the formula $NDI = ((1 \times M1 + (2 \times M2) + (3 \times M3) + (4 \times M4))/N$, where M1–M4

are the number of cells with 1–4 nuclei, and N is the number of scored cells (Fenech 2000).

Antioxidant activity

Scavenging DPPH radicals

The free radical scavenging activity of the extract was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH). The method used was similar to that of Dorman et al. (2004) but was modified in its details. The stock solution of the extract was prepared in 5 % DMSO to achieve a concentration of 1000 μg/mL. Further, two-fold dilutions were made to obtain concentrations of 500, 250, 125 and 62.5 μg/mL. Diluted solutions of the extract (1 mL each) were mixed with 2 mL of methanol solution containing the DPPH radical (0.05 mg/mL) in cuvettes. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was then measured at 517 nm in a Jenway spectrophotometer (Bibby Scientific Limited, Stone, UK) against methanol as a blank. Ascorbic acid was used as a standard. The DPPH radical concentration was calculated using the following equation:

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

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

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

Reducing power

The reducing power of the extract was determined according to Oyaizu et al's method (1986). The stock solution of extract was prepared in 5 % DMSO to achieve a concentration of 1000 μg/mL. Further, two-fold dilutions were made to obtain concentrations of 500, 250, 125 and 62.5 μg/mL. One millilitre of each extract was mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %). The mixtures were incubated in an incubator (Sutjeska, Zagreb, Croatia) at 50 °C for 20 min. Trichloroacetic acid (10 %, 2.5 mL) was then added to the mixture, which was centrifuged using a centrifuge (HermLe, Wehingen, Germany). Finally, the upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL; 0.1 %). The absorbance of the solution was measured at 700 nm in a spectrophotometer (Bibby Scientific Limited, Stone, UK). The blank was prepared with all the reaction agents except the extract. A higher absorbance of the reaction mixture indicated that the reducing power was increased. Ascorbic acid was used as a positive control.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of the extract was detected according to Nishimiki et al's method (1972). The stock solution of the extract was prepared in 5 % DMSO to achieve a concentration of 1000 μg/mL. Further, two-fold dilutions were made to obtain concentrations of 500, 250, 125 and 62.5 μg/mL. 0.1 mL of each extract was mixed with 1 mL nitroblue tetrazolium (NBT) solution (156 μM in 0.1 M phosphate buffer, pH 7.4) and 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 μM in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 μL of phenazine methosulphate (PMS) solution (60 μM in 0.1 M phosphate buffer, pH 7.4). The mixture was incubated at room temperature for 5 min, and the absorbance was measured at 560 nm in spectrophotometer (Bibby Scientific Limited, Stone, UK) against a blank sample (phosphate buffer). Decreased absorbance indicated increased superoxide anion radical scavenging activity. Ascorbic acid was used as a standard. The percentage inhibition of superoxide anion generation was calculated using the following formula:

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

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

The inhibition concentration at 50 % inhibition (IC_{50}) was the parameter used to compare the radical

scavenging activity. A lower IC_{50} meant better radical scavenging activity.

Determination of total phenolic compounds

The total soluble phenolic compounds in the extract were determined with the Folin-Ciocalteu reagent according to Slinkard and Singleton's method (1997) using pyrocatechol as a standard phenolic compound. Briefly, 1 mL of the extract (1 mg/mL) in a volumetric flasc was diluted with distilled water (46 mL). One millilitre of Folin-Ciocalteu reagent was added and the contents of the flask were mixed thoroughly. After 3 min, 3 mL of sodium carbonate (2 %) was added and was then allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer (Bibby Scientific Limited, Stone, UK) against a blank consisting of all the reaction agents except for the extract. The total concentration of phenolic compounds in the extract was determined as a microgram of pyrocatechol equivalent (PE) per milligram of dry extract by using an equation obtained from a standard pyrocatechol graph as follows:

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

Total flavonoid content

The total flavonoid content was determined using the Dowd's method (Meda et al. 2005). Two millilitres of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (1 mg/mL). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in a spectrophotometer (Bibby Scientific Limited, Stone, UK). A negative control, without any extract was used as the blank. The total flavonoid content was determined as a microgram of rutin equivalent (RE) per milligram of dry extract by using an equation that was obtained from a standard rutin graph as follows:

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

Antimicrobial activity

Microorganisms and media

The following bacteria were used as test organisms in this study: *Staphilococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 10987), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC 29906). All bacteria used were obtained from the American Type Culture Collection (ATCC). The bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Candida albicans* (ATCC 10259), *Fusarium oxysporum* (DBFS 292), *Penicillium purpurescens* (DBFS 418) and *Trichoderma harsianum* (DBFS 379). All fungi were from the mycological collection maintained by the Mycological Laboratory within the Department of Biology of Kragujevac University's Faculty of Science (DBFS). The fungal cultures were maintained on potato dextrose (PD) agar, except for *Candida albicans* that was maintained on Sabouraud dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4 °C and subcultured every 15 days.

