Cytotoxic activity of supercritical CO_2 extract of old man's beard in L929 fibrosarcoma cell line

ANA ŽUGIĆ[1,*], ALEKSANDRA ISAKOVIĆ[2], IVICA JEREMIĆ[2,3], SNEŽANA SAVIĆ[4], AND VANJA TADIĆ[1]

[1] *Institute for Medicinal Plants Research "Dr. Josif Pančić", Tadeuša Košćuška 1, Belgrade, Serbia*
[2] *University of Belgrade, Institute of Biochemistry, School of Medicine, Pasterova 2, Belgrade, Serbia*
[3] *University of Belgrade, Institute of Rheumatology, Resavska 69, Belgrade, Serbia*
[4] *University of Belgrade, Faculty of Pharmacy, Department of Pharmaceutical Technology and Cosmetology, Belgrade, Serbia*
[*] *Corresponding author: azugic@mocbilja.rs*

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Old man's beard (*Usnea barbata*) is one of the most investigated lichens which biological properties (such as antimicrobial, anti-inflammatory and cytotoxic activities) have been proven in several scientific studies. Stated properties of old man's beard have been attributed mainly to its major secondary metabolite - a dibenzofuran derivative, usnic acid. The aim of the current study was to examine cytotoxic activity of the quantified supercritical CO_2 extract and pure usnic acid in parallel against L929 fibrosarcoma cells. Our results indicated the tested extract to be more toxic towards the investigated cell line compared to usnic acid *per se*, despite the high amount of this compound (81.41% (w/w)) revealed in the extract using HPLC. Such result may be connected to the unidentified compounds present in the supercritical CO_2 extract that could be responsible for apoptosis and oxidative stress in the exerted cytotoxicity of the investigated extract, but not usnic acid. Our study supports further investigations of supercritical CO_2 extract of *U. barbata* as a prospective therapeutic agent with potential relevance in the treatment of sarcoma.

Key words: *Usnea barbata; usnic acid; supercritical CO_2 extract; L929 cells; fibrosarcoma; cytotoxicity*

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1. INTRODUCTION

Old man's beard (*Usnea barbata* (L.) Weber Ex F. H. Wigg., Parmeliaceae) is one of the most investigated lichen species, growing on trees and branches of different woody and conifer species native to Asia, Europe and North America. It has been used as folk remedy for bronchitis, pleuritis, and other respiratory diseases, different urinary infections and wound healing (Hager, 1979; Ingólfsdóttir, 2002; Madamombe and Afolayan, 2003). Also, old man's beard has been recognized by Commission E according to which it is recommended in the treatment of mild inflammation of oral and pharyngeal mucosa in the form of lozenge (PDR, 2007). Additionally, this lichen is also classified in the Homeopathic Pharmacopoeia of the United States for usage in headaches and sunstrokes (Madamombe and Afolayan, 2003). Recent research has shown that *U. barbata* exhibits a wide range of biological properties. Among them, the best-studied is antimicrobial activity, which has been documented in several scientific studies (Madamombe and Afolayan, 2003; Ranković et al., 2012; Weckesser et al., 2007; Žugić et al., 2015).

Also, in a study of Engel et al. (2007), anti-inflammatory activity of *U. barbata*, mediated by the ability to inhibit the production of prostaglandin and cyclooxygenase-2 (COX-2), was demonstrated. Ranković et al. (2012) confirmed antioxidant and also cytotoxic activity of this lichen against FemX human melanoma cells and LS174 colon cancer cells, further shown to be related to the ability to induce apoptosis in the examined tumor cells. To this end, apoptosis as a mechanism of cell death was also proposed in our recent research for B16 mouse melanoma cells treated with commercially available supercritical extract of *U. barbata*, while in C6 glioma cells treated with the same extract autophagy seemed to play a crucial role in the exerted cytotoxicity (Žugić et al., 2016). Bearing this in mind, the aim of the current study was to further investigate cytotoxic activity of this quantified extract against L929 fibrosarcoma cell line. Taking into account that usnic acid is usually held responsible for the biological activities of *U. barbata* including its well-established toxic effects in a variety of cancer cells (Bačkorová et al., 2011; 2012; Bessadottir et al., 2012; Brisdelli et al., 2013; Chen et al., 2014) it was of interest to perform stated experiments using pure usnic acid, in parallel. This was especially interesting considering that in our previous research pure substance (usnic acid) was shown to exhibit weaker cytotoxic activity towards B16 mouse melanoma and C6 glioma cells compared to the supercritical extract of *U. barbata* used in this investigation as well, although it was present

