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Original paper

In vitro **antigenotoxic activities of lichens** *Cetraria islandica* **and** *Lasallia pustulata*

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Abstract

It has long been known that lichens are popular in folk medicine due to biological activities such as antitumor, antimicrobial and antioxidant. Thus, in this study we aimed to investigate antigenotoxic effects of *C. islandica* and *L. pustulata* methanol extracts on cultured human peripheral blood lymphocytes (PBLs). Genome damage was determined using cytokinesis-block micronucleus (MN) assay. Peripheral blood was collected by venipuncture from healthy donors and the known mutagen mitomycin C (MMC) was used to determine antigenotoxic properties of the tested concentrations of methanol extracts from the lichens (12.5, 25, 50, 100 and 200 µg/ml). The tested extracts dose dependently decreased MMC-induced MN frequencies, but significantly only in the highest concentrations (100 and 200 µg/ml). Both extracts dose dependently decreased nuclear division index (NDI) in MMC-treated PBLs. There were significantly lower NDI values in the treatments with all tested concentrations of both extracts, except two lowest of *L. pustulata* extract, in comparison to the untreated cells ($P<0.05$). Our results suggest that C. *islandica* and *L. pustulata* lichen extracts should be recommended for use in the protection against anticancer drugs and safely in higher concentrations because they are more effective than.

Keywords *Cetraria islandica*, *Lasallia pustulata*, lichens, antigenotoxic activity, human peripheral blood lymphocytes.

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Introduction

Lichens are complex organisms consisting of a symbiotic association of a fungal and an algal partner [1]. More than 20,000 known species of lichens have been identified to inhabit diverse ecosystems ranging from arctic tundra to desert climates [2]. They are ubiquitous on barks, stems, leaves and in soil, but often grow in habitats less favorable for higher plants [3]. Some lichen species have historically been used as food dyes, in the production of alcohol and perfume industry [4]. Moreover, for a long time, these organisms have been used in traditional medicine in the treatments of numerous infectious diseases, such as stomach diseases, diabetes, coughs, pulmonary tuberculosis, wound curing, and dermatological diseases [5, 6]. The use of lichens in medicine is based on the fact that they contain unique and varied biologically active secondary metabolites that may be pharmacologically potent [7].

Lichen's secondary metabolites, called lichen acid, are synthetized mostly by the fungal partner. Although produced within the hyphae, they are deposited as crystals on the surface. Previous studies show that they have very different biological activities such as antibacterial, antiviral, antioxidant, anti-proliferative, cytotoxic, anti-inflammatory and antipyretic activities [8, 9]. The secondary metabolites are soluble in water and can usually be isolated from the lichen crystals using organic solvents [10]. More than 1000 such secondary metabolites have been detected and isolated [11]. Numerous researches determined different activities for lichens, for different extraction solvents used, but many times is found that the methanol extract showed the strongest activity because in methanol dissolves the largest number of lichen secondary metabolites which are responsible for the activity of the lichen [12, 13].

Cetraria islandica (L.) commonly known as Iceland moss is the most commonly utilized lichen and has been included among the drugs listed in 50 pharmacopoeias or dispensatories of the period 1840s in Europe [14], also been screened for various biological activity. In traditional medicine *C. islandica* was used to treat mild inflammation of the oral and pharyngeal mucosa, dyspepsia and loss of appetite and in European folk medicine in cancer treatment [15]. Also, some earlier studies have shown that methanol extract of *C. islandica* collected from Iceland exhibited cancer chemopreventive and cytotoxic activity [16], as well in cultured tissue exhibited activity towards tyrosine inhibition and gram +ve bacteria such as *Bacillus subtilis*, *Propionibacterium acnes* and *Staphylococcus aureus* [17]. In our previous study we showed that only higher tested concentrations of methanol extract obtained from *Cetraria islandica* (50, 100 and 200 μg/ml) induced genotoxic effects in human PBLs of healthy donors, using micronucleus test [18].

Lasallia pustulata is a large, foliose species, which contains the lichen substances gyrophoric acid, arabitol, mannitol and umbilicarin [19]. There is very little literature data on the biological activity of *L. pustulata*, but there is mostly data on its taxonomy. Our previous published results showed that methanol extract of *L. pustulata* exhibits an antioxidant, antimicrobial and anticancer activities *in vitro* [20].

