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Antiproliferative activity of lichen extracts on murine myeloma cells

Doriana Triggiani[1], Donatella Ceccarelli[1], Antonio Tiezzi[1], Tommaso Pisani[2], Silvana Munzi[2], Carlo Gaggi[2] & Stefano Loppi[2]*

[1] *Department of Environmental Science, University of Tuscia, Largo dell'Universitŕ snc, I-01100 Viterbo, Italy*
[2] *Department of Environmental Science, University of Siena, Via P.A. Mattioli 4, I-53100 Siena, Italy; e-mail: loppi@unisi.it*

Abstract: In the present study we report some preliminary results concerning the evaluation of antiproliferative activity on murine myeloma cells (P3X63-Ag8.653) of crude extracts of two common lichen species, *Evernia prunastri* and *Xanthoria parietina*.
The results were evaluated by means of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, which is commonly used to assess the activity of living cells through mitochondrial dehydrogenases. They indicated that extracts of *E. prunastri* had no effect, while those of *X. parietina* significantly affected murine myeloma cell proliferation, with a reduction down to 75% for methanolic extracts. This opens perspectives for deeper investigations extended also to other mammalian cell lines.

Key words: *Evernia prunastri*; *Xanthoria parietina*; antitumoural effect

Introduction

Secondary metabolites produced by lichens, so-called lichen compounds, have a wide range of effects such as antibiotic, antimycobacterial, antiviral, antiinflammatory, analgesic, antipyretic, antiproliferative and cytotoxic (Müller 2001). The most investigated lichen secondary metabolite is surely usnic acid, which showed tumour-inhibitory activity for Lewis lung carcinoma (Kupchan & Kopperman 1975), and inhibiting properties on the proliferation of Ishikawa K-562 and HEC-50 cells (Cardarelli et al. 1997), and is well known for its antibiotic properties (Cocchietto et al. 2002). Some lichen compounds have been shown to exhibit lipoxigenase inhibitory activity (Bucar et al. 2004; Ingólfsdóttir et al. 1994) and to have anti-proliferative effects against malignant cell lines (Ingólfsdóttir et al. 2002; Kristmundsdóttir et al. 2002). In addition, other studies have shown that lichen metabolites exhibit in vitro inhibitory effects against peptide leukotriene formation (Gissurarson et al. 1997) and against leukotriene-B_4 biosynthesis (Kumar & Müller 1999).

The search for new potential anti-cancer compounds has involved several lichen metabolites (Ding et al. 1994; Yamamoto et al. 1995). However, although the activities of bioactive compounds of lichens have been recognized, from the pharmaceutical point of view their therapeutic potential is still largely unexploited (Müller 2001). In this view, recently we have started studies for enlarging knowledge on possible pharmacological properties of lichen compounds. In the present study we report some preliminary results concerning the evaluation of antiproliferative activity on murine myeloma cells of crude extracts of two common lichen species, *Evernia prunastri* (L.) Ach. and *Xanthoria parietina* (L.) Th.Fr.

Material and methods

Lichen collection

Thalli of *E. prunastri* and *X. parietina* were collected from a remote area of Tuscany, central Italy, transported to the laboratory and air-dried at room temperature.

Lichen extracts

Three solvents, i.e. water, methanol and ethanol, were used for the extraction of bioactive compounds from *E. prunastri* and *X. parietina* thalli.

For preparation of water extracts, both species were weighed and homogenized in distilled water at a constant ratio of 10:1 mL/g. After 60 min of extraction in the dark at room temperature, samples were centrifuged at 2 500 rpm for 5 min and the supernatants concentrated three times in an evaporator.

For preparation of methanol and ethanol extracts, both species were weighed and homogenized in methanol and ethanol, respectively, at a constant ratio of 10:1 mL/g. After 60 min of extraction in the dark, samples were centrifuged at 2500 rpm for 5 min at room temperature. Supernatants were dried in an evaporator and the resulting residues resuspended in 3 mL of deionized water.

Samples were then centrifuged at 10000 rpm for 3 minutes, filtered through a 0.22 μm cellulose syringe filter and stored at -20 °C until testing.