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37 °C on Müller-Hinton agar substrates and brought up by dilution according to the 0.5 McFarland standard (bio-Mérieux, Marcy l'Etoile, France) to approximately 10^8 CFU/mL. Suspensions of fungal spores were prepared from freshly mature (3- to 7-day-old) cultures that grew at 30 °C on a PD agar substrate. The spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically (Bibby Scientific Limited, Stone, UK) at 530 nm, and were then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS (1998).

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined using 96-well micro-titer plates (Spektar, Čačak, Serbia), by the broth microdilution method (Sarker et al. 2007). A series of dilutions with concentrations ranging from 40 to 0.156 mg/mL of the extract was used in the experiment against every

microorganism tested. The starting solutions of extract was obtained by measuring off a certain quantity of the extract and dissolving it in DMSO. Two-fold dilutions of the extract were prepared in a Müller-Hinton broth for bacterial cultures and a SD broth for fungal cultures. The MIC was determined with resazurin. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing color of resazurin was defined as the MIC for the tested microorganism at a given concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria and ketoconazole in the case of fungi. A DMSO solution was used as a negative control for the influence of the solvents.

Cytotoxic activity

Cell lines

The human melanoma FemX and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (ATCC). Both cancer cell lines were maintained in the recommended RPMI-1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 10 % heat-inactivated (56 °C) fetal bovine serum, L-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), and 25 mM HEPES and was adjusted to pH 7.2 by bicarbonate solution. The cells were grown in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

Treatment of cell lines

A stock solution (100 mg/mL) of the test sample, made in dimethylsulfoxide (DMSO), was dissolved in the corresponding medium to the required working concentrations. Neoplastic FemX cells (5000 cells per well) and neoplastic LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates, and 24 h later, after cell adherence, five different, double diluted, concentrations of the investigated sample were added to the wells. The final concentrations applied to target cells were 200, 100, 50, 25 and 12.5 μg/mL except for the control wells, where only nutrient medium was added to the cells. The nutrient medium was RPMI 1640 medium, supplemented with L-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), 10 % heat inactivated (56 °C) fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 h.

Determination of cell survival (MTT test)

The effect of the test samples on cancer cell survival was determined by the MTT test (microculture tetrazolium test), according to Mosmann (1983) with modification by Ohno and Abe (1991), 72 h after the addition of the test sample, as described earlier. 20 μL of MTT solution (5 mg/mL PBS) was added to each well. The samples were incubated for a further 4 h at 37 C in 5 % CO_2 in a humidified air atmosphere. 100 μL of 10 % SDS was then added to the extract of the insoluble product formazan, resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of light absorbance, which was then read in an ELISA plate reader (Tecan 200, USA) at 570 nm. Absorbance (A) at 570 nm was measured 24 h later. To get cell survival S (%), was used the following equation: $S(\%) = (As - Ab) \times 100/(Ac - Ab)$. Therefore, A of a sample (As) with cells grown in the presence of various concentrations of the investigated test samples was divided with control optical density (the A of control cells (Ac) grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank (Ab) was always subtracted from A of the corresponding sample with target cells. The IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50 %, compared with a vehicle-treated control. *cis*-diamminedichloroplatinum (*cis*-DDP) was used as a positive control. All of the experiments were done in triplicate.

Statistical analysis

All the results are shown as mean ± standard deviation (SD). The results were compared using the Student's t test. Concentration–response (MN and NDI) relationships were determined by using Pearson correlation coefficients. The level of significance was set at $p < 0.05$.

