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Effect of *Hypogymnia physodes* Extracts and their Depsidones on Micronucleus Distribution in Human Lymphocytes

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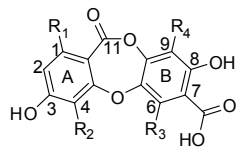
Three lichen depsidones, physodalic acid (**1**), physodic acid (**2**), and 3-hydroxy physodic acid (**3**), were isolated from *Hypogymnia physodes* diethyl ether extract using column chromatography, and their structures determined by comparing their UV,^{1}H and ^{13}C NMR spectroscopic and MS data with those given in the literature, as well as with data computed by CHEM draw ultra 11 software. The contents of **1**, **2** and **3** were determined in the methanol (**ME**), acetone (**AE**), and diethyl ether (**EE**) extracts using reversed–phase high performance liquid chromatography with photodiode array detection. The extracts, isolated depsidones **1-3** and **EE** fraction **F23** (consisting of 90% **2** and **3**, in the ratio 5.5: 1) were evaluated for their *in vitro* effects on chromosome aberrations in peripheral human lymphocytes using the cytochalasin-B blocked micronucleus (CBMN) assay in doses of 1 µg/mL and 2 µg/mL of final culture solution. The frequency of MN was scored in binucleated cells, and nuclear proliferation index was calculated. It was found that **1**, **2**, **3**, **F23**, and **EE** at 1.0 µg/mL exerted a beneficial effect on lymphocyte cells giving a significant decrease of the frequency of MN in comparison with the positive control Amifostin WR-2721.Among the tested depsidones at a concentration of 1 µg/mL, **3** exhibited the most prominent effect decreasing the frequency of MN by 30.3%, followed by **2** (28.2%) and **1** (22.0%). The extracts were less effective than the isolated depsidones.

Keywords: *Hypogymnia physodes* (L.) Nyl., Parmeliaceae, Depsidones, Human lymphocytes, Micronucleus test.

Lichens have been used as important remedies in the traditional medicines of many countries [1]. Increasing interest in the biological activities of lichens has resulted in numerous published review papers [1-10]. Most works refer to the biological activity of the crude extracts of lichens, among which some relate to extracts of *Hypogymnia physodes* [11-16], while a few record the biological activities of pure secondary metabolites isolated from this lichen species [12, 16-21].

According to our best knowledge, there is no published data related to the effect of *H. physodes* extracts and their constituents on micronucleus (MN) expression in peripheral blood lymphocytes. Micronuclei result from lesions/adducts at the level of DNA or chromosomes, or at the level of proteins directly or indirectly involved in chromosome segregation (e.g. tubulin). The cytokinesis-block micronucleus technique enables micronuclei to be specifically scored in cells that have completed nuclear division and is, therefore, not influenced by variations in cell division kinetics, and it has been shown to be a sensitive and reliable index of chromosome damage [22].

Continuing our investigation of the chemical composition [23] and biological activities [13, 21] of *H. physodes*, herein we report the isolation of the main constituents of the diethyl ether extract and their effect on micronucleus distribution in peripheral blood lymphocytes. The obtained results were compared with those obtained with the methanol (**ME**), acetone (**AE**), and diethyl ether (**EE**) extracts of the studied lichen. Three lichen acids, physodalic acid (**1**), physodic acid (**2**), and 3-hydroxy physodic acid (**3**) (Figure 1), were isolated from *H. physodes* diethyl ether extract [24-26]. Relative proportions of **1-3** in the *H. physodes* extracts are given in Table 1.



Physodalic acid (**1**):
R_2=CHO; R_1=CH_3; R_4=CH_2OCOCH_3; R_3=CH_3

Physodic acid (**2**):
R_2=H; R_1=$CH_2COCH_2(CH_2)_3CH_3$; R_4=H; R_3=C_5H_{11}

3-Hydroxyphysodic acid (**3**):
R_2=OH; R_1=$CH_2COCH_2(CH_2)_3CH_3$; R_4=H; R_3=C_5H_{11}

Figure 1: The structures of physodalic acid (**1**), physodic acid (**2**) and 3-hydroxy-physodic acid (**3**)

Table 1: The content (%) of physodalic acid (**1**), physodic acid (**2**) and 3-hydroxy physodic acid (**3**) in the studied *H. physodes* extracts.

