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ISOLATION AND QUANTITATIVE ANALYSIS OF PHYSALIN D IN THE FRUIT AND CALYX OF *PHYSALIS ALKEKENGI* L.

Eszter Laczkó-Zöld,[1] Péter Forgó,[2] István Zupkó,[3]
Eşianu Sigrid[1] and Judit Hohmann[2,4]*

[1]Department of Pharmacognosy, University of Medicine and Pharmacy Târgu Mureş, 540139 Târgu Mureş, Romania
[2]Department of Pharmacognosy, University of Szeged, H-6720 Szeged, Hungary
[3]Department of Pharmacodynamics and Biopharmacy, University of Szeged, H-6720 Szeged, Hungary
[4]Interdisciplinary Centre of Natural Products, University of Szeged, H-6720 Szeged, Hungary

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Physalin D was isolated from the methanol extract of *Physalis alkekengi* L. fruits by combination of different chromatographic methods (CPC, TLC, HPLC). The structure was elucidated based on ^{1}H and ^{13}C NMR spectral analysis with the aid of 2D-correlation spectroscopy (^{1}H, ^{1}H-COSY, HSQC and HMBC) and comparison with literature data. The quantity of physalin D in mature and immature fruits and calyces was determined by RP-HPLC-UV method. Among the studied samples, immature calyx showed the highest content of physalin D (0.7880 ± 0.0612%), while mature calyx contained 4 times less amount (0.2028 ± 0.016%). The physalin D content of the fruit was much lower; immature fruits contained 0.0992 ± 0.0083% physalin D and mature fruits 0.0259 ± 0.0021%. The antiproliferative activity of the $CHCl_3$ extract and its fractions was tested on three cancer cell lines (HeLa, MCF-7 and A431). The antiproliferative activity of physalin D is discussed with regard the published data.

Keywords: Physalin D – steroids – cytotoxic activity – *Physalis alkekengi* – Solanaceae

INTRODUCTION

The genus *Physalis*, which belongs to the family Solanaceae, includes about 120 species, mainly distributed in Mexico, Europe, and Southeast and Central Asia. The fruit of the most widely used plant *Ph. alkekengi* var. *franchetii* (Mast) Makino (Chinese lantern) is popular as fruit and food additive, and is also used in the traditional Chinese medicine for the treatment of different diseases [25]. *Ph. alkekengi* L., known as "winter cherry" grows in western-Asia, Middle- and South-Europe; this is the only species of the genus occurring in the Carpathian-Basin [2, 23]. Previous studies have focused on the antileukemic, antitumor, immunomodulator, antiprotozoal, molluscicidal, antihepatoma, and antinociceptive activities of *Physalis* species [1].

Physalis species are known to contain different type of compounds, including physalins, alkaloids, flavonoids, carotenoids, vitamins and polysaccharides, among which physalins are the most active representative of secondary metabolites of the

*Corresponding author; e-mail address: hohmann@pharm.u-szeged.hu

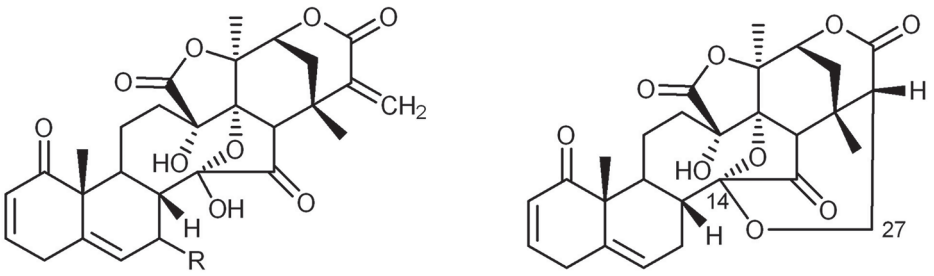

Fig. 1. Structures of physalins

genus. Physalins are steroidal constituents, they possess a 16,24-cyclo-13,14-secoergostan framework, and are classified into types A and B according to the presence or absence of a C(14)-O-C(27) epoxy linkage, respectively (Fig. 1) [5, 19].