Results

The frequencies of MN and NDI in the PBLs of healthy donors treated with different concentrations of the methanolic extract from the lichen are presented in Table 1. The presence of five tested concentrations of extract did not significantly affect the MN frequency compared to the control untreated peripheral blood lymphocytes PBLs ($p > 0.05$). The lichen extract at the concentrations tested concentrations did not modify the NDI values in comparision to untreated cell culture ($p > 0.05$). The correlation between MN and different concentrations was negative and without statisticaly significance ($r = -0.237$, $p > 0.05$). No significant positive correlation was detected between the NDI and the concentrations of extract tested ($r = 0.164, p > 0.05$). The analyses of the distribution of MN revelead that BN cells with 1 MN were predominant, folowed by cells with 2 MN while cells with more MN were not found.

The extract tested had antioxidant activity against various oxidative systems in vitro as a shown in Tables 2 and 3.

DPPH radical scavenging and superoxide anion radical scavenging of the extract studied summarized in Table 2, while its reducing power is shown in Table 3. The tested extract revealed lower antioxidant activities than ascorbic acid. The IC_{50} values were 395.56 and 523.71 μg/mL for DPPH radicals and superoxide anion radicals scavenging activity, respectively. The values measured for absorbance for reducing power in the tested extract varied from 0.1327 to 0.7702. As shown in Table 3, reducing power was concentration dependant (high concentrations exhibit high reducing power).

The total phenolic and flavonoid constituents of the tested extract are given in Table 4. The amount of total phenolics and flavonoids in the extract were 84.33 μg PE/mg and 49.62 μg RE/mg, respectively.

The antimicrobial activity of the tested extract against the microorganisms tested is shown in Table 5.

The methanol extract from the lichen tested showed relatively similar antibacterial and antifungal activity. The MIC for the extract in the tested bacteria and fungi were 0.625–20 mg/mL. The lowest measured MIC

Table 1 The frequency of micronuclei (MN) and nuclear division index values (NDI) in peripheral blood lymphocytes (PBLs) of healthy donors after the treatments with four concentrations of methanolic extracts from *Lasallia pustulata* in vitro

Treatments	Concentration (μg/mL)	No of analyzed BN cells	MN/1000 BN cells (X ± SD)	BN with MN (%)	Distribution of MN		NDI
					1 MN (%)	2 MN (%)	
Untreated cultures	0	3000	9.00 ± 1.73	26 (0.86)	25 (0.83)	1 (0.03)	1.65 ± 0.07
L. pustulata	12.5	3000	9.00 ± 1.00	25 (0.83)	23 (0.77)	2 (0.06)	1.66 ± 0.06
L. pustulata	25	3000	9.33 ± 1.16	26 (0.86)	24 (0.80)	2 (0.06)	1.61 ± 0.10
L. pustulata	50	3000	8.67 ± 1.53	25 (0.83)	24 (0.8)	1 (0.03)	1.68 ± 0.04
L. pustulata	100	3000	9.33 ± 1.53	28 (0.93)	28 (0.93)	/	1.68 ± 0.09
L. pustulata	200	3000	7.67 ± 1.53	21 (0.70)	19 (0.63)	2 (0.07)	1.68 ± 0.08

Table 2 DPPH radical scavenging activity and superoxide anion scavenging activity of methanol extract of *Lasallia pustulata*

	DPPH radical scavenging activity IC_{50} (μg/mL)	Superoxide anion scavenging activity IC_{50} (μg/mL)
Lasallia pustulata	395.56	523.71
Ascorbic acid	6.42	115.61

Table 3 Reducing power of methanol extract of *Lasallia pustulata*

Absorbance (700 nm)					
	1000 μg/mL	500 μg/mL	250 μg/mL	125 μg/mL	62.5 μg/mL
L. pustulata	0.7702	0.6289	0.3869	0.2578	0.1327
Ascorbic acid	2.1131	1.6543	0.9572	0.4784	0.2472

Table 4 Total phenolics and flavonoid content of methanol extract of *Lasallia pustulata*

Phenolics content (μg PE/mg of extract)	Flavonoid content (μg RE/mg of extract)
84.33	49.62

Table 5 Minimum inhibitory concentration (MIC) of methanol extract of *Lasallia pustulata*

Microorganisms	Methanol extract	S	K
Staphylococcus aureus	2.5[a]	31.25	–
Bacillus subtilis	0.625	15.62	–
Bacillus cereus	1.25	15.62	–
Escherichia coli	5	62.5	–
Proteus mirabilis	2.5	62.5	–
Aspergillus flavus	10	–	7.81
Candida albicans	5	–	3.9
Fusarium oxysporum	5	–	3.9
Penicillium purpurescens	20	–	15.62
Trichoderma harsianum	10	–	7.81

Antibiotics: *K* ketoconazole, *S* streptomycin

[a] Minimum inhibitory concentration (MIC); values given as mg/mL for extract and as μg/mL for antibiotics. Values are the mean of three replicate

value (0.625 mg/mL) was related to the *Bacillus subtilis* species.

