Food and Chemical Toxicology 51 (2013) 160–164

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Effect of four lichen acids isolated from *Hypogymnia physodes* on viability of rat thymocytes

Voja Pavlovic [a,*], Igor Stojanovic [b], Milka Jadranin [c], Vlatka Vajs [c], Iris Djordjević [d] Andrija Smelcerovic [b], Gordana Stojanovic [e]

[a] Institute of Physiology, Faculty of Medicine, University of Nis, Bulevar Dr. Zorana Djindjica 81, 18000 Nis, Serbia
[b] Department of Pharmacy, Faculty of Medicine, University of Nis, Bulevar Dr. Zorana Djindjica 81, 18000 Nis, Serbia
[c] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoseva1 2, 11000 Belgrade, Serbia
[d] Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobodjenja 18, 11000 Belgrade, Serbia
[e] Department of Chemistry, Faculty of Science and Mathematics, University of Nis, Visegradska 33, 18000 Nis, Serbia

ARTICLE INFO

Article history:
Received 30 March 2012
Accepted 27 April 2012
Available online 9 May 2012

Keywords:
Hypogimnia physodes
Lichen acids
Isolation
Cytotoxicity
Oxidative stress
Mitochondrial membrane potential

ABSTRACT

Four lichen acids, physodalic acid (**F1**), physodic acid (**F2**), 3-hydroxyphysodic acid (**F3**), and isophysodic acid (**F4**), were isolated from *Hypogymnia physodes* methanol extract using preparative reversed-phase high performance liquid chromatography and their structures were determined by UV, MS, ^{1}H NMR and ^{13}C NMR. This is the first report on the isolation of **F4** from *H. physodes*. Isolated rat thymocytes were cultivated with increasing **F1**–**F4** concentrations (0.1, 1, 10 μg/well) and proliferative activity, viability, ROS (reactive oxygen species) production and MMP (mitochondrial membrane potential) disturbances were evaluated. Obtained results show significantly decreased thymocytes proliferation was observed when cells were treated with **F1** (1 μg, $p < 0.05$; 10 μg; $p < 0.001$), **F2** (10 μg, $p < 0.05$) and **F3** compound (10 μg, $p < 0.05$). Significantly increased cytotoxicity was detected when cells were incubated with **F1** (1 μg, $p < 0.05$; 10 μg, $p < 0.01$), **F2** (10 μg, $p < 0.05$) and **F3** compound (10 μg, $p < 0.001$). Increased H2DCF-DA fluorescence intensity, when cells were treated with **F1** (1 μg, $p < 0.001$; 1 μg, $p < 0.01$; 10 μg, $p < 0.001$) and **F2** (1 μg, $p < 0.05$; 10 μg, $p < 0.01$) compound, indicating the increase of intracellular ROS production. Simultaneously, increased ROS levels were followed with significantly decreased MMP when thymocytes were cultivated with **F1** (0.1 μg, $p < 0.001$; 1 μg, $p < 0.001$; 10 μg, $p < 0.001$) and **F2** compound (10 μg, $p < 0.001$). Thymocytes exposure to increased (0.1, 1, 10 μg) concentrations of **F3** and **F4** compounds did not result with significant alterations in MMP and intracellular ROS production. We have shown that higher **F1** and **F2** concentrations induce thymocytes toxicity mainly through induction of oxidative stress, while cytotoxicity effect of **F3** is followed with altered antioxidant/oxidant balance. The rigid 11*H*-dibenzo[*b*,*e*][1,4]dioxepin-11-one ring in the depsidone structure may play a important role for the examined biological activities.

1. Introduction

Lichens, as symbiotic organisms of fungi and algae, synthesize more than 1000 unique secondary metabolites different from those synthesized by higher plants (Stocker-Wörgötter, 2008). They have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world (Huneck, 1999; Upreti et al., 2005; Gupta et al., 2007). Typical lichen secondary metabolites are extracellular compounds produced by fungal partner of lichen symbiosis and they are stored on the surface of both mycobiont (fungus), as well as photobiont (alga/cyanobacterium) partners of symbiosis. Backor et al. (2010) demonstrated phytotoxicity of secondary metabolite (usnic acid) from lichens on the algal partner resulted in decrease of cell division. This can be even one of the key processes for maintaining balance between the symbionts forming the lichen thalli as lichens are long living but slowly growing organisms.

Depsidones are one of the classes of secondary metabolites which are mostly produced in lichens. They have been reported to possess many biological activities, such as antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic, as was reviewed recently (Stojanović et al., 2012).