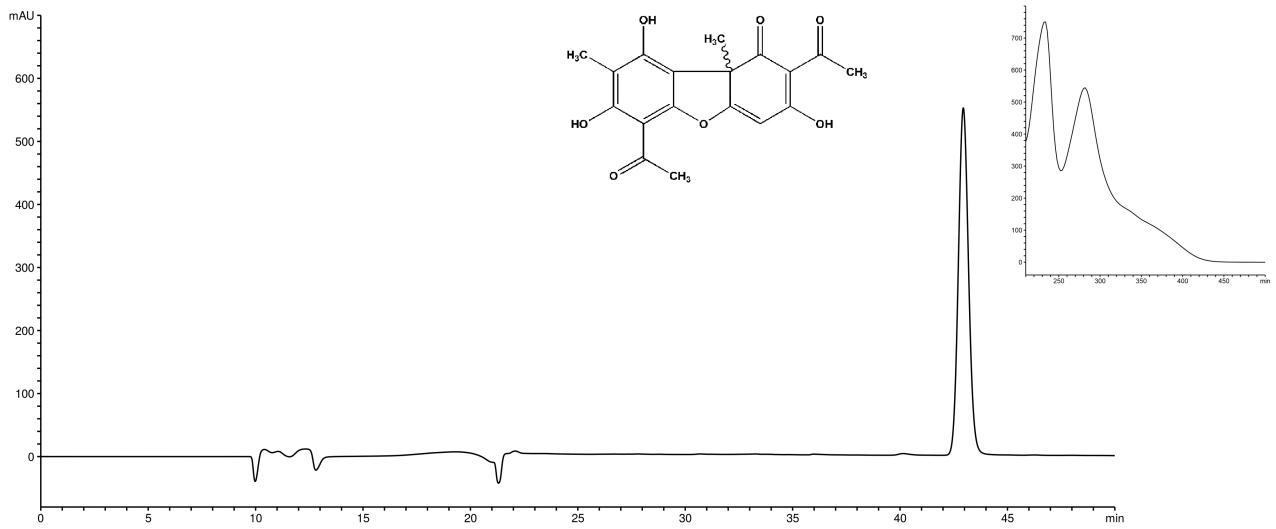

Fig. 1. HPLC-DAD chromatogram of the supercritical CO_2 extract of *U. barbata* (λ=282 nm); chemical structure and UV spectra of usnic acid.

in the stated extract in the high amount. Such discrepancy was attributed to the production of reactive oxygen species (ROS) induced by the extract, but not pure usnic acid (Žugić et al., 2016). In line with the aforementioned, in the first part of the study, chemical characterization of the supercritical extract of *U. barbata* included identification and quantification of usnic acid as a known active/analytical marker of this lichen, which was achieved by means of HPLC. In the second part of the study, cytotoxic activity of the extract and usnic acid was evaluated in the fibrosarcoma cell line L929. Observed cytotoxic effect of the stated samples was additionally assessed for apoptosis and autophagy through analysis of number of cells in different phases of cell cycle and formation of acidic cytoplasmic vesicles, respectively. Also, taking that oxidative stress may contribute to the cytotoxicity of substances, measurement of reactive oxygen species was performed, as well.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Analytical grade reagents methanol, ethanol, H_3PO_4, trypsin, EDTA, phosphate-buffered saline, acetate buffer, para-nitrophenyl phosphate (PNPP), Triton-X, NaOH, propidium iodide, acridine orange and dihydrorhodamine-DHR123 were purchased from Sigma Aldrich, Germany. Acetonitrile (HPLC grade) was purchased from Merck, Germany. Water (HPLC grade) was produced from double distilled water using Simplicity® UV Water Purification System (Millipore, France). Reference HPLC standard usnic acid (purity > 98%), used for both chemical and analysis of cytotoxic effects, was purchased from Santa Cruz Biotechnology, USA. Extract of *U. barbata* (SCE) was purchased from Flavex, Germany and according to manufacturers' claims it was obtained by the method of supercritical CO_2 extraction [drug:extract ratio (DER) 62 -100:5]. The extract was quantified based on the determined amount of usnic acid (as described later on).