The antimicrobial activity was compared to streptomycin (a standard antibiotic) and ketoconazole (a standard antimycotic). The results showed that streptomycin and ketoconazole had stronger activity than the extract as shown in Table 5. In a negative control, DMSO had no inhibitory effect on the organisms tested.

The anticancer activity of the lichen extract against the cell lines tested is shown in Table 6. The IC_{50} against FemX and LS174 cell lines was 46.67 and 71.71 μg/mL respectively. As shown in the table, the positive control (Cis-DDP) had a slightly better anticancer activity than the sample tested.

Discussion

Numerous studies have so far been investigated a diverse range of lichens for their biological activities including antioxidant, antibacterial, anticancer, antifungal and antigenotoxic effects (Halama and Van Haluwin 2004; Odabasoglu et al. 2004; Zeytinoglu et al. 2008; Paudel et al. 2008; Ranković et al. 2011; Turkez et al. 2012a).

Table 6 Growth inhibitory effects of methanol extract of *Lasallia pustulata* on FemX and LS 174 cell lines

Test sample	FemX IC_{50} (μg/mL)	LS 174
Lasallia pustulata	46.67 ± 1.78	71.71 ± 3.68
Cis-DDP	0.94 ± 0.35	2.3 ± 0.31

In order to broaden our knowledge about the properties *L. pustulata* and its safety, it is very important to evaluate its effects on human genetic material. At this point, the micronucleus assay is used most frequently for assessing chromosomal damage in human peripheral blood lymphocytes (Fenech 2000) The micronuclei are small nuclei clearly separeted from the main nuclei, which contain acentric fragments/chromatids or whole chromosomes that are left outside of the daughter nuclei after cell division. The micronucleus test is very often used in vitro cytogenetic studies as a useful indicator of the genetic damage due to exposure to different medicinal plants (Alves dos Santos et al. 2008; Miloševic-Djordjevic et al. 2013). Beside measuring the genotoxic effect, this test also provides information about the cytostatic effects of the agent using the nuclear division index (NDI) value (Fenech 2007).

In our current study of the methanolic extract of *L. pustulata* in the concentrations tested (12.5–200 μg/mL) did not modify the frequency of MN or NDI, suggesting that the lichen exctract had no genotoxic or anticancer potential on human lymphocytes in vitro. Similarly, numerous studies have been reported that lichen extracts had no genotoxic effects on human lymphocytes in vitro (Geyikoglu et al. 2007; Zeytinoglu et al. 2008; Turkez et al. 2012a, b, Turkez and Dirican 2012).

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

Free radical scavenging is one of the numerous mechanisms for antioxidation (Sini and Devi 2004). The antiradical activity of the extract was studied by screening its ability to bleach stable DPPH radicals. This method is based on the formation of the non-radical form of DPPH-H in the presence of an alcoholic DPPH solution and a hydrogen donating antioxidant (AH) by the reaction DPPH + AH → DPPH-H + A (Anandjiwala et al. 2008).

The reducing power of the methanol extract of *L. pustulata* also may indicate its potential antioxidant activity. The reducing features are mainly related to the presence of reductones. Gordan (1990) found that the antioxidant effect of reductones is based on the destruction of the free radical chain by the donation of a hydrogen atom. The reduction of ferric ion (Fe3+) to ferrous ion (Fe2+) is measured by the strength of the green–blue color of the solution which absorbs light at 700 nm. The results presented here indicate that the marked ferric reducing power activity of the extract is due to the presence of polyphenols. These may act in a similar way as reductones in their reaction with free radicals, turning them into more stable products and aborting free radical chain reactions (Sasikumar et al. 2010).

The superoxide radical scavenging activity of the methanol extract of *L. pustulata* was estimated based on its ability to destroy the superoxide radical produced from the PMS/NADH reaction. A decrease in absorbance at 560 nm with antioxidants present indicates that the superoxide anion in the reaction mixture disappeared (Gulcin et al. 2004).