In determining genotoxic effects of physical, chemical and biological agents, the micronucleus test in human peripheral blood lymphocytes (PBLs) is an attractive cytogenetic end point for assessing *in vitro* [21, 22] and *in vivo* exposure [23, 24]. Micronuclei indirectly reflect chromosome damage because they are formed of chromosome/chromatid fragments or whole chromosomes, which are lost during cell division.

The objective of the present study was to investigate the antigenotoxic activities of methanol extracts obtained from lichens *Cetraria islandica* and *Lasallia pustulata* against the mitomycin C-induced genotoxicity in human peripheral blood lymphocytes (PBLs) *in vitro*, using cytokinesis-block micronucleus (MN) assay.

Material and Methods

1. Collection and identification of lichen samples

Lichen samples of *Cetraria islandica* (L.) Ach., and *Lasallia pustulata* (L.) Merat, were collected from Kopaonik, Serbia, in September of 2011, and authenticated by Dr B. Ranković and Dr M. Kosanić at the Faculty of Science, University of Kragujevac. Determination of the investigated lichens was accomplished using standard keys [25, 26]. The voucher specimen of the lichen (Voucher no. 148 and 153) was deposited at the Mycological Laboratory within the Department of Biology and Ecology at the Faculty of Science, University of Kragujevac, Serbia.

2. Preparation of lichen extracts

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

3. Blood samples

Peripheral blood was collected by venipuncture from three healthy donors aged 30-34 years, nonsmokers, who had not been exposed to known mutagens. Blood was kept at room temperature for the shortest time, within 2 hours. The experiments were conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki).

4. Cytokinesis-block micronucleus (MN) assay

Cytokinesis-block MN assay was performed as described by FENECH [27]. Whole heparinised blood (0.5 ml) was added to 5 ml of peripheral blood karyotyping medium with phytohemagglutinin (GIBCO^{TM} PB-Max^{TM}, Invitrogen, California, USA). Cultures were incubated for 72 hours at 37°C. Cytochalasin-B (Sigma, St. Louis, MO, USA) dissolved in DMSO was added to cultures 44 hours after the beginning of incubation, in a concentration of 4.0 μg /ml. The cultures were re-incubated and harvested 28 hours later. First, the cells were collected by

centrifugation and then treated with cold (4°C) hypotonic solution (0.56% KCl). Then, the cells were fixed three times with methanol and glacial acetic acid at a 3:1 ratio. The cells suspensions were dropped onto slides and stained with 2% Giemsa.

The analysis of MN was performed using a light microscope (Nikon E50i) at 400 x magnification following the criteria for MN scoring in binucleated (BN) cells only, described by FENECH [27]. Each slide was scored for the number of MN and the distribution of binucleate (BN) cells with 1 or more MN. The frequency of micronucleated BN cells found in 3000 BN cells per tested concentration of extracts (1000 per donor) was scored as well as the proportion of mononucleated, binucleated, trinucleated and tetranucleated cells per 500 cells. Nuclear division index (NDI) was calculated using the formula NDI = ((1 x M1 + (2 x M2) + (3 x M3) + (4 x M4))/N, where M1 - M4 are the number of cells with 1 to 4 nuclei, and N is the number of the scored cells FENECH [27].

2.4.1. *In vitro* antigenotoxicity assessment

Methanol *C. islandica* and *L. pustulata* extracts in the same five concentrations (12.5, 25, 50, 100 and 200 µg/ml) were added separately to lymphocyte cultures 24 hours after the beginning of incubation. Mitomycin C (MMC, Sigma, St. Louis, MO, USA) was added to cell cultures in the concentration of 0.5 µg/ml concomitantly with lichen extract and alone (positive control).

5. Statistical analysis

SPSS Statistics 20 package was used for statistical analysis, applying the paired *t*-test for comparison mean MN frequencies and NDI values between different treatments. A *P* value of less than 0.05 was considered statistically significant. All data are expressed as mean ± standard deviation (S.D.). The relationship between the tested concentrations of extracts and MN and NDI was determined by Pearson correlation coefficients.

Results and Discussion

The results of *in vitro* combined activities of methanol extracts obtained from *C. islandica* and *L. pustulata* and MMC in cultured human PBLs are shown in Tables 1 and 2, and Figures 1-4.

