

## Original Article

# Effects of Depsidones from *Hypogymnia physodes* on HeLa Cell Viability and Growth

(*Hypogymnia physodes* / anti-proliferative activity / HeLa cells / physodic acid / 3-hydroxyphysodic acid / physodalic acid)

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**Abstract.** The anti-proliferative activity of *Hypogymnia physodes* methanol extracts (ME) and its main constituents, physodalic acid (P1), physodic acid (P2), and 3-hydroxy physodic acid (P3), was tested on human cancer HeLa cell lines. Three lichen depsidones, P1, P2 and P3, were isolated from *H. physodes* ME using column chromatography and their structures were determined by UV, ESI TOF MS, <sup>1</sup>H and <sup>13</sup>C NMR. The content of P1, P2 and P3 in ME was determined using reversed-phase high-performance liquid chromatography with photodiode array detection. P1–3 represented even 70 % of the studied extract. The HeLa cells were incubated during 24 and 72 h in the presence of ME and depsidones P1, P2 and P3, at concentrations of 10–1000 µg/ml. Compounds P2 and P3 showed higher activity than compound P1. Half maximal inhibitory concentrations (IC<sub>50</sub>, µg/ml) of P1, P2, P3 and ME for 24-h incubation were 964, 171, 97 and 254 µg/ml, respectively, while for 72-h incubation they were 283, 66, 63 and 68 µg/ml. As far as we know, this is the first report on the effect of *H. physodes* ME and their depsidones on HeLa cells.

## Introduction

Depsidones are secondary metabolites mainly present in the lichens, even though they have been found in

some fungi and higher plants. Their structure consists of two phenolic acids linked together by both ester and ether bonds giving the rigid 11H-dibenzo[b,e][1,4]dioxepin-11-one ring (Legaz et al., 2011; Stojanović et al., 2012). Correche and Carrasco (2002) found that nine depsidones (pannarin, 1'-chloro pannarin, salazinic acid, psoromic acid, fumarprotocetraric acid, lobaric acid, vicanicin, stictic acid and variolaric acid) exhibited a significant cytotoxic activity on cell cultures of rat lymphocytes. Among the tested depsidones greater toxicity was observed in the structures with neighbouring groups that can form a hydrogen bond. Lobaric acid showed an anti-proliferative effect on the following cancer cell lines: Capan-1, Capan-2 and PANC-1, T47-D, PC-3, NCI-H1417, NIH:OVCAR-3, AGS, WiDr, HL-60, K-562, JURKAT, ZR-75-1, and K-562 (Ogmundsdottir et al., 1998; Haraldsdottir et al., 2004). Variolaric acid and α-alectoronic acid were active against B16 murine melanoma cells (Millot et al., 2007). Micheletti et al. (2009) found that salazinic acid and its di-O-alkyl derivatives showed cytotoxic activity in cell lines HCT-8, SF-295 and MDA/MB-435. Diploicin showed cytotoxic activities against the B16 murine melanoma and HaCaT human keratinocyte cell lines (Millot et al., 2009). Pannarin inhibited growth of DU-145 (Russo et al., 2006) and M14 cell lines (Russo et al., 2008).

Some depsidones isolated from fungi (Khumkomkhet et al., 2009; Abdou et al., 2010) and higher plants (Xu et al., 2000; Ito et al., 2001; Permana et al., 2001, 2005) also showed cytotoxic activity against various cancer cell lines. Atrovirisidone isolated from roots of *Garcinia atroviridis* (Permana et al., 2005) and botryorhodine isolated from endophytic fungus *Botryosphaeria rhodina* showed some cytotoxicity on HeLa cells (Abdou et al., 2010). Reports concerning the biological activities of *Hypogymnia physodes* depsidones in rat thymocytes (Pavlovic et al., 2013) and micronucleus distribution in human lymphocytes (Stojanović et al., 2013) and anti-proliferative effects of *H. physodes* methanol extracts on HCT-116 cell lines (Mitrović et al., 2011) have been

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Abbreviations: 5-FU – 5-fluorouracil, ME – methanol extracts, P1 – physodalic acid, P2 – physodic acid, P3 – 3-hydroxy physodic acid.

published recently. Pavlović et al. (2013) showed that higher concentrations of physodalic acid and physodic acid induce thymocyte toxicity mainly through induction of oxidative stress, while the cytotoxic effect of 3-hydroxyphysodic acid is not followed by altered anti-oxidant/oxidant balance.