2.2. Usnic acid assay

Usnic acid was assayed using Hewlett Packard HPLC model 1200; column Zorbax Eclipse XDB-C18 600 Bar (4.6 × 100 mm, 1.8 µm). The mobile phase A consisted of 99% H_2O and 1% H_3PO_4, while B was acetonitrile. Flow rate was 0.1 mL/min, and elution was as follows: 11-55 %B, 0–5 min; 55-80 % B, 5–10 min; 80 %B, 10–12 min; 80-100 %B, 12–20 min; 100 %B, 20-35 min, 100-11 %B, 35-40 min, 11 %B, 40-55 min. Samples (in triplicate) were prepared in the following procedure: 5.0 mg of the extract was dissolved in 50 ml methanol then filtered through 0.45 µm PTFE syringe filters into glass HPLC vials and analyzed as described above.

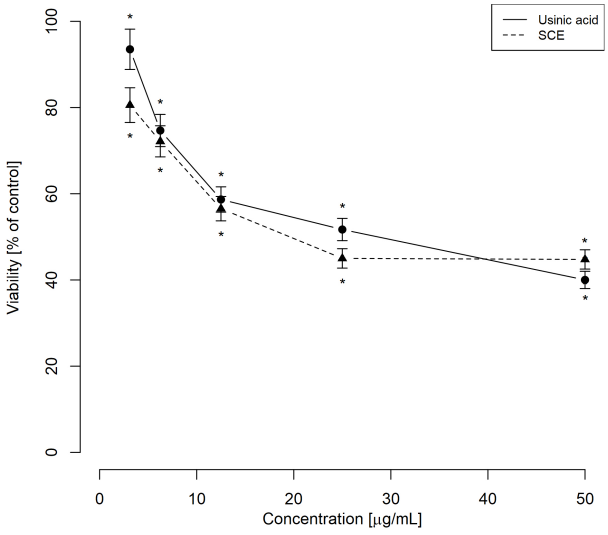

Fig. 2. Antitumor dose-dependent action of usnic acid (UA) and SCE on L929 cell line viability, where UA and SCE were used in double diluted concentration gradient (50-3.125 µg/mL). *P<0.05 refers to control - untreated cells.

2.3. Evaluation of the cytotoxic activity

2.3.1. Cell cultures

The mouse fibrosarcoma cell line L929 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2, in a HEPES buffered RPMI 1640 cell culture medium supplemented with fetal calf serum (5%), L-glutamine, 2-mercaptoethanol, pyruvate, and antibiotics (all