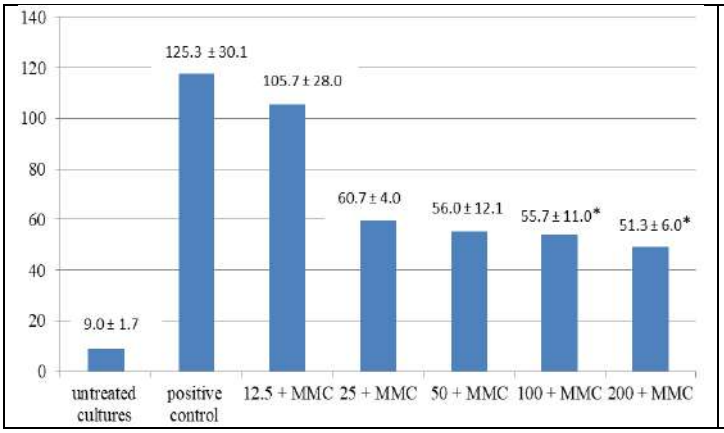

Figure 1. The micronuclei frequencies in cultured peripheral blood lymphocytes after the combined treatments with different concentrations of *Cetraria islandica* extract and mitomycin C (0.5 µg/ml) (*statistical significance).

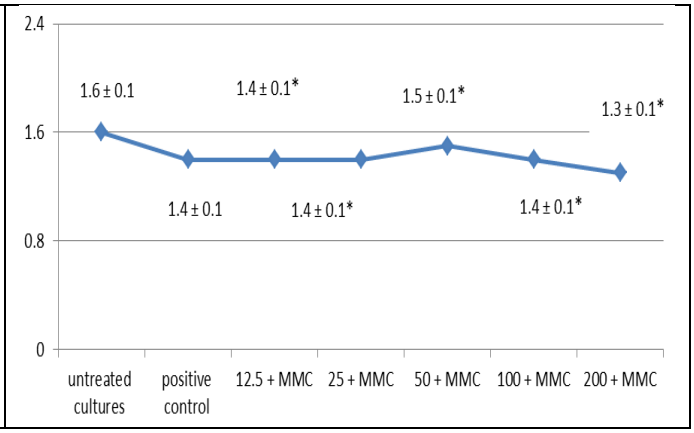

Figure 2. The nuclear division index (NDI) of cultured peripheral blood lymphocytes after the treatments with different concentrations of *Cetraria islandica* extract and mitomycin C (0.5 µg/ml) (*statistical significance).

Table 1. The distribution of micronuclei in cultured peripheral blood lymphocytes (PBLs) of healthy donors after the combined treatments with methanol *Cetraria islandica* extract and mitomycin C (0.5 µg/ml)

Treatments	Extract µg/ml + MMC	No of analyzed BN cells	BN with MN (%)	Distribution of MN			
				1MN (%)	2MN (%)	3MN (%)	4MN (%)
Untreated cells	0	3000	26 (0.9)	25 (0.8)	1 (0.0)	/	/
Positive control	MMC	3000	315 (10.5)	266 (8.9)	37 (1.2)	12 (0.4)	/
C. islandica	12.5+MMC	3000	278 (9.3)	244 (8.1)	30 (1.0)	3 (0.1)	1 (0.0)
	25+MMC	3000	172 (5.7)	162 (5.4)	10 (0.3)	/	/
	50+MMC	3000	156 (5.2)	144 (4.8)	12 (0.4)	/	/
	100+MMC	3000	156 (5.2)	145 (4.8)	11 (0.4)	/	/
	200+MMC	3000	145 (4.8)	136 (4.5)	9 (0.3)	/	/

Figures 1 and 2 show the effects of concomitant treatments with five concentrations of *C. islandica* extract and MMC on MN frequency and NDI. MMC alone significantly increased the MN frequency in PBLs in comparison to untreated PBLs ($P<0.05$). All tested concentrations of this extract decreased the MMC-induced MN frequencies dose dependently (r=-0.591, $P<0.05$) but significantly only at the highest tested concentrations (100 and 200 µg/ml) compared to positive control cells, with probability $P<0.05$ (Figure 1). There was significant dose dependent decrease of NDI (r=-0.525, $P<0.05$) in MMC-induced PBLs at all tested extract concentrations in comparison to the untreated PBLs (Figure 2). The difference in the NDI was observed between the untreated and positive control PBLs (MMC alone), too.

The analysis of MN distribution revealed that all tested concentrations of the extract decreased both the number of MMC-induced BN cells with MN and the number of MMC- induced MN in BN cells, but significantly only in the treatments with 100 and 200 µg/ml concentrations of extract. The greatest number of micronucleated BN cells had 1 MN in both MMC alone (positive control) and in combined treatments with MMC and different concentrations of the extract. The BN cells with 2 MN were less present in all treatments. Cells with 3 and 4 MN were present only in two treatments of PBLs: MMC alone and in combination with the lowest tested concentration of the extract (12.5 µg/ ml).