We found that physodalic acid, physodic acid, and 3-hydroxy physodic acid at a concentration of 1 µg/ml exerted a beneficial effect on lymphocyte cells, causing a significant decrease in the frequency of micronucleus in comparison with the positive control, Amifostin WR-2721. Among the tested depsidones, 3-hydroxy physodic acid exhibited the most prominent effect, decreasing the frequency of micronucleus by 30.3 %, followed by physodic acid (28.2 %) and physodalic acid (22.0 %) (Stojanović et al., 2013). *H. physodes* methanol extract demonstrated significant inhibition of HCT-116 cell growth in a dose- and time-dependent manner (Mitrović et al., 2011).

Since HeLa cells are used as a common research model, especially in the examination of anti-proliferative effects, the objective of this study was to investigate the effect of *H. physodes* methanol extract (ME) and its depsidones, physodalic acid (P1), physodic acid (P2), and 3-hydroxy physodic acid (P3) on HeLa cell lines. To the best of our knowledge there are no data on the effect of *H. physodes* ME and its depsidones on HeLa cells.

## Material and Methods

### Lichen material

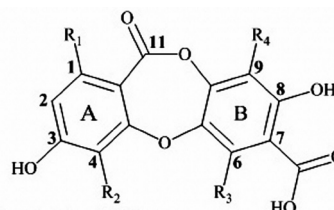
Lichen *Hypogymnia physodes* (L.) Nyl., (syn: *Parmelia duplicata* var. *douglasicola* Gyelnik, *Parmelia physodes* (L.) Ach., *Parmelia oregana* Gyelnik; common names: monk's-hood lichen, hooded tube lichen, puffed lichen) growing on the *Prunus domestica* tree was collected at the locality: Donje Vlase (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. A voucher specimen was deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Nis (voucher number 6576).

### Preparation of lichen extracts

The mixture of powdered air-dried lichen material (10 g) and methanol (200 ml) was sonicated for 30 min, then left at room temperature overnight, and filtered (Tay et al., 2004). The extract dry residue was obtained using a rotary evaporator with the water bath set at 40 °C. The ME extracts yield was 10.8 % (w/w).

### Isolation and identification of depsidones (1–3)

Isolation of P1–P3 was done by column chromatography according to the known procedure (Lukač and Onderka, 2006). The purity of the isolated depsidones was determined by HPLC-DAD and amounted to 93.8, 95.6, and 94.5 % for P1, P2, and P3, respectively.



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):  
 $R_2=OH$ ;  $R_1=CH_2COCH_2(CH_2)_3CH_3$ ;  $R_4=H$ ;  
 $R_3=C_5H_{11}$

Fig. 1. The structures of physodalic acid (P1), physodic acid (P2) and 3-hydroxyphysodic acid (P3).

Compounds P1–P3 were identified as physodalic acid (P1; 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 (P2; 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 (P3; 3,4,8-trihydroxy-11-oxo-1-(2-oxoheptyl)-6-pentyl-11*H*-dibenzo[*b,e*][1,4]dioxepin-7-carboxylic acid) (Fig. 1) by comparing their UV, MS, 1H- and 13C-NMR data with those given before (Jones et al., 1976; Yoshimura et al., 1994; Millot et al., 2007), as well as with data computed by CHEM draw ultra 11 software.

### HPLC analyses

HPLC was carried out using the HPLC system 1200 series with a diode array detector. The separations were achieved on a Zorbax XDB C18 (5 µm, 150 mm × 4.6 mm) column (Agilent Technologies, Wilmington, DE). The mobile phase was 80% aqueous methanol (v/v) that contained 0.2% formic acid. The mobile phase was pumped at the 0.5 ml.min<sup>-1</sup> flow rate, while the injection volume was 5 µl (1 mg of the dry extract dissolved in 1 ml of methanol), at 25 °C. The chromatograms were recorded at 254 nm. Methanol was of HPLC grade, and was purchased from Merck (Darmstadt, Germany). Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA). The constituents of the extracts were identified by comparison of their retention times and by co-injection of the isolated substances. Relative representation of the studied components based on DAD peaks was 28.7 %, 26.5 % and 14.8 % for P1, P2 and P3, respectively.