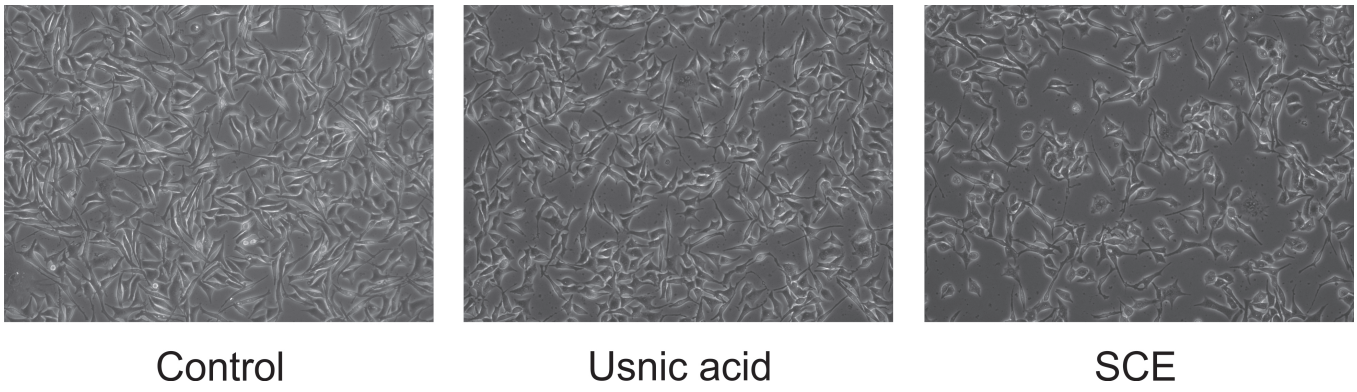

Fig. 3. Morphology of L929 cells treated with IC_{50} concentrations of usnic acid and SCE (25 µg/mL) investigated with bright-field microscopy (magnification 200×).

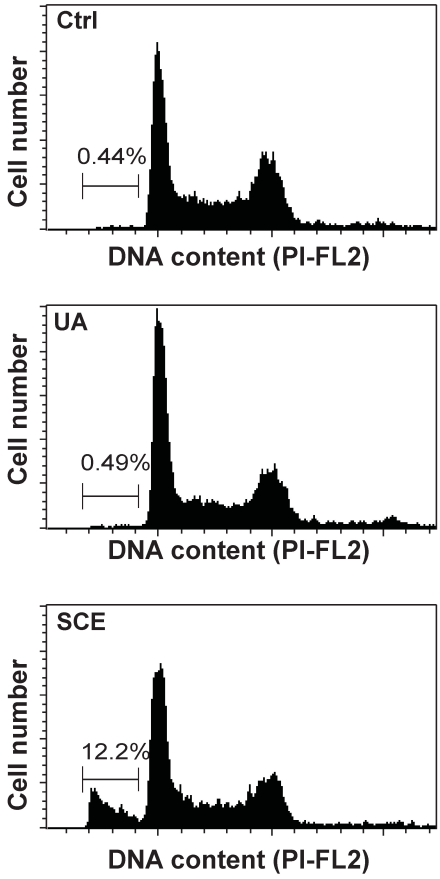

Fig. 4. The effects of usnic acid (UA) and SCE extract on cell cycle progression of L929 cells. Numbers on histograms in the percentage of cells in subGo phase of cell cycle under the treatment with UA (25 µg/mL) and SCE (25 µg/mL).

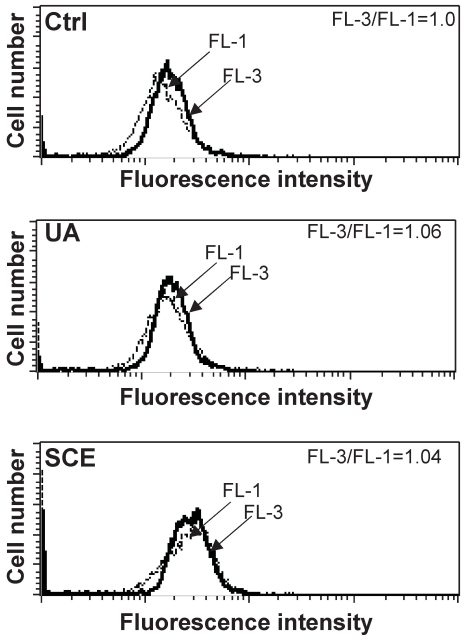

Fig. 5. The effects of usnic acid (UA) and SCE extract on the induction of autophagy determined by flow cytometry.

from Sigma, St. Louis, MO). The cells were prepared for experiments using the conventional trypsinization procedure with trypsin/EDTA and incubated in 6-well plates for the flow cytometric analysis (3 × 105 cells/well) or in 96-well flat-bottom plates (2 × 104 cells/well) for the acid phosphatase viability assay.

