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RESEARCH ARTICLE

The anti-genotoxic effect of some lichenic acids

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Abstract: Four lichen secondary metabolites; diffractaic acid, lecanoric acid, lobaric acid and vulpinic acid were evaluated for the anti-genotoxic effects on human lymphocytes *in vitro* using single cell gel electrophoresis (SCGE) or comet assay. Carbon tetrachloride, a known mutagenic agent was used as the positive control in the comet test system. The results revealed that at all concentrations, the tested compounds mediated a significant decrease in total DNA damage (comet assay) as compared with positive controls ($p > 0.05$). The most effective doses of lichen acids were found to be 100 µgmL^{-1} of diffractic acid, lobaric acid and vulpinic acid, and 50 µgmL^{-1} of lecanoric acid. In addition, it was determined that the most effective among the tested compounds was vulpinic acid.

Keywords: Comet assay, diffractaic acid, lecanoric acid, lichen compounds, lobaric acid, vulpinic acid.

INTRODUCTION

Lichens are symbiotic connections between one or more inhabitant photosynthetic partners (algae or cyanobacteria) and an associated fungus. Countless studies have revealed an extensive range of biological activities of lichen secondary metabolites (Karunaratne *et al*., 2005; Lauinger *et al*., 2013). They have been used for medicinal purposes including anti-tumor, anti-bacterial, anti-fungal, anti-proliferative, anti-inflammatory, anti-mutagenic, cytotoxic and anti-oxidant activities (Foden *et al*., 1975; Müller, 2001; Oksanen, 2006). On the other hand, although many of the metabolites from 60 lichen species have been studied for determining their biological properties, and diffractaic acid, lecanoric acid, lobaric acid and vulpinic acid have been purified and identified, their therapeutic potential has not yet been fully explored.

Karaosmanoglu *et al.* (2015) reported that doses of 200 − 450 µM of vulpinic acid was cytotoxic and apoptotic in HepG2 and F2408 cells. Another study demonstrated that *Vulpicida canadensis* has a high cytotoxic effect in HepG2 and MCF-7, and vulpinic acid was determined as the major component in this lichen (Fernández-Moriano *et al*., 2015). Recent research has shown that vulpinic acid has anti-angiogenic, anti-microbial and anti-proliferative effects (Koparal, 2015). Different researchers have emphasised that lobaric acid possessing anti-oxidant activity is also a tubulin polymerisation inhibitor (Ravaglia *et al*., 2014). Thadhani *et al.* (2012) found that lobaric acid exhibits high anti-microbial activity against *Escherichia coli* bacteria.

Ingólfsdóttir *et al.* (1998) reported that lobaric acid obtained from *Stereoculon alpine* has anti-microbacterial activity and anti-proliferative effects in T-47D, ZR-75-1 and K-562 cells. Şahin *et al.* (2015) found that *Ramalina* species (*R. farinacea, R. fastigiata* and *R. fraxinea*) have anti-oxidant capacity, which was derived from evernic, fumarprotocetraric, lecanoric, sticic and usnic acids. Some researchers determined that evernic, vulpinic, psoromic and (+) - usnic acids have anti-parasitic and anti-fungal effects (Halama & Van Haluwin, 2004; Lauinger *et al*., 2013). In another study it was shown that the diffractic acid isolated from *Usnea longissima* lichen

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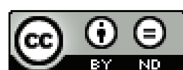
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has anti-hepatotoxic, anti-tumor and insecticidal effects (Karagoz *et al.*, 2014). Also, it was found that diffractaic, usnic, norstictic and psoromic acids were cytotoxic in UACC-62 cell culture (Brandão *et al*., 2013).

Anti-mutagenic properties of diffractaic acid, lecanoric acid, lobaric acid and vulpinic acid have not been reported up to the present. The purpose of this study was to investigate the anti-mutagenic and mutagenic activities of the above acids using the comet assay.

METHODOLOGY

The comet assay method of Singh *et al.* (1988) was modified and used to determine the genotoxic effects of lichen acids.

Slide preparation

One percent normal boiling grade agarose (NMA) was prepared in 50 mL PBS solution and 80 µL of NMA was dropped to each well on the slide. One percent low melting grade agarose (LMA) was also prepared in 50 mL PBS solution. The prepared LMA solution was placed in 5 mL volumes in the balcony tubes and stored at +4 °C.