The antioxidative nature of the methanol extract of *L. pustulata* might depend on its phenolics. Phenolic components are potential antioxidants and can donate hydrogen to free radicals and so stop the chain reaction of lipid oxidation at the initial stage. This ability of phenolic compounds to scavenge radicals comes due to the presence of their phenolic hydroxyl group (Sawa et al. 1999). Flavonoids are a group of natural compounds and are also the most important natural phenolics. In most lichens, phenols are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals (Shanab et al. 2011). Numerous researchers have found a high correlation between the antioxidative activities of lichens and their phenolic content (Behera et al. 2009; Ranković et al. 2012).

In the literature, there is currently no data on the antioxidant activity of *L. pustulata,* but the antioxidant activity of some other lichens has been studied by other researchers. For example, Kosanić et al. (2013) found antioxidant activity in the acetone extracts of *Evernia prunastri* and *Pseudoevernia furfuraceae.* Manojlović et al. (2012) explored the antioxidant properties of *Umbilicaria cylindrica*. Compared with their results, the results of this research suggest that the methanol extract of *L. pustulata* showed relatively powerful antioxidant activity.

Numerous lichens, such as *Parmelia reticulata, Toninia candida, Usnea barbata* have been screened for antimicrobial activity in a search for new antimicrobial agents (Ranković et al. 2012; Goel et al. 2011), but, for the first time, in this study we investigated the antimicrobial activity of the methanol extract of *L. pustulata*. In comparison with the results obtained in experiments with other lichens, we noticed that the methanol extract of *L. pustulata* showed relatively strong antimicrobial activity.

Lichens produce many secondary metabolites called lichen acids, which are unique to lichens and these substances are responsible for the antimicrobial activity of lichens. The possible mechanisms of antimicrobial action of lichen substances include inhibition of protein synthesis (translation), inhibition of nucleic acid synthesis or antimetabolite activity. Protein synthesis inhibitors act at the ribosome inhibiting the synthesis of proteins of the microorganisms to occur, misreading the sequence of amino acids, and thus inhibit the functioning of the cells of microorganisms. Nucleic acid inhibitors act by inhibiting the production of nucleic acids (DNA and RNA), while antimetabolites prevent a cell of microorganisms from carrying out a metabolic reaction, wherefore the cells become unable to function normally (Maciąg-Dorszyńska et al. 2014; Kosanić and Ranković 2015). *L. pustulata* contains gyrophoric acid, umbicilin, pustulan, arabitol, mannitol, sucrose, trehalose, amino acids, and enzymes (Culberson 1969). In this study we examined the methanol extract of the lichen *L. pustulata.* It is necessary to understand that extracts are mixtures of natural compounds, and their antimicrobial activity is not only a result of the different activities of individual components but may be the result of their interactions, which can have different effects on the overall activity of extracts.

In these experiments, the extract examined at the same concentrations showed a slightly stronger antibacterial than antifungal activity. These results could be expected due to the fact that numerous tests proved that bacteria are more sensitive to the antibiotic compared to fungi (Hugo and Russell 1983). The reason for different sensitivities between fungi and bacteria can be found in different permeabilities of the cell wall. The cell wall of the gram-positive bacteria consists of peptidoglycans (murein) and teichoic acids, while the cell wall of gram-negative bacteria consists of lipopolysaccharides and lipopolyproteins

(Heijenoort 2001) whereas, the cell wall of fungi consists of polysaccharides, such as hitchin and glucan (Farkaš 2003).

The importance of lichens as anticancer agents has been shown in recent years, suggesting that lichens can be used as biological agents in the treatment of cancer. In this study, the results clearly demonstrate that the methanol extract of the lichen studied induced significant anticancer effect on the cancer cell lines tested. Until now, only a few researchers have proven that lichen extracts have some anticancer activity. Kosanić et al. (2013) reported a significant anticancer effect for *Evernia prunastri* and *Pseudoevernia furfuraceae*. Manojlović et al. (2010) explored the anticancer properties of *Thamnolia vermicularis*. Triggiani et al. (2009) also found strong anticancer activity for *Xanthoria parietina.*

Conclusion

In conclusion, it can be stated that the extract tested had a certain level of antioxidant, antimicrobial and anticancer activities in vitro. Our results revealed that *L. pustulata* extract was safe to use at all concentrations tested. Further investigation is necessary to find the definite mechanisms of action of the activities tested, as well to find out which of the bioactive compounds have a protective effect.