As a part of our study of the chemical composition (Stojanović et al., 2011) and biological activities (Stojanović et al., 2010) of *Hypogimnia physodes*, herein we isolated physodalic acid (**F1**), physodic acid (**F2**), 3-hydroxyphysodic acid (**F3**), and isophysodic

* Corresponding author. Tel.: +381 18 4570029; fax: +381 18 4238770.
E-mail address: vojapav@yahoo.com (V. Pavlovic).

0278-6915/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fct.2012.04.043

acid (**F4**), which represent 70% of the total methanol extract of *H. physodes*. Lichen acids **F1**, **F2** and **F3** belong to the class of depsidones. **F4** is rearranged isomer of **F2**. There are little data about biological activities of isolated compounds. Osawa et al. reported that **F1** and **F2** inhibited mutagenicity of a heterocyclic amine in *S. typhimurium* TA 98 (Osawa et al., 1991). **F2** demonstrated the antimicrobial activity (Ranković et al., 2008). **F2** and **F4** were active against HIV-1 integrase (Neamati et al., 1997).

Since the effect of **F1**–**F4** on the cells of the immune system has not been documented before, in our study we tried to evaluate the potential effect of those compounds on rat thymocytes and to correlate the examined biological effects with the structure of the studied compounds.

2. Experimental

2.1. Lichen material

Lichen *Hypogymnia physodes* (L.) Nyl., (syn: *Parmelia duplicata* var. *douglasicola* Gyelnik, *Parmelia physodes* (L.) Ach., *Parmelia oregana* Gyelnik; common names: Monk's-hood lichen, Hooded tube lichen, Puffed lichen) growing on a *Prunus domestica* tree was collected on the locality: Donje Vlase (Grcke Pojate), northern slopes of Selicevica Mt. (SI Serbia) – N lat: 43°16′18,6″; E long 21°55′07,6″; altitude 354 m in September 2011. The lichen material was air-dried for 10 days and stored at ambient temperature (25 ± 2 °C) without exposure to direct sunlight. A voucher specimen was deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš (voucher number 6576). Taxonomical identification was performed by Dr. Bojan Zlatković, Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš.

2.2. Preparation of lichen extracts

The mixture of powdered air-dried lichen material (10 g) and methanol was sonicated for 30 min and left at room temperature overnight (Tay et al., 2004). The extract were filtered, evaporated until dray residues were obtained, by using a rotary evaporator with the water bath set at 40 °C. The extract yield was 10.8% (w/w).

2.3. Isolation and identification *F1–F4* acids

Preparative HPLC was carried out using the HPLC system 1200 series with a semipreparative diode array detector, and a Zorbax Eclipse XDB-C18 Semi-Prep, 5 μm, 9.4 × 250 mm column. The mobile phase, methanol:water:formic acid (80:20:0.2, v:v:v), was pumped at 4.0 mL min^{-1} flow rate, while the injection volume was 50 μL (50 mg of the dry extract dissolved in 1 mL of methanol), at 25 °C. Twenty injections gave 14 mg **F1**, 12 mg **F2**, 7.5 mg **F3** and, 1.5 mg **F4,** which represent 70% of the total methanol extract. The retention times of **F1**, **F2**, **F3**, and **F4** were 7.19, 11.84, 6.58 and 8.26 min, respectively.

The UV spectra were recorded from the HPLC chromatogram at 254 nm from 210 to 400 nm. Exact mass measurement of molecular ions of analytes was performed with a time of fligt (TOF) mass spectrometer in negative polarity mode. The NMR spectra of isolated compounds were recorded on a Varian Gemini 200 (^{1}H at 200 MHz, ^{13}C at 50 MHz) spectrometer, using d_6-DMSO as the solvent.

2.4. Animals

Experiments were performed on adult male Wistar rats (150–180 g), 8–10 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions and in accordance with national animal protection guidelines.

2.5. Preparation of thymocytes

Rat thymocytes were isolated as described previously (Pavlovic et al., 2007; Cekic et al., 2011). The viability of the isolated cells, as determined by trypan blue dye exclusion test, was always over 95%. Isolated thymocytes were counted and adjusted to a density of 5 × 10^6 cells/ml of culture medium (CM). CM was prepared using RPMI 1640 (Sigma, St. Louis, Mo., USA), according to the manufacturer's instructions. CM containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS).

