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Full Length Research Paper

HPLC analysis and cytotoxic potential of extracts from the lichen, *Thamnolia vermicularis* var*. subuliformis*

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The aim of this study was to investigate metabolites of the lichen *Thamnolia vermicularis* (Sw.) Schaer var*. subuliformis* (Ehrh.). Chloroform, ethyl acetate and methanol extracts of the lichen *Thamnolia vermicularis* var*. subuliformis* were prepared and a high performance liquid chromatographic (HPLC) method was developed for the characterization of depsides in these extracts. All the tested extracts of *T. vermicularis* var*. subuliformis* contain depsides baeomycesic, lecanoric acid, barbatic acid and squamatic acid, but in different amounts and relations. Components of this lichen were identified by relative retention time and spectral data. Some preliminary results concerning the evaluation of cytotoxic activity on HeLa cell of the chloroform, ethyl acetate and methanol extracts of the lichen *T. vermicularis* var*. subuliformis* are reported in the present study. *In vitro* cytotoxicity of the extracts on human cancer cell lines Hela was examined. Viability HeLa cells treated with the ethyl acetate and chloroform extracts of *T. vermicularis* var. *subuliformis* decreased with increasing concentrations of these extracts. IC_{50} value after 24 h for the ethyl acetate extract was 162.50 ± 5.80 µg/ml, while for the chloroform extract this value was 159.32 ± 5.16 µg/ml. All the tested concentrations of the ethyl acetate and chloroform lichens extract after 72 h of treatment of HeLa cells showed a cytotoxic effect. The methanolic extract containing the lowest content of baeomycesic and squamatic acid showed the lowest activity.

Key words: *Thamnolia vermicularis var. subuliformis*, cytotoxic activity, high performance liquid chromatographic (HPLC), depsides.

INTRODUCTION

Until now, several hundred secondary metabolites including depsides, depsidones, naphthoquinones, anthraquinones, pulvinates, chromones and dibenzofurans have been detected in lichens (Huneck and Yoshimura, 1996). Slow growth and often harsh living conditions, make production of protective metabolites, a necessity to lichens, and many secondary constituents are believed to serve as antigrowth, antimicrobial or antiherbivore agents (Hale, 1983; Manojlovic et al., 2002, 2005; Rankovic et al., 2008). A large number of lichen species have proved to be source of these metabolites for food and pharmaceutical industries. As far as is known, every lichen species produces some unique secondary compounds, often in remarkably large quantities. Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects. Some findings related to cytotoxicity

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have already been presented by our group (Vasiljevic et al., 2009). Lichens produce some characteristic depside derivatives, which are yet to be found in higher plants (Yoshimura et al., 1994; Huneck and Yoshimura, 1996). Previous works show that the lichen extract may possess antitumor and antiproliferative activity (Carderelli et al., 1997; Ogmundsdottir et al., 1998; Perry et al., 1999; Ingolfsdottir et al., 2000; Ingolfsdottir, 2002; Kristmundsdottir et al., 2002; Bezivin et al., 2003; Lin et al., 2003; Bucar et al., 2004; Zeytinoglu et al., 2004; 2008). Lichen extracts obtained by different type of lichen extraction showed a different cytotoxic effect (Bezivin et al., 2003). Many constituents in lichens have strong effect on proliferation of normal and cancer cells as protolichesterinic-, lobaric- and usnic acids (Carderelliet al., 1997; Ingolfsdottir et al., 1997; Ogmundsdottir et al., 1998; Ingolfsdottir, 2002; Lin et al., 2003; Bucar et al., 2004).

Thamnolia vermicularis belongs to the family *lcmadophilaceae* and occurs as two varieties, the var. *vermicularis* and the var. *subuliformis. T. vermicularis* (Sw.) Schaer. var. *subuliformis* (Ehrh.) Schaer. (Purvis et al., 1992), previously named *T. subuliformis* (Ehrh.) W. Culb. (Hale, 1983) is arctic-alpine lichens and belongs to the group of sterile lichen (Tehler, 1996). Thallus of this lichen is white, hollow pointed, elongated or worm-like branches.