Blood sampling

Heparinised human blood from 4 healthy non-smoking donors between age of 25 and 30, with no history of X-ray exposure and antibiotic treatment was used. The donors also signed informed consent forms. Lymphocytes were obtained by ficoll density centrifugation method from heparinised whole blood. Histopaque (600 mL) was added onto 1 mL of peripheral blood diluted with PBS. After centrifugation, the supernatant was discarded and 1640-RPMI medium was added onto the pellet. Ten milliliter samples (approximately 10000 lymphocytes) were separated and 20 µL trypan blue solution was added. The number of cells in the haemocytometer was calculated. CCl_4 (in concentrations of 5 µM) and lichen acids were added to the cultures. The experiments were performed on 7 groups as follows:

Culture 1: Control
Culture 2: 5 µM CCl_4
Culture 3: 25 $\mu g mL^{-1}$ lichen acids
Culture 4: 5 µM CCl_4 + 12.5 $\mu g mL^{-1}$ lichen acids
Culture 5: 5 µM CCl_4 + 25 $\mu g mL^{-1}$ lichen acids
Culture 6: 5 µM CCl_4 + 50 $\mu g mL^{-1}$ lichen acids
Culture 7: 5 µM CCl_4 + 100 $\mu g mL^{-1}$ lichen acids

For comet demonstration, the cultures were incubated at 37 ºC for 3 h. The pellets obtained after centrifugation were suspended in 100 µL PBS. Then 100 µL of LMA solution was added to the suspension. Agar was mixed with lymphocytes by pipetting immediately before solidification and 80 µL of this final mixture was spread over previously prepared slides. It was covered with lamella and kept in the refrigerator for 10 min. The coverslip was slowly removed from the slides, removed from the refrigerator and 90 µL LMA was added. The slides were closed again for 25 min and after the slides were dried, the cell and nucleus were lysed. After the lysing phase, the slides were left in alkaline electrophoresis buffer for 30 min to separate the DNA strands. Subsequently, the DNAs were run in the buffer solution at 300 mA, 24 volts for 30 min. All of these operations were done in yellow light. The slides were then neutralised. After the neutralisation process was completed, the comets were counted by staining. Ethidium bromide stain (5 $\mu g mL^{-1}$), a fluorescent dye, was used for staining. For each slide, 80 µL of dye was added, and 100 DNA images were evaluated with a 40-magnification fluorescent microscope to asses DNA damage. DNA damage was examined at a scale of 0 to 4 according to the formation of the tail (Collins, 2004). The scale used was as follows: 0, no tail; 1, comet tail < half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus; 4, comet > twice the width of the nucleus. The visual score for each class was calculated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score characterising the degree of DNA damage in the entire study groups was the sum of the scores in the five comet classes. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU), as reported by Collins (2004).

Statistical analysis

The statistical analysis of comet results was performed using the Mann–Whitney U-test and one-way ANOVA. A level of probability of $p < 0.05$ was taken as indicating statistical significance. All experiments were performed in three replicates and data was compared for reproducibility. For these procedures, SPSS v15.0 version for Windows was used. The results are expressed as the mean ± SE.

RESULTS AND DISCUSSION

Mutagenic and anti-mutagenic effects of diffractaic, lecanoric, lobaric and vulpinic acids were examined by using comet assay test systems and a single dose of CCl_4,

Table 1: The effects of lichenic acids and CCl_4 on comet assay

	DNA damage level			
	Diffractaic acid	Lecanoric acid	Lobaric acid	Vulpinic acid
Control	6.72 ± 0.64[a]	6.72 ± 0.64[a]	6.72 ± 0.64[a]	6.72 ± 0.64[a]
CCl_4 (5 µM)	116.35 ± 1.28[e]	116.35 ± 1.28[e]	116.35 ± 1.28[e]	116.35 ± 1.28[e]
Lichen acid (25 µgmL^{-1})	12.20 ± 0.45[ab]	19.48 ± 0.36[ab]	17.26 ± 0.86[ab]	11.26 ± 0.96[a]
Lichen acid (12.5 µgmL^{-1}) + CCl_4 (5 µM)	97.08 ± 0.17[de]	102.26 ± 0.90[e]	91.42 ± 1.58[de]	73.38 ± 1.26[cd]
Lichen acid (25 µgmL^{-1}) + CCl_4 (5 µM)	93.36 ± 1.23[d]	94.60 ± 0.42[d]	82.12 ± 0.74[d]	61.78 ± 0.78[c]
Lichen acid (50 µgmL^{-1}) + CCl_4 (5 µM)	80.10 ± 2.26[c]	78.46 ± 1.30[cd]	79.30 ± 1.62[d]	39.12 ± 1.92[b]
Lichen acid (100 µgmL^{-1}) + CCl_4 (5 µM)	76.03 ± 1.52[cd]	82.40 ± 0.15[d]	74.12 ± 0.84[cd]	26.58 ± 0.32[ab]

Carbon tetrachloride (CCL_4) was used as positive control for human blood cells. Values of DNA damage level (a, b, c, d, e) are significantly different compared to negative control ($p < 0.05$)

which is a known mutagenic agent. A single dose of CCl_4 caused a significant increase in DNA strand breaks that lead to DNA migration. All tested concentrations of metabolites were not found to be mutagenic or toxic to human lymphocyte cells. According to the findings of this study, it was evident that exposure to the four metabolites significantly decreased the mutagenic activities.