### Cell culture

The effect of lichen extracts on viability and proliferation was examined in the HeLa cell line. HeLa cells were cultivated in DMEM (PAA Laboratories GmbH, Pasching, Germany), which was supplemented with 10% FCS (Gibco, Paisley, Great Britain), penicillin (100 U/mL), streptomycin (100 µg/ml), and 4 mM L-glutamine.

HeLa cells were seeded into sterile 96-well plates. The cells were incubated for 24 h in the atmosphere saturated with aqueous vapour, at 37 °C with 5% CO<sub>2</sub> in 100 µl of culture medium. After that, 100 µl of lichen extracts was added in proper concentrations per wells. In order to examine cytotoxicity, the cells were incubated with the lichen extracts for 24 h. To examine the cytostatic effect, the cells were incubated with lichen extracts for 72 h. Culture medium without lichen extracts was a negative control. 5-Fluorouracil (5-FU) was used as positive control in both incubation periods, while saponin was used in the 24-h incubation period.

The experiments were performed in triplicates at minimum.

### MTT test

Assessment of cell viability and proliferation was made through a colorimetric assay, using MTT. Yellow tetrazole of MTT is reduced to purple formazan by mitochondrial succinate dehydrogenase of metabolically active cells.

After 24 and 72 h of incubation culture medium and extracts were removed from wells and the cells were washed with PBS, and finally 100 µl PBS/well was added. After that, 20 µl of MTT was added to each well and plates were incubated at 37 °C for 3 h. After the incubation time, supernatant was removed and formazan was dissolved in 100 µl of isopropanol/well. The absorbance was read at 540 nm in a multichannel spectrophotometer (Multiskan Ascent N°354, Thermo Labsystems, Vantaa, Finland). Absorbance of the negative control was regarded as 100 % and percentage values of the extracts were calculated as follows: absorbance of the extracts/absorbance of the negative control \* 100 % = percent of the extract compared to the negative control.

### Data analysis

Results are presented as mean ± SD. Significant differences between the groups were analysed in SPSS 20 with Student's *t*-test. Statistical difference was considered significant at  $P < 0.05$  and  $P < 0.01$ . The concentration of the sample required to inhibit cell growth by 50 % (IC<sub>50</sub>) in comparison with the growth of the cell control was determined by regression analysis.

## Results

Three compounds were isolated from *H. physodes* ME and identified as P1, P2, and P3 (Fig. 1).

MTT assay was used to investigate the cytotoxicity of isolated depsidones (P1–P3) and ME in HeLa cells at a concentration of 10–1000 µg/ml for incubation periods of 24 and 72 h. The results are presented in Fig. 2.

Compounds P2 and P3 showed a similar profile of activity on HeLa cells with a significant decrease in cell viability (24-h incubation) at concentrations ranging from 40 to 400 µg/ml ( $P < 0.05$ ;  $P < 0.01$ ). HeLa cells were less sensitive to P1 and ME. A significant increase in activity was observed in the concentration range of