In addition to the low molecular weight depsides, baeomycesic acid and squamatic acid described from *T. vermicularis* var. *subuliformis* (Asahina et al., 1954; Ingolfsdottir et al., 1997), a novel cold water soluble rhamnopyranosylgalactofuranan polysaccharide named thamnolan, has been isolated from the water extract of this lichen and shown to be immunologically active in *in vitro* tests (Olafsdottir et al., 1999). *T. vermicularis* has been commonly used as a tea with the local name “snow tea” in some part of China. Ethnic people from Yunnan Province China, use this species of lichens also as foods (Wang et al., 2001). A closely related chemical variant *T. vermicularis* var. *vermicularis* (Purvis et al., 1992) has been used in China for the treatment of psychic disorders, high blood pressure and inflammatory conditions of the respiratory tract (Hanssen and Schadler, 1985). It has been believed to counteract inflammation and has been used in traditional Chinese medicine for hundreds or thousands of years (Wang et al., 2001). Chemical constituents from *T. vermicularis* var. *subuliformis* have shown various biological activities, such as anti-tumour and immunomodulating activities (Olafsdottir et al., 1999, 2000, 2003).

In the course of our studies on phytochemistry and biological activity of lichens and their metabolites, the chloroform, ethyl acetate and methanol extracts of the lichen *T. vermicularis* (Sw.) Schaer. var. *subuliformis* (Ehrh.) were prepared and crude extracts were screened for their cytotoxic activity. A High-performance liquid chromatographic (HPLC) method was used for the characterization of depside metabolites in the extracts examined.

MATERIALS AND METHODS

Collection and identification of lichen samples

The lichen *T. vermicularis* (Sw.) Schaer. var. *subuliformis* (Ehrh.) Schaer. was collected from Mt Suva Planina, in southeast Serbia during July, 2008. The identification of the chemotype *Thamnolia* is made by the fact that *T. vermicularis* var. *subuliformis* contain baeomycesic and squamatic acids and fluorescent in UV light, while *T. vermicularis* var. *vermicularis* contain thamnolic acid and nonfluorescent in UV light (Culberson, 1963; Thomson, 1984). The collected materials were tested with ultraviolet light and were found to be UV+.

The collected plant material was deposited in the herbarium of the Institute of Botany and Jevremovac Botanical Garden, Faculty of Biology, University of Belgrade (BEOU). Voucher specimens: Serbia: Mt Suva planina, paek Trem, 1100 – 1500 m a.s.l. 43° 11.303 N, 22° 09.9 EO (Vasiljević, P., Jušković, M., 16268/08, BEOU, 31 May, 2008).

Preparation of lichen extracts

The lichen material (*T. vermicularis*) was air-dried at room temperature (26°C) for one week, after which it was grinded to a uniform powder. After that, lichen material (3 × 10 g) was extracted with 100 ml chloroform, ethyl acetate and methanol (12 h each at room temperature). The obtained extracts were filtered through a Whatman filter paper No. 42 (125 mm) and concentrated using a rotary evaporator with the water bath set at 40°C. The chloroform, ethyl acetate and methanol extracts were used for cytotoxic testing. Small parts of obtained extracts were used for HPLC analysis.

HPLC studies

Determination of depsides by HPLC

High-performance liquid chromatography (HPLC) analysis was carried out on Agilent 1200 series HPLC instrument with: C18 column (C18; 25 cm × 4.6 mm, 10 m) and UV Spectophotometric detector with solvent methanol-water-phosphoric acid (80:20:0.9, v/v/v). Methanol was of HPLC grade, and was purchased from Merck (Darmstadt, Germany). Phosphoric acid was analytical grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The sample injection volume was 10 µl. The flow rate was 1.0 ml/min. The constituents of the chloroform, ethyl acetate and methanol extracts were identified by comparison of their retention times and absorption spectra (200 - 400 nm). The method was validated according to the ICH guidelines (ICH, 1994, 1996).

Cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, PAA Laboratories) supplemented with 10% fetal bovine serum (Gibco), penicillin G/streptomycin at 100 µg/ml and 2 mM L glutamine (Sigma). Incubation was performed at 37°C in a humidified atmosphere with 5% CO_2.

Cell viability assay

Lichen extracts were dissolvent in dimethylsulfoxide (DMSO) and serially diluted DMEM to obtain concentrations 50, 100, 150, 200 µg/ml. HeLa cells were seeded at a density of 1×10^5 cell/well into 96-well plates and routinely cultured for 24 h. After 24 h, extracts were added in serial concentrations, and reincubated for 24 and72 h. All experiments were repeated three times.