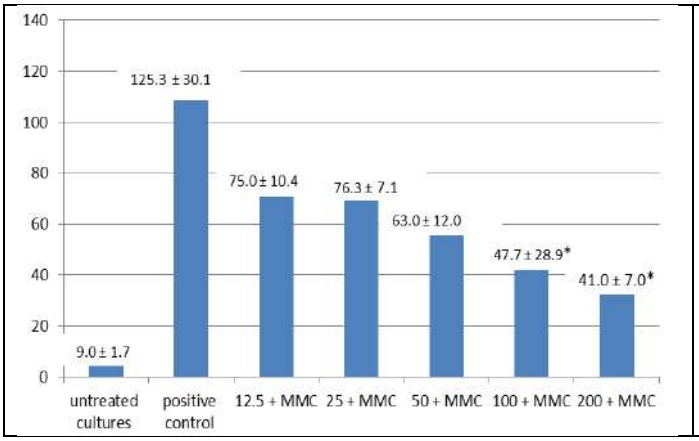

Figure 3. The micronuclei frequencies in peripheral blood lymphocytes after the combined treatments with different concentrations of extract from *Lasallia pustulata* and mitomycin C (0.5 µg/ml) *in vitro* (*statistical significance).

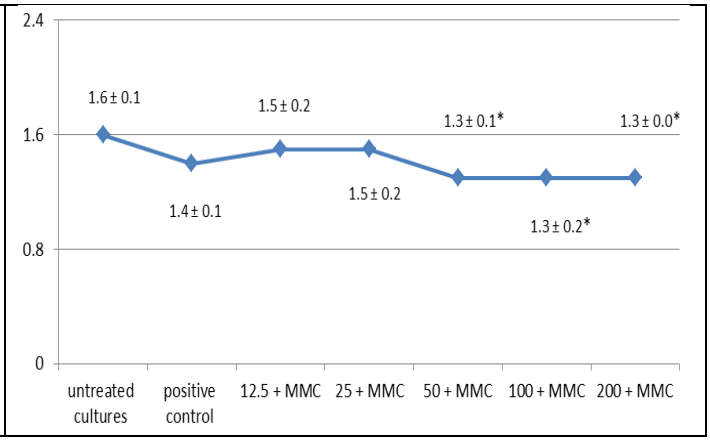

Figure 4. The nuclear division index (NDI) of peripheral blood lymphocytes after combined treatments with different concentrations of extract from *Lasallia pustulata* and mitomycin C (0.5 µg/ml) *in vitro* (*statistical significance).

Figures 3 and 4 represent the results of combined effects of five tested concentrations *L. pustulata* extract and MMC on MN frequency and NDI. It was observed that the extract reduced the MMC-increased MN frequency in PBLs in a dose dependent manner (r=-0,805, $P<0.001$), but a statistically significant decrease was found only at 100 and 200 µg/ml concentrations, with probability $P<0.001$ (Figure 3). A significant dose dependent decrease of NDI values in MMC-treated PBLs (r=-0,659, $P<0.01$) was obtained. Concentrations 50 to 200 µg/ ml of the extract significantly lowered NDI in comparison to the untreated cells ($P<0.05$) (Figure 4).

Table 2. The distribution of micronuclei in peripheral blood lymphocytes (PBLs) of healthy donors after *in vitro* combined treatments with different concentrations of *Lasallia pustulata* extract and mitomycin C (0.5 µg/ml)

Treatments	Extract µg/ml + MMC	No of analyzed BN cells	BN with MN (%)	Distribution of MN			
				1MN (%)	2MN (%)	3MN (%)	4MN (%)
Untreated cells	0	3000	26 (0.9)	25 (0.8)	1 (0.0)	/	/
Positive control	MMC	3000	315 (10.5)	266 (8.9)	37 (1.2)	12 (0.4)	/
L. pustulata	12.5+MMC	3000	197 (6.6)	169 (5.6)	28 (0.9)	/	/
	25+MMC	3000	208 (6.9)	188 (6.3)	19 (0.6)	1 (0.0)	/
	50+MMC	3000	150 (5.0)	118 (3.9)	25 (0.8)	7 (0.2)	/
	100+MMC	3000	128 (4.3)	115 (3.8)	11 (0.4)	2 (0.1)	/
	200+MMC	3000	113 (3.8)	105 (3.5)	6 (0.2)	2 (0.1)	/

The analysis of MN distribution (Table 2) revelead that micronucleated BN cells with 1 MN were predominant, followed by significantly fewer cells with 2 MN, while the cells with 3 MN were found in the cultures treated with 25-200 μg/ml lichen extract and MMC and in positive control (MMC).