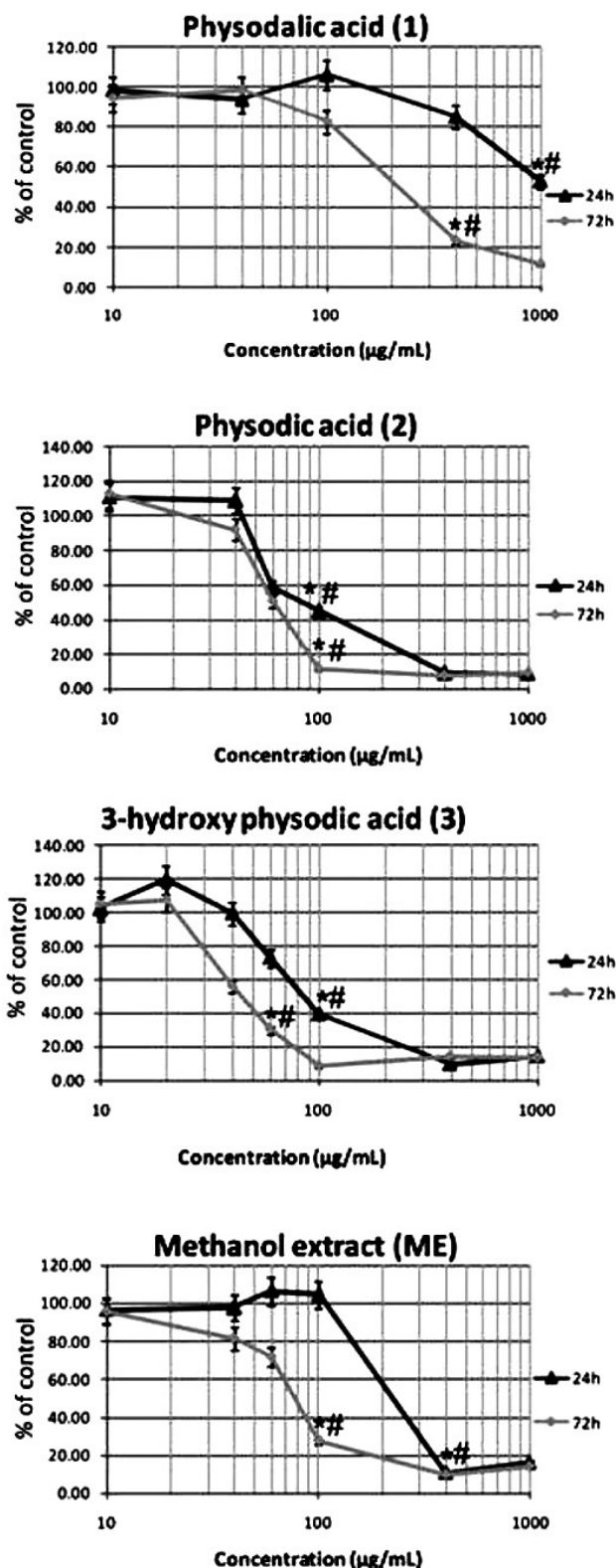


Fig. 2. The effect of physodalic acid (1), physodic acid (2), 3-hydroxyphysodic acid (3), and methanol extract (ME) on viability and proliferation of HeLa cells determined by MTT test. Results are presented as mean percentage ± SD. \* –  $P < 0.05$ , # –  $P < 0.01$  compared to the control.

200–1000 and 100–1000 µg/ml, respectively. IC<sub>50</sub> values for P1, P2, P3 and ME were 964, 171, 97 and 254 µg/ml (Table 1). These values were significantly higher than

Table 1. The inhibitory effects of depsidones (P1-P3), methanol extract (ME) and 5-fluorouracil (5-FU) on HeLa cells after 24-h and 72-h incubation (expressed as  $IC_{50} \pm SD$ ,  $\mu\text{g/ml}$ )

Sample	$IC_{50} \pm SD$ ( $\mu\text{g/ml}$ )	
	24 h	72 h
Physodalic acid (P1)	964.38 $\pm$ 104.23	282.91 $\pm$ 5.43
Physodic acid (P2)	170.50 $\pm$ 72.81	65.96 $\pm$ 2.40
3-Hydroxy physodic acid (P3)	97.35 $\pm$ 4.89	63.41 $\pm$ 9.12
Methanol extract (ME)	253.54 $\pm$ 13.11	67.74 $\pm$ 6.76
5-Fluorouracil (5-FU)	8.34 $\pm$ 0.72	0.84 $\pm$ 0.11

$IC_{50}$  of 5-FU and saponin (8.34 and 0.22  $\mu\text{g/ml}$ , respectively).

The extended incubation period (72 h) significantly increased the activity of P1 at concentrations of 100, 400 and 1000  $\mu\text{g/ml}$ , activity of P2 at concentration of 100  $\mu\text{g/ml}$ , and activity of P3 at concentrations of 40 and 100  $\mu\text{g/ml}$ . The  $IC_{50}$  values observed for P1, P2, P3 and ME after 72-h incubation (283, 66, 63 and 68  $\mu\text{g/ml}$ , respectively (Table 1) were several times lower than those after 24-h incubation, maintaining the same inter-relationship, but still higher than  $IC_{50}$  of 5-FU (0.84  $\mu\text{g/ml}$ ), known anti-cancer drug which acts principally as a thymidylate synthase inhibitor.

Except for compound P1, all other samples showed a slight decrease in the activity at the concentration of 1000  $\mu\text{g/ml}$  compared to the concentration of 400  $\mu\text{g/ml}$ .