Compds	Methanol extract (**ME**)	Diethyl ether extract (**EE**)	Acetone extract (**AE**)	Diethyl ether extract fraction (**F23**)
Physodalic acid (**1**)	28.7	34.9	38.7	0.0
Physodic acid (**2**)	26.5	30.7	22.8	75.9
3-Hydroxy physodic acid (**3**)	14.8	16.5	12.4	14.1
Total	70.0	82.1	73.9	90.0

According to the current and previous studies of *H. physodes*, specimens from different sites do not show qualitative differences in terms of their main extract constituents [7, 27].The extracts (**ME**, **AE** and **EE**), depsidones **1-3** and **EE** fraction **F23** consisting of **2** and **3** were tested for *in vitro* protective effect on chromosome aberrations in peripheral human lymphocytes using the cytochalasin-B blocked micronucleus (CBMN) assay in doses of 1.0 and 2.0 µg per mL of final culture solution. The frequency of MN was scored in binucleated cells, and nuclear proliferation index

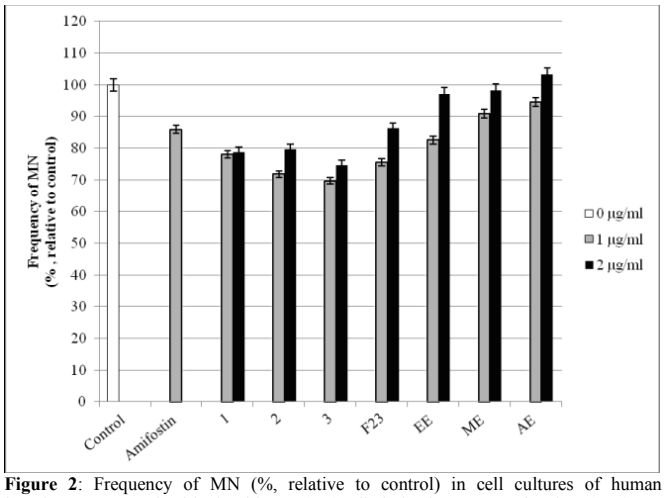


Figure 2: Frequency of MN (%, relative to control) in cell cultures of human lymphocytes treated with depsidones (**1-3**), diethyl ether extract fraction (**F23**), *H. physodes* diethyl ether, methanol and acetone extracts (**EE**, **ME** and **AE**), and Amifostin.

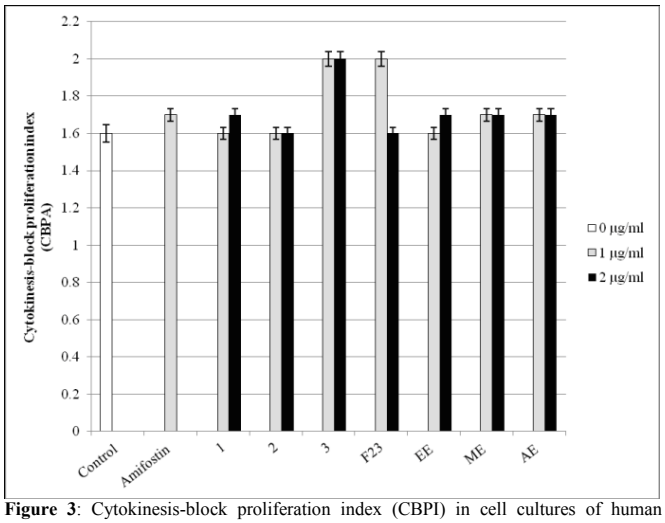


Figure 3: Cytokinesis-block proliferation index (CBPI) in cell cultures of human lymphocytes treated with depsidones (**1-3**), diethyl ether extract fraction (**F23**), *H. physodes* diethyl ether, methanol and acetone extracts (**EE**, **ME** and **AE**) and Amifostin.

was calculated. Amifostine (radioprotectant, previously known as WR-2721) was used as positive control. The results are shown in Figures 2 and 3.

The cell cultures treated with Amifostine at a concentration of 1 µg/mL exhibited a 14.1% decrease in the frequency of MN, compared with the control cell cultures. Treatment with depsidones **1-3** at a concentration of 1 µg/mL showed a 22.0, 28.2 and 30.3% (respectively) decrease in the frequency of MN, while concentrations of 2 µg/mL gave decreases in MN frequency of 21.3, 20.3 and 25.3%, respectively. However they were still more efficient than Amifostine at the concentration of 1 µg/mL. Since the number of micronuclei serves as an indicator of DNA damage, it can be concluded that **1-3** exert a beneficial effect on human lymphocyte cells. The activity of the investigated depsidones is similar to that of flavonoids [28], furanocoumarins [29] and ellagitannins [30].

The extracts and **F23** were less effective in decreasing the frequency of MN relative to the pure substances. Even more, **AE** at the concentration of 2 µg/mL stimulated the frequency of micronuclei by 3.3%.