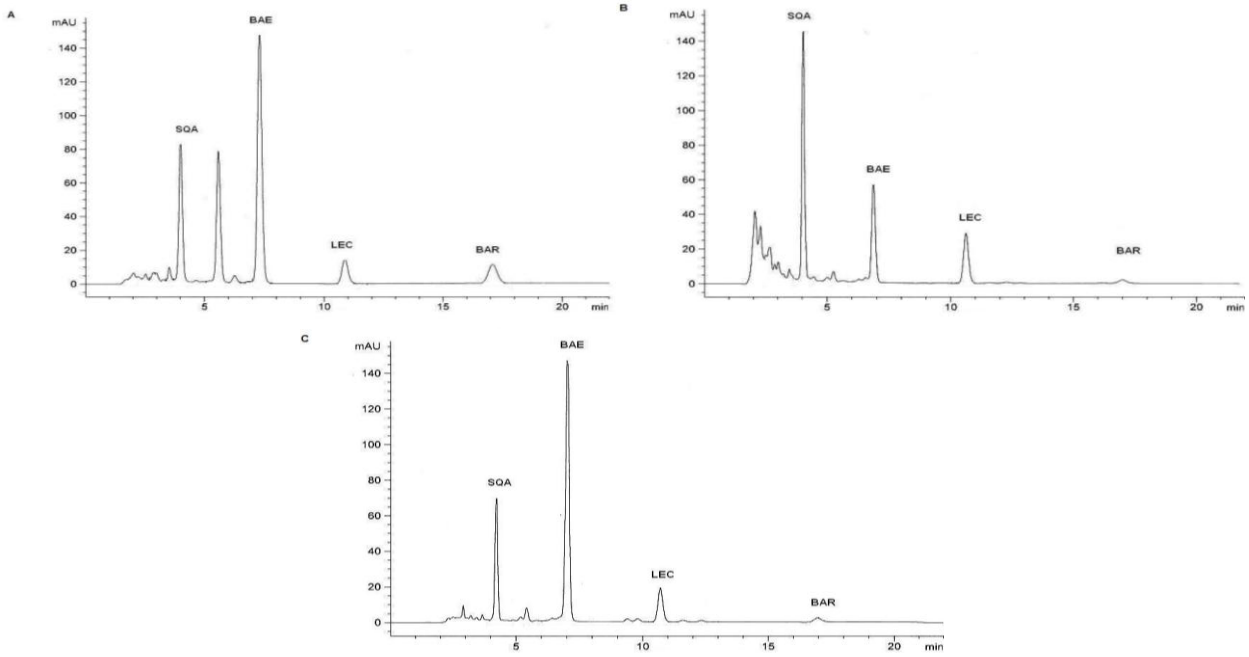

Figure 1. HPLC chromatograms of the ethyl acetate (A), methanol (B) and chloroform (C) extracts of *T. vermicularis*. SQA = squamatic acid; BAE = baeomycesic acid; LEC = lecanoric acid; BAR = barbatic acid.

The effects of tested samples on the variability of these cells were assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The tetrazolium salt was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml. After 24 h or 72 h exposure to lichen extract, 20 µl of a MTT stock solution were added to each well and incubated 4 h. The formazan crystals were dissolved with 100 µl acidic isopropanol (0.04 M HCl in absolute isopropanol). The absorbance was read by a Multiscan Ascent No354 (Thermo Lab Systems) ELISA reader at a wavelength of 540 nm. Viable cell percentage is calculated by the formula:

$$\% \text{ viable cells} = A_c\text{-}A_b/A_c\text{-}A_b \times 100$$

(The absorbance of the treated cells – the absorbance of the blank)/(The absorbance of the control – the absorbance of the blank) x 100.

Data analysis

The concentration of sample required to inhibit cell growth by 50% (IC_{50}) in comparison with the growth of a cell control, was determined from the dose-response curves. All assays were done in triplicates. Values obtained were expressed as mean ± standard deviation.

RESULTS

Figure 1 shows the HPLC chromatogram of the ethyl acetate (A), methanol (B) and chloroform (C) extracts of *T. vermicularis* var. *subuliformis* collected from Serbia. Squamatic acid (SQA) and baeomycesic acid (BAE) were the major beta-orcinol depsides presented in chloroform extract. The t_R values for squamatic and baeomycesic acid amount to 4.23 and 7.02 min, respectively. In addition to these most abundant compounds, depsides lecanoric acid (LEC) (t_R = 10.70 min) and barbatic acid (BAR) (t_R = 17.20 min) were also identified in extract tested. The amounts of depsides in this extract were presented in the following order: BAE > SQA > LEC > BAR.