2.6. Cell culture

Isolated thymocytes were cultivated in 96-well round-bottom plates (Falcon, BD, USA), containing a 100 μl of cell suspension (5 × 10^5 cells) in each well. Cells were treated with increasing concentrations (0.1, 1, 10 μg/well) of **F1**–**F4**, diluted in appropriate amounts of pure methanol. Control samples were cultured in CM or with appropriate amounts of pure methanol. For further evaluation of the proliferative activity, thymocytes were treated with optimal (5 μg/ml) concentration (Pavlovic et al., 2006) of ConA (Pharmacia Fine Chemicals AB, Uppsala, Sweden). All cell cultures were done in triplicates and cultivated for 24 h in an incubator (Galaxy, Wolf laboratories, USA) with 5% CO_2 at 37 °C.

2.7. Cell proliferation

The proliferation of rat thymocytes was estimated by Cell Counting Kit (CCK-8, Sigma–Aldrich, St. Louis, Mo., USA), according to the manufacturer's guidelines. This assay is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenase in viable cells. The absorbance at 450 nm of each well was measured with a Perkin-Elmer microplate reader (Wallac Victor^{2}V, Turku, Finland). For each sample, basal intensity values were subtracted from those obtained after different treatments and results were presented as ratio for comparison with control samples (Pavlovic et al., 2012).

2.8. Analysis of cell viability

Cell viability of rat thymocytes, after cultivation period, was evaluated by CCK-8 assay as was previously described (Hori et al., 2002). Ten microliter of reaction mixture was added in each well. After 2 h of incubation, the solubilized formazan product was quantified spectrophotometrically, by using a microplate reader Perkin-Elmer. Absorbance was measured at 450 nm. For each sample, basal intensity values were subtracted from those obtained after different treatments. Absorbances were presented as ratio for comparison with control samples.

2.9. Measurement of intracellular reactive oxygen species (ROS) production

A redox-sensitive probe 2′,7′-dichlorofluorescin diacetate (H2DCF-DA, Sigma–Aldrich, St. Louis, Mo., USA) was used to determine changes in overall cellular ROS levels, as described previously (Das et al., 2005; Boldogh et al., 2003). The change in fluorescence (excitation 485 nm; emission 530 nm) was measured using a Perkin-Elmer fluorimeter (Wallac Victor^{2}V, Turku, Finland). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and results were presented as ratio of mean fluorescence intensity (MFI).

2.10. Determination of mitochondrial membrane potential

Changes in mitochondrial membrane potential (MMP) of thymocytes, treated with **F1**–**F4**, were evaluated by uptake of lipophilic cation Rhodamine 123 (Sigma–Aldrich, St. Louis, Mo., USA) into mitochondria, as previously described (Wang et al., 2007; Pathak and Khandelwal, 2006). The fluorescence of intracellular Rhodamine 123 (excitation 485 nm; emission 530 nm) was measured by Perkin-Elmer fluorimeter, as published earlier (Yang et al., 2008). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and results were presented as ratio of mean fluorescence intensity (MFI).

2.11. Statistical analysis

Results are presented as mean ± SD. Significant differences between the groups were analyzed with Student's *t*-test.

3. Results

Compounds **F1**–**F4** were identified as physodalic acid (**F1**; 9-(acetoxymethyl)-4-formyl-3,8-dihydroxy-1,6-dimethyl-11-oxo-11*H*-dibenzo[*b*,*e*][1,4]dioxepine-7-carboxylic acid), physodic acid (**F2**; 3,8-dihydroxy-11-oxo-1-(2-oxoheptyl)-6-pentyl-11*H*-dibenzo[*b*,*e*][1,4]dioxepine-7-carboxylic acid), 3-hydroxy physodic (**F3**; 3,4,8-trihydroxy-11-oxo-1-(2-oxoheptyl)-6-pentyl-11*H*-dibenzo[*b*,*e*][1,4]dioxepin-7-carboxylic acid), and isophysodic acids (**F4**; 4,6-dihydroxy-3-(6-hydroxy-1-oxo-3-pentyl-1H-isochromen-8-yloxy)-2-pentylbenzoic acid) (Fig. 1) by comparing their UV, MS, ^{1}H and ^{13}C NMR data with those given before (Yoshimura et al., 1994; Jones et al., 1976; Millot et al., 2007) as well as with data computed by CHEM3D ultra 10 software. A perfect match was observed. This is the first report of **F4** isolation from *H. physodes*.

The effects of increasing concentrations (0.1, 1, 10 μg) of **F1**–**F4** compound on the proliferation of rat thymocytes, triggered by optimal (5 μg/ml) concentration of ConA, were tested 24 h after