Reducing the frequency of MN decreases in the order **EE** (17.4%), **ME** (9.1%) and **AE** (5.4%) at the concentration of 1 µg/mL. The total content of **3** and **2** in the **EE**, **ME** and **AE** decreases in the same order (47.2, 41.3 and 35.3%, respectively). The **EE** fraction **F23**, containing in total 90.0% of **3** and **2** (in ratio 1:5.5), reduces the frequency of micronuclei by 24.5%. The frequency of MN is statistically significant (p<0.05) between control and cell cultures treated with **2** and **3** (at both examined concentrations), and **F23** at a concentration of 1 µg/mL. Comparing the effects of Amifostine (1 µg/mL) and the examined samples there is a statistically significant difference in the frequency of MN (p<0.01) for cell cultures treated with **F23** (1 µg/mL), **ME** (2 µg/mL) and **AE** (2 µg/mL). Previous papers showed that treatment of lymphocytes with seed extracts of grape, raspberry, and blackberry induced a significant decrease in the frequency of micronuclei [31,32].

The effect of samples on cell proliferation was investigated by determining the cytokinesis-block proliferation index (CBPI). The comparable CBPI values of the investigated samples and control suggested that they did not show a statistically significant inhibitory effect on lymphocyte cell proliferation at the applied concentrations. *H. physodes* methanol extract had an IC_{50}value of 253.7 µg/mL in the experiment on colon cancer adenocarcinoma cell line HCT-116 [14]. Significantly decreased proliferation was observed when rat thymocyte cells were treated with **1**, **2**, and **3** [21].

In summary, we have shown that lichen depsidones **1-3** and *H. physodes* extracts at a concentration of 1 µg/mL were highly active in reducing the amount of DNA damage in comparison with the positive control (Amifostin). Among the tested compounds, compound **3** exhibited the most prominent effect decreasing the frequency of MN by 30.3%. It is interesting that all examined depsidones and extracts at the concentration of 1 µg/mL showed better beneficial effect on lymphocyte cell cultures than at a concentration of 2 µg/mL.

This first study on the impact on the micronucleus distribution in human lymphocytes of depsidones **1**, **2** and **3**, and extracts containing them as their main constituents, provide evidence of their protective effect on cytogenetic damage in human lymphocytes treated *in vitro*. Taking into account that the synthetic protector (Amifostin), used in the treatment of humans, decreases the frequency of MN by about 16% [33], these results could be important in identifying substances that can potentially contribute to human health.

Experimental

Lichen material: *Hypogymnia physodes* (L.) Nyl., (syn: *Parmelia duplicata* var. *douglasicola* Gyelnik, *P. physodes* (L.) Ach., *P. oregana* Gyelnik; common names: Monk's-hood lichen, Hooded tube lichen, Puffed lichen), growing on a *Prunus domestica* tree, was collected in the locality of 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. 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).

Preparation of lichen extracts: The mixture of powdered air-dried lichen material (10 g) and solvent (methanol, acetone and diethyl ether) was sonicated for 30 min, then left at room temperature overnight, and filtered [34]. Extract dry residues were obtained using a rotary evaporator with the water bath set at 40°C. The **ME**, **AE** and **EE** total extract yields were 10.8, 7.3 and 5.2 % (w/w), respectively.

Isolation and identification of depsidones (1-3): Isolation of **1-3** was achieved by CC according to a known procedure [35]. Compounds **1-3** were identified as physodalic acid (**1**; 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 (**2**; 3,8-dihydroxy-11-oxo-1-(2-oxoheptyl)-6-pentyl-11*H*-dibenzo[*b*,*e*][1,4]dioxepine-7-carboxylic acid), and 3-hydroxy physodic acid (**3**;3,4,8-trihydroxy-11-oxo-1-(2-oxoheptyl)-6-pentyl-11H-dibenzo [b,e][1,4]dioxepin-7-carboxylic acid) by comparing their UV, ^{1}H- and ^{13}C-NMR spectroscopic and MS data with those given in the literature [24-26], as well as with data computed by CHEM draw ultra 10 software. UV,^{1}H- and ^{13}C-NMR spectroscopic and MS data are available as supplementary information. The purity of the isolated depsidones was determined by HPLC-DAD and amounted to 93.8, 95.6, and 94.5% for **1**, **2**, and **3**, respectively.

Cytokinesis-block MN assay: This test was performed as previously described [29]. The cell culture lymphocytes were treated with 1 μg/mL and 2 μg/mL of the studied extracts (**ME**, **EE**, **AE**), **EE** fraction **F23** and isolated depsidones **1-3**. Amifostine (S-{2-[(3-aminopropyl)amino]ethyl} dihydrogenphosphorothioate; 98%, Marligen-Biosciences, USA), 1 μg/mL, was added to one cell culture serving as a positive control. The results are expressed as the means ± SD of 3 independent experiments.

Statistical analysis: The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at $p<0.05$.

Supplementary data: UV, ^{1}H- and ^{13}C-NMR and mass spectra of the isolated compounds, as well as the relevant data on the cytokinesis-block MN assay (Table 2) are available as supplementary data.

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