## Discussion

In this study two incubation periods (24 and 72 h) were used for HeLa cells with different concentrations of three depsidones (P1, P2 and P3) isolated from the ME of *H. physodes*, as well as the initial methanol extract. HeLa cells are a very useful tool for examination of cytotoxic and anti-proliferative activity of various pharmacologically active substances (Liu et al., 2012; Guo et al., 2013). The incubation period of 24 h was used to examine the short-term effect of the tested substances on the viability of HeLa cells, in order to evaluate their possible cytotoxic activity. Comparing the  $IC_{50}$  values of the investigated depsidones, the minimum value was found for compound P3, followed by depsidone P2, and compound P1 showed the least toxic activity. The initial ME had the  $IC_{50}$  value 3.8 times lower than the  $IC_{50}$  of the least toxic acid P1, but was 1.4 and 2.6 times higher than  $IC_{50}$  for P2 and P3, respectively. The period of 72-h incubation of HeLa cells with the test substance was used to evaluate the effect on cell growth in culture, i.e. on cell proliferation. It is expected that, under normal conditions, during this period the cells divide more times, which multiplies the number of cells. For HeLa cells the doubling time is about 24 h, which means that in this period they undergo at least one division. Substances that reduce cell viability and/or suppress the division mechanism will lead to a reduction in the number of cells in culture that will manifest itself as lower MTT reduction. The reduction is performed by

mitochondrial enzymes and the produced formazan is a direct consequence of mitochondrial activity of the cells. Since mitochondrial activity occurs only in living cells, it is possible to measure the number of viable cells by the amount of produced formazan.

When comparing the incubation period of 72 h with that of 24 h it is evident that the toxic effects were more pronounced over a longer period. A longer incubation period with stable substances such as depsidones showed cumulative effects over time.  $IC_{50}$  values for samples P2, P3 and ME for 72 h incubation were very similar because of the demonstrated time-dependent toxicity, which was very high in all the three above-mentioned specimens after 72 h of incubation. Low acute toxicity and expressed cumulative or specific toxicity of the examined substances on cell division is a good recommendation for further investigation.

It is worthwhile to mention that the observed  $IC_{50}$  values for compounds P2 and P3 after 72-h incubation were similar to  $IC_{50}$  for dibenzofuran derivative usnic acid, the most tested lichen secondary metabolite and effective anti-cancer agent against various cell lines (Bačkorová et al., 2011).

There was a notable difference in short-term cytotoxicity and anti-proliferative activity of compounds P2 and P3 compared to compound P1, which had the lowest activity among them. A possible explanation for the demonstrated differences could be found in the structure of the tested substances. Compounds P1–P3 belong to depsidones, which contain a rigid 11H-dibenzo[b,e][1,4]dioxepin-11-one ring. It has been shown (Correche and Carrasco, 2002) that this structure is relevant for cytotoxic activity and also for inhibition of HIV-1 integrase (Neamati et al., 1997). Regarding the correlation between the structure and the examined biological effect, the length of alkyl chains of the 11H-dibenzo[b,e][1,4]dioxepin-11-one core may play a key role in the obtained biological response. Namely, positions 1 and 6 in compounds P2 and P3 are substituted with long non-polar substituents, unlike compound P1 containing methyl groups in both positions, and this may be responsible for the similarity of action of P2 and P3, and their differences compared to the activity of P1. This would be consistent with the observation of Micheletti et al. (2009) that elongation of the alkyl chain causes an increase in the cytotoxic activity of salazinic acid di-O-alkyl derivatives in cell lines HCT-8, SF-295 and MDA/MB-435.

Half maximal inhibitory concentrations after 24-h incubation for the studied ME is almost identical to that determined for the *H. physodes* methanol extract activity in HCT-116 cells (254 µg/ml) (Mitrović et al., 2011). On the other hand, the IC<sub>50</sub> value after 72-h incubation for our ME (68 µg/ml) was lower than that determined for methanol extract investigated by Mitrović et al. (2011), which was 102 µg/ml.

In conclusion, physodic acid (P2) and 3-hydroxy physodic acid (P3), as well as the examined *H. physodes* ME, demonstrated a significant decrease in the viability and proliferation of HeLa cells, which is of interest for further investigation of their biological activity, including potential anticancer activity, taking into account that cancer is a proliferative disease. The presented results are a new contribution to the existing knowledge on the biological activity of lichens and their depsidones and enable more reliable determination of the activity-structure correlation.

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