The ethyl acetate and methanol extracts of *T. vermicularis* contain the same depsides as the chloroform extract but in different amounts and relations. Baeomycesic acid was dominant depside in the chloroform and ethyl acetate extracts. The most abundant depside of the methanol extract was squamatic acid. The amounts of depsides in ethyl acetate and methanol extracts were present in the following order: BAE > SQA > unknown depside > BAR > LEC and SQA > BAE > LEC > BAR (traces), respectively.

These four compounds were determined by comparison of their t_R values and their UV absorption spectra with the chromatogram and spectra of the standard substances (Figure 2). The values of retention times and absorbance maxima are shown in Table 1. The absorbance spectral data also corresponded with those in the literatures (Huneck and Yoshimura, 1996; Yoshimura at al., 1994). The UV spectra of the identified depsides were presented in Figure 3.

Cytotoxic screenings of three lichen extracts were evaluated on HeLa cells. Our current findings show that measured absorbance cells exposed to extracts are

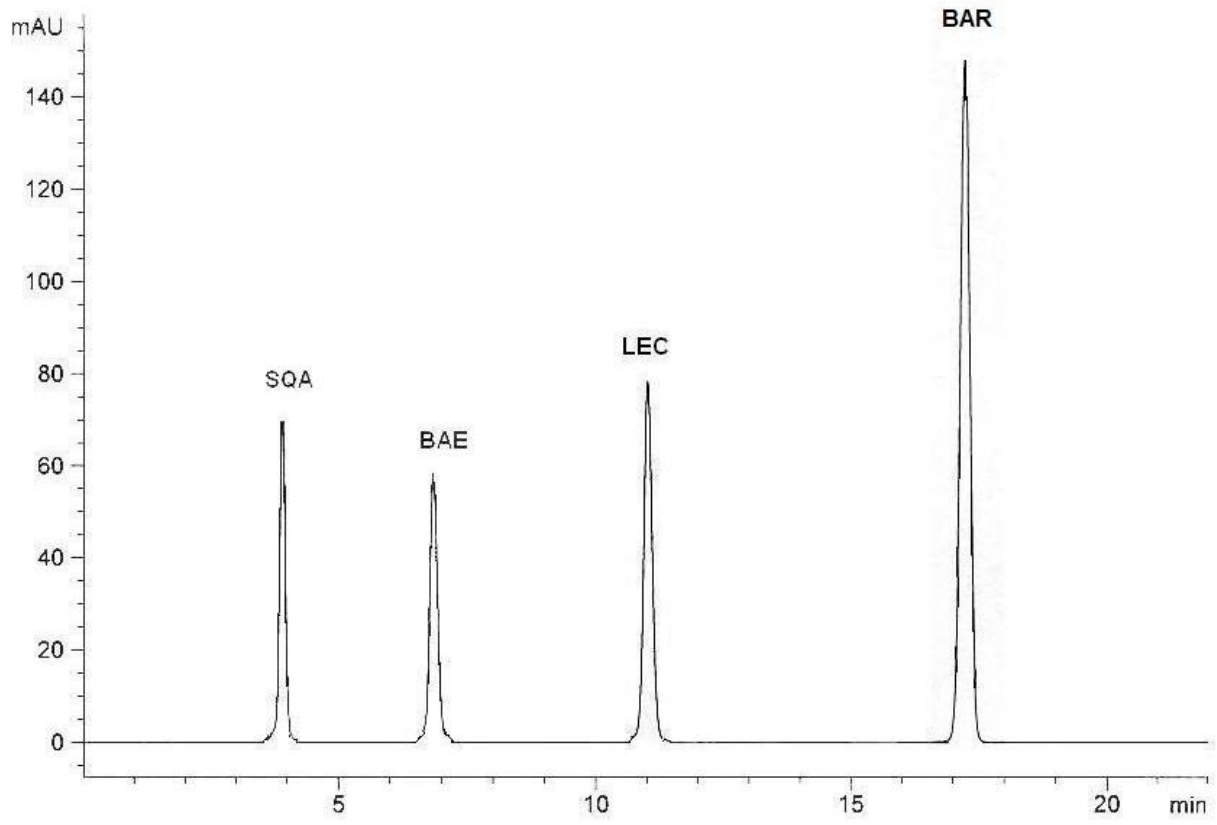

Figure 2. HPLC chromatogram of mixed standards.

Table 1. Identification of the depsides by HPLC-UV.