* Corresponding author

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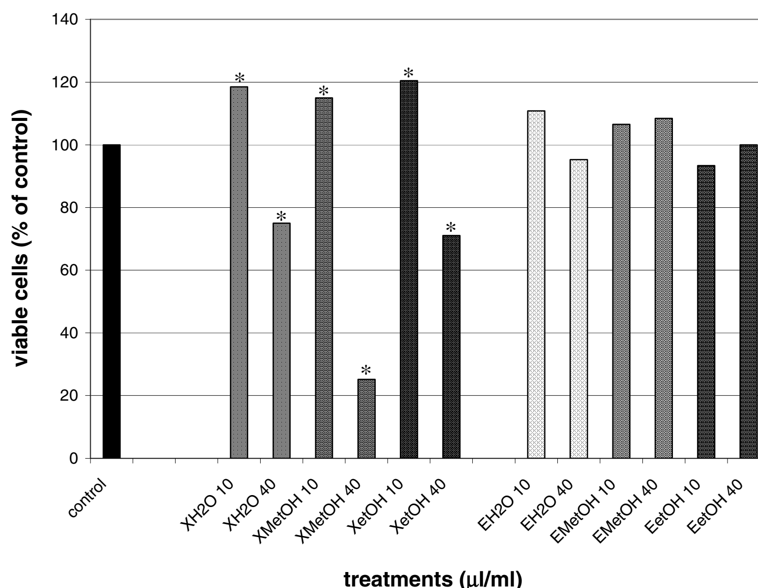

Fig. 1. Control, untreated cells; XH2O, water extracts from *X. parietina*; XmetOH, methanolic extracts from *X. parietina*; XetOH, ethanolic extracts from *X. parietina*; EH2O, water extracts from *E. prunastri*; EMetOH, methanolic extracts from *E. prunastri*; EetOH, ethanolic extracts from *E. prunastri*. Each extract was administered in aliquots of, respectively, 10 and 40 μL. * = Treatment statistically different from the control (one-way ANOVA, Dunnett test, $P < 0.05$).

Murine myeloma cell culture
Murine myeloma P3X63-Ag8.653 cell line is derived from the Balb/c strain of mice. This myeloma cell line was propagated and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics such as penicillin and streptomycin, and glutamine 2 mM (complete DMEM) in a 37°C humidified incubator in an atmosphere of 5% CO_2 in the air. 10000 cells/well were seeded into a 24-well culture plate containing 1 mL of complete DMEM per well and incubated for 20 h in the absence (control) or presence of the extracts. The three lichen extracts were added, respectively, at concentrations of 10 and 40 μL/mL.

Cell proliferation assay
The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] system is a commonly used test to assess the activity of living cells through mitochondrial dehydrogenases (Mosmann 1983). After 24 h of treatment with water, methanol and ethanol extracts, 100 μL from each of the 24 wells were drowned and plated in 96 wells/plates. To each well, 10 μL of MTT solution was added and the cells were further incubated for 4 h at 37°C. The blue MTT-formazan product was solubilised by the addition of 100 μL DMSO and the absorbance was measured at 595 nm using a Victor III spectrophotometer. The anti-proliferative activity was expressed as % of viable cells vs. untreated cells (control). Results for each experimental condition are the mean of 3 replicate wells.

Statistical analysis
Significance of differences between control and treated cells was checked by one-way ANOVA, using the Dunnett test for post-hoc comparisons. Prior to the analysis, data not matching a normal distribution (Shapiro-Wilk W test at the 95% confidence interval) were transformed using the Box-Cox method. Homogeneity of variances was checked with the Levene test.

Results

The results of treatments of murine myeloma cells with lichen extracts, evaluated by means of the MTT test, are shown in Fig. 1. Extracts of *X. parietina* always significantly affected murine myeloma cell proliferation, whereas extracts of *E. prunastri* had no substantial effect. Ten μL of *X. parietina* extract, irrespective whether it was water, methanol or ethanol, induced a 15–20% increase in cell proliferation compared to control (untreated cells), whereas 40 μL of extract, again irrespective whether it was water, ethanol or methanol, induced a consistent reduction in cell proliferation. In particular, around 70% of leaving cells were detected after treatment with both water and ethanolic extracts, and such reduction was especially remarkable for the methanolic extract, which reduced cell proliferation down to ca. 25% compared with the control.