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References

Alves dos Santos R, Cabral TR, Cabral IR, Antunes LM, Pontes Andrade C, Cerqueira dos Santos Cardoso P, de Oliveira Bahia M, Pessoa C, Martins do Nascimento JL, Rodríguez Burbano R, Takahashi CS (2008) Genotoxic effect of Physalis angulata L. (Solanaceae) extract on human lymphocytes treated in vitro. Biocell 32:195–200

Anandjiwala S, Bagul MS, Parabia M, Rajani M (2008) Evaluation of free radical scavenging activity of an ayurvedic formulation, *Panchvalkala*. Indian J Pharm Sci 70:31–35

Bates ST, Cropsey GW, Caporaso JG, Knight R, Fierer N (2011) Bacterial communities associated with the lichen symbiosis. Appl Environ Microbiol 77:1309–1314

Behera BC, Verma N, Sonone A, Makhija U (2009) Optimization of culture conditions for lichen *Usnea ghattensis* G. Awasthi to increase biomass and antioxidant metabolite production. Food Technol Biotech 47:7–12

Culberson CF (1969) Chemical and botanical guide to lichen products. University of North Carolina Press, Chapel Hill, NC

Dobson F (2000) Lichens an illustrated guide. The Richmond publishing Co. Ltd, England

Dorman HJ, Bachmayer O, Kosar M, Hiltunen R (2004) Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. J Agric Food Chem 52:762–770

Farkaš V (2003) Structure and biosynthesis of fungal cell walls: methodological approaches. Folia Microbiol 48:469–478

Fenech M (2000) The in vitro micronucleus technique. Mutat Res 455:81–95

Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2:1084–1104

Geyikoglu F, Turkez H, Aslan A (2007) The protective roles of some lichen species on colloidal bismuth subcitrate genotoxicity. Toxicol Ind Health 23:487–492

Goel M, Dureja P, Rani A, Uniyal PL, Laatsch H (2011) Isolation, characterization and antifungal activity of major constituents of the himalayan lichen *Parmelia reticulata Tayl.*†. J Agric Food Chem 59:2299–2307

Gordan MH (1990) Food antioxidants. Elsevier, London, New York

Gulcin I, Kurfrevioglu OI, Oktay M, Buyukokuroglu ME (2004) Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica L.*). J Ethnopharmacol 90:205–215

Halama P, van Haluwin C (2004) Antifungal activity of lichen extracts and lichenic acids. Biocontrol 49:95–107

Heijenoort J (2001) Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiology 11:25–36

Hugo WB, Russell AD (1983) Pharmaceutical microbiology, 3rd edn. Blackwell Scientific Publications, Oxford

Kosanić M, Ranković B (2015) Lichen secondary metabolites as potential antibiotic agents. In: Ranković B (ed) Lichen secondary metabolites bioactive properties and pharmaceutical potential. Springer International Publishing, Switzerland, pp 81–104

Kosanić M, Manojlović N, Janković S, Stanojković T, Ranković B (2013) *Evernia prunastri* and *Pseudoevernia furfuraceae* lichens and their major metabolites as antioxidant, antimicrobial and anticancer agents. Food Chem Toxicol 53:112–118

Maciąg-Dorszyńska M, Węgrzyn G, Guzow-Krzemińska B (2014) Antibacterial activity of lichen secondary metabolite usnic acid is primarily caused by inhibition of RNA and DNA synthesis. FEMS Microbiol Lett 353:57–62

Manojlović N, Vasiljević P, Jusković M, Najman S, Janković S, Milenkovic-Andjelković A (2010) HPLC analysis and anticancer potential of extracts from the lichen, *Thamnolia vermicularis var.* Subuliformis. J Med Plants Res 4:817–823

Manojlović NT, Vasiljević PJ, Masković PZ, Jusković M, Bogdanović-Dusanović G (2012) Chemical composition, antioxidant and antimicrobial activities of lichen *Umbilicaria cylindrica* (L.) delise (Umbilicariaceae). Evid Based Complement Alternat Med 2012:1–8

Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG (2005) Determination of the total phenolic, flavonoid and proline contents in burkina fasan honey, as well as their radical scavenging activity. Food Chem 91:571–577