2.3.2. Determination of cell viability

Cell viability was measured by using acid phosphatase assay exactly as previously described (Yang et al., 1996). Shortly, after 24-h treatment with old man's beard extract/usnic acid, adherent cells were washed twice with phosphate-buffered saline (PBS), and 100 µL of reaction mixture (0.1 M acetate buffer pH 5.5, containing para-nitrophenyl phosphate (PNPP) and 0.1% Triton-X) was added to each well. After 90 minutes, the reaction was stopped by adding 50 µL of 0.1M NaOH. The absorbance of the developed yellow color, which was directly proportional to the viability of the cells, was measured by an automated microplate reader at 405 nm. The results were presented as percent of the control value (untreated cells), which was arbitrarily set to 100%.

2.3.3. Cell cycle analysis

Cell cycle analysis was performed as previously described (Mijatovic et al., 2005) by measuring DNA content in ethanol fixed cells. The amount of DNA was measured by propidium iodide fluorescence and analyzed by Cell Quest Pro software.

2.3.4. Intracellular acidification measurement by acridine orange staining

The appearance of acidic autophagic vesicles was detected by flow cytometry, as previously described by Paglin et al. (2001). After the treatment with the investigated samples, cells were trypsinized, washed and incubated for 15 minutes at 37 °C with 1 µM acridine orange. Acridine orange stained nuclei are fluorescent green, while autophagic lysosomes are fluorescent orange/red (acidic pH). The increase in red vs. green (FL3/FL1) fluorescence ratio, reflecting autophagy, was determined by flow cytometry and Cell Quest Pro software.

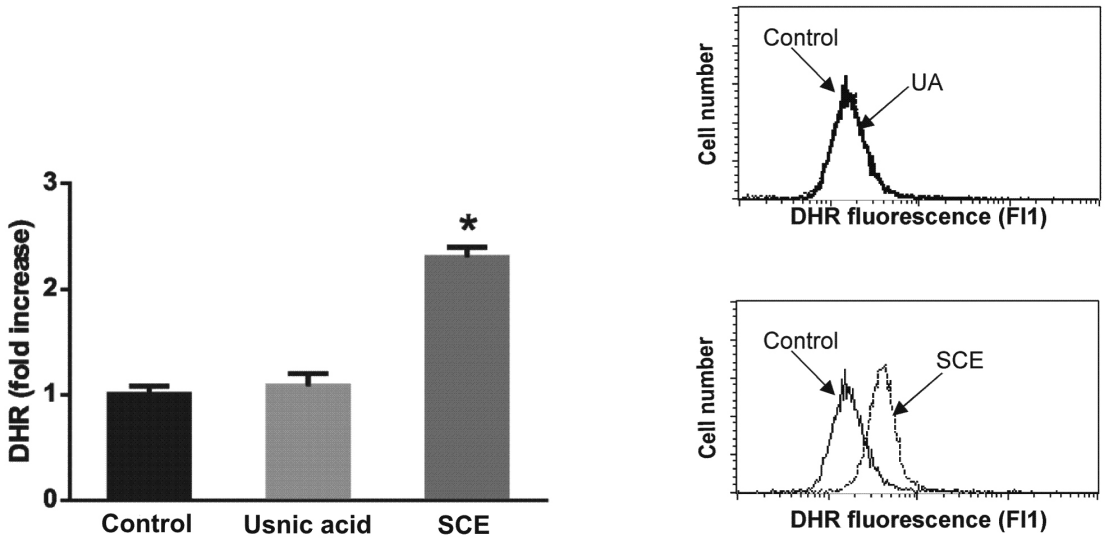

Fig. 6. The effects of usnic acid (UA) and SCE extract on the ROS production: relative increase in DHR fluorescence compared to control as untreated cells (left); representative histograms of mean fluorescence intensity increase under the 24h treatment with UA and SCE (right).