Discussion

Evernia prunastri is extremely rich in lichen compounds, which can constitute up to 20% of its weight (Barnes et al. 1992), and *X. parietina* contains well established antioxidants such as peroxidases and superoxide desmutase (SOD), as well as glutathione (GSH) and anthraquinones (Silberstein et al. 1996). Both species have been investigated to evaluate their possible bioactive use in pharmaceutical and biomedical fields and in particular as anticancer agents. Chemically induced carcinogenesis has two successive stages: initiation, when normal cells change to dormant tumour cells, and promotion, when dormant cells change to tumour cells. An assay for inhibition of tumour promotion measures the inhibition of Epstein-Barr virus

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(EBV) activation: promoters activate EBV in latently-infected human B-lymphoblastoid cells (Raji cells) and EBV produces early antigens, which can be detected by fluorescence-antibody analysis. The inhibitory effect of acetone extracts of *E. prunastri* on EBV was almost twice that of *X. parietina* (Yamamoto et al. 1995).

Tumour promoters bind receptors in cell membranes and cause pharmacological reactions such as the generation of superoxide anions, which act as protection against microorganism attacks, but their excess can damage tissues and cause tumours. The excess of superoxide anions is normally neutralized by SOD activity, which is in turn related to the inhibition of tumour promotion. The SOD activity of *X. parietina* is higher than that of *E. prunastri* by about 30% (Yamamoto et al. 1993). In fact, *X. parietina* is a species tolerant to air pollution and exposure to air pollutants results in an increase in the activity, while *E. prunastri* is a species sensitive to air pollution and the peroxidases and SOD activity is decreased by exposure to air pollutants (Deltoro et al. 1999). Furthermore, the response to oxidative stress (e.g. high light influx, SO_2 pollution, paraquat treatment) in lichens can follow several routes, such as production of crystalline deposits of calcium oxalate (Modenesi 1993; Modenesi et al. 1998), increased synthesis of ascorbic acid (Caviglia & Modenesi 1999), and usnic acid (Caviglia et al. 2001). In *X. parietina*, the orange coloured cortical anthraquinone compound parietin synthesised by the mycobiont protects the lichen photobiont against oxidation by excessive solar radiation (Gauslaa & McEvoy 2005). However, parietin did not show any inhibitory activity on platelet-type 12(S)-lypoxygenase, which is involved in malignant cell growth (Bucar et al. 2004).

To evaluate if different solvents had selective extraction of possible bioactive chemical compounds in lichens, we used three solvents with different chemico-physical properties, which are routinely used for analogous studies with higher plants (Basle et al. 2005; Lapornik et al. 2005; Ozturk et al. 2007); as a consequence, the use of water, methanol, and ethanol allowed us to carry out chemical extraction of both polar and non polar lichen compounds.

When the extracts were administered to murine myeloma cells, different behaviours were detected by MTT assays. *Evernia prunastri* extracts did not significantly affect the process of cell proliferation, enhancing or inhibiting cell proliferation, suggesting that further attention should not be addressed to additional evaluation on myeloma murine cells. On the contrary, *X. parietina* extracts affected murine myeloma cells in a dose-dependent way, since different behaviours were detected following treatment with 10 and 40 μL. Low extract volumes are in fact able to enhance cell proliferation, whereas higher extract volumes induce the inhibition of cell proliferation. In the case of methanolic extract, the inhibition activity is very consistent compared with the other two solvents assayed, probably because of the chemical nature of bioactive compounds: this opens perspectives for deeper investigations extended also to other mammalian cell lines. It is reasonable to suggest that the high content of antioxidants in *X. parietina* extracts is responsible for the inhibition of myeloma murine cells proliferation. Presently, chemical investigations are running in our lab to investigate the molecular nature of bioactive compounds of *X. parietina*.

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