Compounds	t_R (min)	Spectral data: Absorbance maxima (nm) UV spectrum	
		Experimental values	Data from reference (Yoshimura et al., 1994)
SQA	4.23	215, 250, 313	214, 248, 312
BAE	7.02	212, 253, 284, 317	212, 252, 284
LEC	10.70	216, 268, 307	212, 270, 304
BAR	17.20	214, 278, 310	214, 276, 310

directly proportional to metabolically active cells. Figure 4 shows that the MeOH extract after 24 h, show no significant inhibitory activity but after 72 h, exposure of cells showed inhibitory activity and IC_{50} value was 60.58 ± 22.24 µg/ml. Viability of HeLa cells treated with ethyl acetate and chloroform extracts decreases with increasing concentration of this extract (Figure 4). IC_{50} value after 24 h for ethyl acetate extracts was 162.5 ± 5.8 µg/ml, while for the chloroform extracts this value was 159.32 ± 5.16 µg/ml. All the tested concentrations of ethyl acetate and chloroform lichen extracts after 72 h of treatment of HeLa cells showed a cytotoxic effect.

DISCUSSION

In the literature, the information about isolation of depsides from the family *Icmadophilaceae* is limited. With respect to the presence of depsides, Asahina (1954) divided *T. vermicularis* into two species. One of them (*T. vermicularis* var. *subuliformis)* contained barbatinic and baeomycesic acid and the other (*T. vermicularis* var. *vermicularis)* contained thamnolic acid. The previous papers also showed that several phenolic compounds, baeomycesic acid, vermicularin and thamnolin (Sun et