2.3.5. Measurement of reactive oxygen species-ROS

Redox sensitive dye dihydrorhodamine-DHR123 was added simultaneously with treatment to cell culture media. After incubation, cells were detached in dark, washed with PBS and intensity of emitted green fluorescence was measured by FACSCalibur flow cytometer (BD) and analyzed with Cell Quest Pro software (Emmendörffer et al., 1990).

2.4. Statistical analysis

The statistical significance of the observed differences was analyzed by the Mann-Whitney U-test or by t-test, or ANOVA followed by the Student-Newman-Keuls test using Statistical Package for the Social Sciences-SPSS version 16.0. A value $P<0.05$ was considered significant.

3. RESULTS AND DISCUSSION

Chemical analysis of the supercritical CO_2 extract of *U. barbata* revealed usnic acid to be the most abundant component of this sample (Figure 1) being present in the high amount of 81.41 % (w/w).

We further aimed at investigating cytotoxic activity of the studied SCE extract of old man's beard, as well as usnic acid. Cytotoxic activity was assessed in L929 cell line and both, SCE extract and usnic acid decreased the viability of L929 cells in the dose-dependent manner (Figure 2).

But, in comparison with usnic acid, SCE exhibited more potent effect when used in 25 µg/mL making its cytotoxic effect more potent than the effect obtained with usnic acid used in the same concentration. This result was also confirmed under morphology analysis of treated cells (Figure 3).

In addition, when SCE and usnic acid were used in maximal investigated concentration (50 µg/mL), the viability of L929 was less than 44.7±1.5 % and 40.0±1.5 % of control, respectively. Such findings are in accordance with our previous study with the same extract (Žugić et al., 2016), suggesting the need for further investigations in order to gain a deeper insight into the mechanisms involved in the demonstrated cytotoxic activity of this sample compared to pure usnic acid.

Further performed cell cycle analysis revealed no changes in DNA content under the treatment with usnic acid while the cells treated with SCE demonstrated increased hypodiploid DNA content (12.2 % in comparison with 0.44 % in control and 0.49 % in UA treatment), indicating DNA fragmentation and possible apoptosis (Figure 4). Apoptosis as a mechanism of cell death has been reported previously for the acetone extract of old man's beard in FemX and LS174 cells (Ranković et al., 2012), and for its supercritical CO_2 extract in B16 cells (Žugić et al., 2016).

Neither SCE nor usnic acid increased the number of acidic vesicles, a morphological characteristic of autophagy, since FL3/FL1 fluorescence intensity, indicating ratio between orange and green fluorescence, was 1 as well as in control cells (Figure 5).

Taking into account that oxidative stress may contribute to the cytotoxicity of a substance throughout several different mechanisms (Fimia and Piacentini, 2009), our further experiments were performed in order to investigate the level of ROS production in the L929 cells after treatment with SCE and usnic acid. As seen in Figure 6, unlike usnic acid, SCE treatment increased for 2.3 times ROS production compared to control cells.

CONCLUSION

Based on these results, and also taking into account our previous findings it could be speculated that the observed more pronounced cytotoxicity of supercritical CO_2 extract of *U. barbata* comprising high amount of usnic acid (81.41 % (w/w)) compared to usnic acid *per se* may be connected to the presence of unidentified compound(s) in the extract that could be responsible for apoptosis and oxidative stress detected in the L929 cells after treatment with the extract. However, additional experiments are needed to support these presumptions and elucidate precise mechanisms involved in the cytotoxicity of the tested extract of *U. barbata*. Presented results support further investigations of supercritical CO_2 extract of *U. barbata* as a prospective therapeutic agent with potential relevance in the treatment of sarcoma.

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