As evident from Table 1, the most effective doses of lichen acids were found to be diffractic, lobaric, vulpinic acid (100 µgmL^{-1}) and lecanoric acid (50 µgmL^{-1}). In addition, it was determined that the most effective lichen acid is vulpinic acid when compared with the negative control. However, the DNA damage decreased progressively with increased diffractic acid, lobaric acid and vulpinic acid concentration. The 50 µgmL^{-1} concentration of lecanoric acid was the most effective dose against DNA damage as mentioned above. The effects of diffractic acid on the frequencies of total chromosomal aberration and the micronucleus have been reported earlier (Demir *et al.*, 2015). In addition, researchers have reported that diffractic acid isolated from *Usnea longissima* lichen has anti-hepatotoxic, anti-tumor and cytotoxic effects (Emsen *et al.*, 2012; Brandão *et al.*, 2013; Karagoz *et al.*, 2014; 2015). It has also been found that diffractic acid from *Usnea longissima* lichen has antiulcerogenic properties obtained against indocyanine-induced gastric lesions formed in rats (Bayir *et al.*, 2006). In addition, some investigators have found changes in anti-proliferative activity and reactive oxygen species (ROS) levels in MCF-7, HeLa, and HCT-116 cell lines exposed to different concentrations ranging from 2.5 − 100 µM diffractic acid and determined that they mediate concentration dependent cytotoxicity (Brisdelli *et al.*, 2013).

The studies with vulpinic acid revealed cytotoxic and apoptotic activities in HepG2, MCF-7 and F2408 cells, in addition to their anti-inflammatory, anti-angiogenic, anti-microbial and anti-proliferative activities (Foden *et al.*, 1975; Fernández-Moriano *et al.*, 2015; Karaosmanoğlu *et al.*, 2015; Koparal, 2015).

The present study describes for the first time the anti-mutagenic effects of lecanoric, lobaric and vulpinic acids. Recently, several studies have demonstrated that lobaric acid possesses anti-microbial activity, anti-proliferative effects and anti-oxidant activity (Ingólfsdóttir *et al.*, 1998; Haraldsdóttir *et al.*, 2004; Thadhani *et al.*, 2011).

CCl_4, a known mutagenic agent was used as the positive control in the comet test system. A single dose

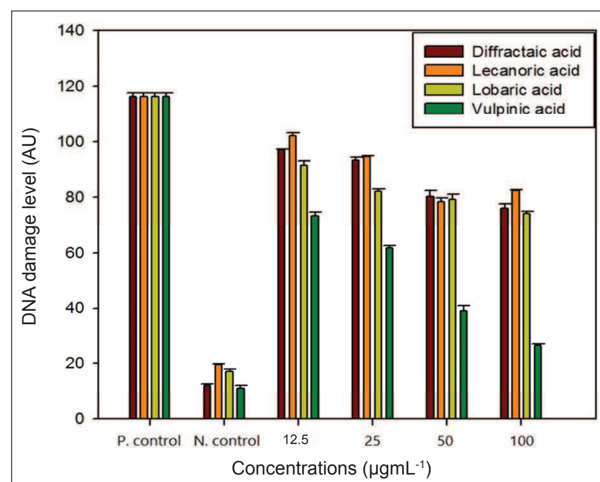

Figure 1: The effects of lichenic acids and CCl_4 on comet assay

of CCl_4 caused a significant increase in DNA strand breaks that lead to DNA migration. On the other hand, the comet tail lengths were reduced by different doses of lichen acid. The most effective doses of lichen acids were found as 100 μgmL^{-1} diffractic acid, lobaric acid, and vulpinic acid and 50 μgmL^{-1} lecanoric acid as seen in Table 1 and Figure 1. In addition, it was determined that the most effective lichen acid is vulpinic acid when compared with the negative control.

CONCLUSION

Based on the results obtained in the present study and those obtained in the investigations described above, the anti-mutagenic effect of the four lichen acids (diffractaic acid, lecanoric acid, lobaric acid and vulpinic acid) could be related to their antioxidants concentrations. This view is supported by the data obtained in recent investigations carried out in the laboratory. These studies indicate that the above mentioned four lichen acids could increase the levels of glutathione (GSH) and superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes and decrease the level of malondialdehyde (MDA) in human lymphocytes *in vitro.*

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