The results of the present study clearly showed that all tested concentrations of both *C. islandica* and *L. pustulata* extracts lowered the MMC-increased MN frequency in a dose dependent manner as well that the highest tested concentrations of both lichens (100 and 200 μg /ml) were the most effective. The obtained results showed that both lichens extracts had protective effect in cultured human PBLs.

In accordance with our findings, recent studies have revealed a protective role of different lichen extracts. Until now, E.KOTAN *&* al [33] studied anti-mutagenic activity of *Cetraria islandica* lichen and they reported that methanol extract of this species suppressed the mutagenic effects of aflatoxin B1. To our knowledge, no report is available on the anti-mutagenic effect of *Lasallia pustulata*, but our cytogenetic findings are in agreement with the results reported by the authors who studied *in vitro* effects of taxonomically similar lichens on human PBLs. H. TÜRKEZ *&* al [21] found the anti-mutagenic effect of aqueous *Peltigera rufescens* extract against the mutagenic damage induced by imazalil. In addition, H. TÜRKEZ *&* al [31] reported that *Xanthoria elegans* is a potential antigenotoxicant. Very recently S. CEKER *&* al [34] reported that *Usnea articulata* and *Usnea filipendula* have strong antigenotoxic effect against aflatoxin B1-induced genotoxic damage and that its effect associated with lichen antioxidant nature.

MMC is a natural antitumor, antibiotic and cytotoxic agent, used against different tumors (gastric and esophageal carcinomas, bladder cancer), but in addition to cancer cells it causes high toxicity in the normal tissues during the therapy. It is known that MMC induces chromosomal aberrations during S phase and several mechanisms of its mutagenic and clastogenic effects are explained by its alkylating activity [29]. On the other hand, MMC produces covalent cross-linkages between opposite DNA strands [30] and it was found to have led to the development of oxidative stress [31] and the production of free radicals when metabolized [32].

The present study of treatment by the lichens extracts and MMC concomitantly, revealed a protective effect of the lichens extracts against chromosome damage induced by MMC.

The exact mechanism by which the lichens extract reduced genotoxic effect against MMC was not clear. There are two possible explanations of the obtained results: 1) increase in DNA repair and 2) induction of apoptotic cell death mechanisms in the cells which collect a large quantity of genetic damage.

In order to get the correct answer we investigated the NDI values. Our results indicated dose dependent decrease of NDI values in the PBLs concomitantly treated with MMC and lichen extract, when compared to the untreated PBLs. Our results showed a decrease in MN frequencies, number of MNs per BN cell and number of micronucleated BN cells, too. This may lead to a conclusion that the tested extracts in the cells which collected a large quantity of MMC-increased genetic damage induced the apoptotic mechanisms of cell death. It can be a general mechanism of protective effect for the both extracts obtained from *C. islandica* and *L. pustulata* lichens.

Some authors showed that significant decrease of NDI in cultures of peripheral blood lymphocytes may be due to the fact that cells with greater chromosomal damage die before cell division [35]. C.J. NATH & T. ONG [36] observed that BN cells with greater number of MN could be the result of the loss of major part of genome, which was enough to stop further division of these cells. Such cells are eliminated by apoptosis, which contributes to understanding the decrease of MN frequency [37]. S. PETROVIĆ *&* al [38] showed inverse relationship between MN and apoptosis, and concluded that damaged cells incapable to undergo apoptosis are mostly visible as binucleated cells carrying MN.

Induction of apoptosis is an important mechanism of chemoprevention and different studies suggest that polyphenols which are present in the lichens extracts have the primary role in the induction of apoptosis [39]. In this respect, polyphenols act as protection against genome instability [40].

Conclusion

The results of this study showed that methanol extracts obtained from *C. islandica* and *L. pustulata* lichens had highly protective effect, which we detected as a reduction in genome damage caused by a known mutagen. Our general conclusion is that methanol *C. islandica* and *L. pustulata* extracts should be recommended for use in the protection against anticancer drugs and safely in higher concentrations because they are more effective than.

Acknowledgements

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Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

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