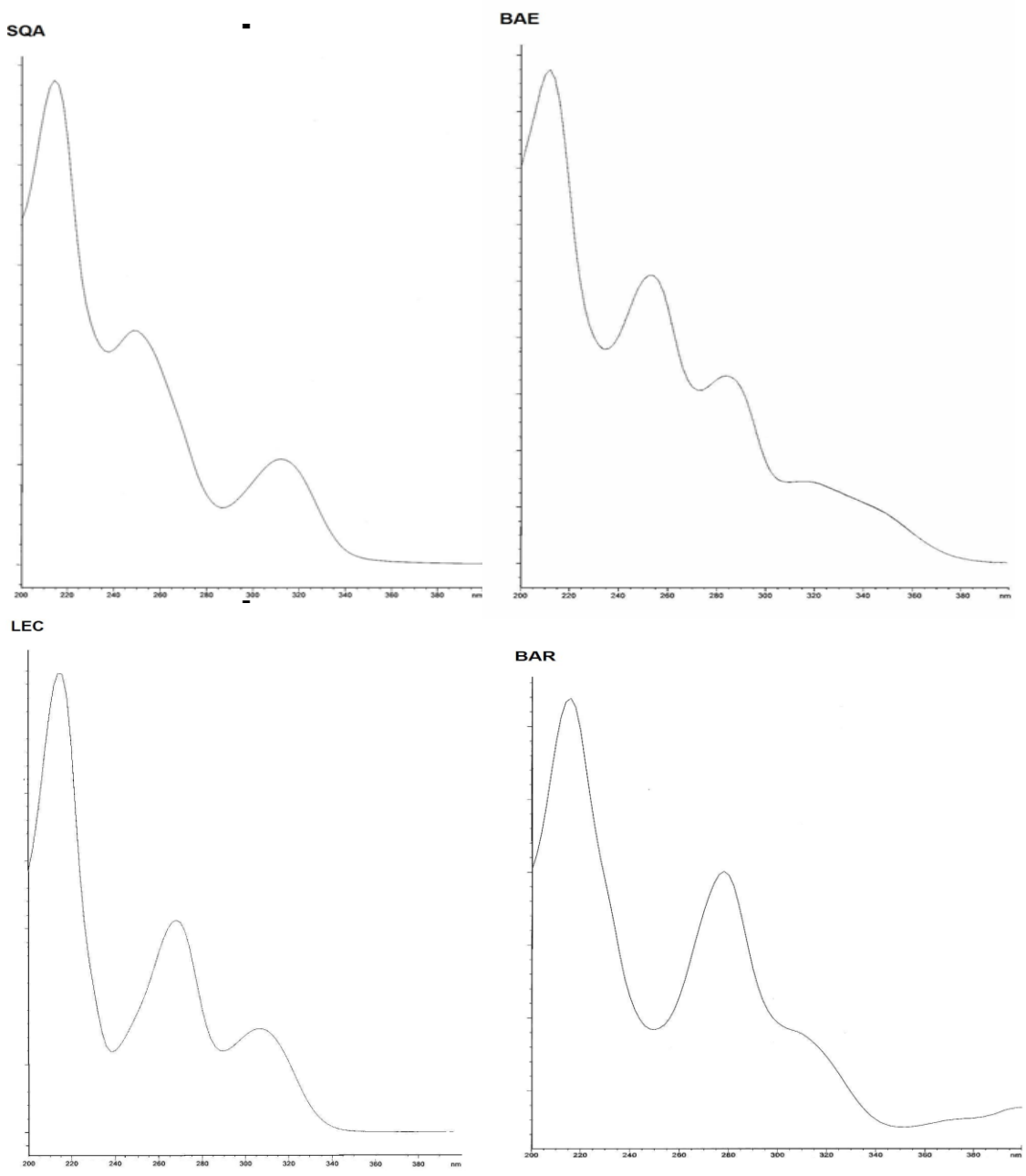

Figure 3. UV spectra of the identified depsides (SQA, BAE, LEC and BAR). The UV spectrum was recorded from the HPLC chromatogram. UV traces were recorded at 254 nm and UV spectrum from 200 to 400 nm.

al., 1985; Jiang et al., 2001) had been isolated from this lichen. But there is no information on which variety has been investigated. Generally, the main problem was that detailed phytochemical analysis of one particular variety of this lichen is shown nowhere. This paper reported for the first time, identification of four depsides in the *T. vermicurlaris* var. *subuliformis*. The depsides were identified as squamatic acid, baeomycesic acid, lecanoric acid and barbatic acid in the methanol, ethyl acetate and chloroform extracts. Lecanoric acid was identified in this species for the first time. Until now, it has been believed that only *T. vermicularis* var. *vermicularis* grows in Serbia. This is the first time to introduce *T. vermicurlaris* var. *subuliformis* from Serbia. Cytotoxic activity of extracts depends on the chemical composition and the relationship of depsides represented in the lichen. The order of cytotoxic activity was as follows: chloroform extract > ethyl acetate extract > methanol extract. The isolation of bioactive components in the extracts would certainly help to ascertain the individual potency of the compounds which could be further exploited for use by the food and pharmaceutical industries. Therefore, further

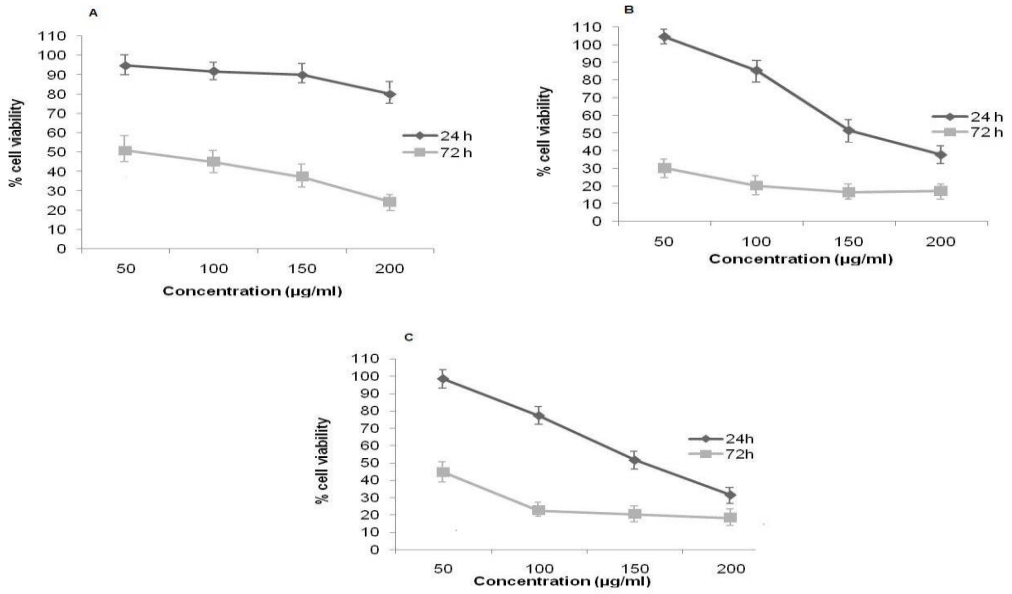

Figure 4. Effects of the MeOH (A), ethyl acetate (B) and chloroform (C) lichen extracts on the viability of HeLa cell lines. Viable cells were determined using the MTT method as described in Materials and Methods. Cells were incubated for 24 or 72 h in the presence of lichen extract. Values are the mean ± SD of data from three independent experiments with three replicates at each point.

work should be focused on the isolation and purification of other active components of the crude extracts of the studied lichen.

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