Milošević-Djordjević O, Stošić I, Stanković M, Grujičić D (2013) Comparative study of genotoxicity and antimutagenicity of methanolic extracts from *Teucrium chamaedrys* and *Teucrium montanum* in human lymphocytes using micronucleus assay. Cytotechnology 65:863–869

Mitrović T, Stamenković S, Cvetković V, Tošić S, Stanković M, Radojević I, Stefanović O, Čomić L, Đačić D, Ćurčić M, Marković S (2011) Antioxidant, antimicrobial and antiproliferative activities of five lichen species. Int J Mol Sci 12:5428–5448

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and anticancerity assays. J Immunol Methods 65:55–63

NCCLS (National Commitee for Clinical Laboratory Standards) (1998) Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi: proposed standard M38-P. NCCLS, Wayne, PA

Nishimiki M, Rao NA, Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 46:849–853

Odabasoglu F, Aslan A, Cakir A, Suleyman H, Karagoz Y, Halici M, Bayir Y (2004) Comparison of antioxidant activity and phenolic content of three lichen species. Phytother Res 18:938–941

Ohno M, Abe T (1991) Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). J Immunol Methods 145:199–203

Oyaizu M (1986) Studies on products of browning reaction prepared from glucoseamine. Jpn J Nutr 44:307–314

Paudel B, Bhattarai HD, Lee JS, Hong SG, Shin HW, Yim JH (2008) Antibacterial potential of Antarctic lichens against human pathogenic Gram-positive bacteria. Phytother Res 22:1269–1271

Purvis OW, Coppins BJ, Hawksworth DL, James PW, Moore DM (1992) The lichen flora of Great Britian and Ireland. Natural History Museum. Publications in association with the British Lichen Society, London

Ranković BR, Kosanić MM, Stanojković TP (2011) Antioxidant, antimicrobial and anticancer activity of the lichens *Cladonia furcata, Lecanora atra* and *Lecanora muralis*. BMC Complement Altern Med 20:97

Ranković B, Kosanić M, Stanojković T, Vasiljević P, Manojlović N (2012) biological activities of *toninia candida* and *usnea barbata* together with their norstictic acid and usnic acid constituents. Int J Mol Sci 13:14707–14722

Sarker SD, Nahar L, Kumarasamy Y (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 42:321–324

Sasikumar JM, Mathew GM, Teepica PDD (2010) Comparative studies on antioxidant activity of methanol extract and flavonoid fraction of *Nyctanthes arbortristis* leaves. Elec J Env Agricult Food Chem 9:227–233

Sawa T, Nakao M, Akaike T, Ono K, Maeda H (1999) Alkylperoxyl radical scavenging activity of various flavonoids and other phenolic compounds: implications for the anti-tumor promoter effect of vegetables. J Agric Food Chem 47:397–492

Shanab SMM, Shalaby EA, El-Fayoumy EA (2011) *Enteromorpha compressa* exhibits potent antioxidant activity. J Biomed Biotechnol 2011:726405. doi:10.1155/2011/726405

Sini H, Devi KS (2004) Antioxidant activities of chloroform extract of *Solanum trilobatum*. Pharm Biol 42:462–466

Slinkard K, Slingleton VL (1997) Total phenolic analyses: automation and comparison with manual method. Am J Enol Vitic 28:49–55

Triggiani D, Ceccarelli D, Tiezzi A, Pisani T, Munzi S, Gaggi C, Loppi S (2009) Antiproliferative activity of lichen extracts on murine myeloma cells. Biologia 64:59–62

Turkez H, Dirican E (2012) A modulator against mercury chloride-induced genotoxic damage: *dermatocarpon intestiniforme* (L.). Toxicol Ind Health 2828:58–63

Turkez H, Aydin E, Aslan A (2012a) *Xanthoria elegans* (Link) (lichen) extract counteracts DNA damage and oxidative stress of mitomycin C in human lymphocytes. Cytotechnology 64:679–686

Turkez H, Aydin E, Sisman T, Aslan A (2012b) Role of *Peltigera rufescens* (Weis) Humb. (a lichen) on imazalil-induced genotoxicity: analysis of micronucleus and chromosome aberrations in vitro. Toxicol Ind Health 28:492–498

Zeytinoglu H, Incesu Z, Tuylu BA, Turk AO, Barutca B (2008) Determination of genotoxic, antigenotoxic and anticancer potential of the extract from lichen *Cetraria aculeata* (Schreb.) Fr. in vitro. Phytother Res 22:118–123