The first physalins were isolated from *Physalis alkekengi* var. *franchetii* (Mast) Makino [10, 13, 20, 21]. A large number of physalins have been characterized in the past years, mostly isolated from roots and aerial part of *Physalis* species [3, 7, 9, 17, 18, 29, 30, 31]. Some physalins were known to exert cytotoxic activity against tumor cells [3, 8, 19]. Moreover, their antimalarial, antimycobacterial, antileishmanial, antinociceptive and chemopreventive activities were also demonstrated [4, 14, 22, 32].

Ph. alkekengi var. *franchetii* (Mast) Makino*, Ph. angulata* L.*, Ph. minima* L.*, Ph. peruviana* L. and *Ph. pubescens* L. are the most studied species and more than 30 physalins have been described from these plants [11, 12, 16, 18, 26, 27, 28, 33]. In case of *Ph. alkekengi*, physalins D, L, N and O were isolated from the herb and calyx [6, 12, 28], and physalin D was found to be the main constituent. No comprehensive study has yet been reported about the content of this compound in the fruits and calyces, therefore our aim was the isolation and quantitative determination of physalin D. With regard to the cytotoxic activity of physalins, the isolation process was guided by *in vitro* cytotoxic assay.

MATERIALS AND METHODS

General

^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 and $CDCl_3$ using TMS as internal standard at 500 and 125 MHz, respectively, on a Bruker Avance DRX500 spectrometer. Centrifugal planar chromatographic (CPC) separation was performed using a Chromatotron® (Harrison Research Model 8924, USA) apparatus. HPLC separation was performed using a Waters Separation Module with a Waters 600 Controller and Waters 2998 PDA detector on a LiChroCART RP18 column (5 µm, 250 × 4 mm). Operation and data acquisition was performed using Empower Pro Software. All chemicals used for extraction and isolation were of analytical or HPLC-

grade (Molar Chemicals Kft, Budapest, Hungary or Merck, Darmstadt, Germany). Silica gel $60GF_{254}$ and TLC plates from Merck (Darmstadt, Germany) were applied.

Plant material

Mature and immature calices-with-fruits of *Ph. alkekengi* were collected from wild plants growing in the Mures district (Romania) in September 2015 and were dried at room temperature. A voucher specimen (No. 632/2015) has been deposited at the Herbarium of the Department of Pharmacognosy, University of Medicine and Pharmacy Târgu Mureş, Târgu Mureş, Romania.

Extraction and isolation

A 200 g dried powdered mature fruit without calyx was macerated in 400 mL 50% MeOH overnight, then extracted with 2000 mL 50% MeOH by percolation, and concentrated in vacuum to approximately 400 mL. This extract was separated by solvent–solvent extraction with *n*-hexane and then with chloroform. The chloroform extract was concentrated under reduced pressure and fractionated using different chromatographic methods.

CPC separation of chloroform extract was performed on silica gel $60GF_{254}$ with gradient system of cyclohexane–EtOAc–EtOH–MeOH of increasing polarity gaining 74 fractions. These fractions were monitored by TLC (silica gel $60F_{254}$, cyclohexane–EtOAc 3:7) and the appropriate fractions were combined in fractions P1 – P7.

Fractions P1 – P7 were tested for antiproliferative activity in order to find active fractions or compounds.

Antiproliferative assay

Antiproliferative effects were measured *in vitro* on three human cell lines (ECACC; Salisbury, UK): HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma) and A431 (skin epidermoid carcinoma). The cells were cultivated in minimum essential medium (Gibco BRL; Paisley, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic mixture in a humified atmosphere of 5% CO_2 at 37 °C. Cells were seeded into a 96-well plate at a density of 5000 cells/well. After the plate stood overnight, the medium containing the tested samples was added. Ten mg/mL stock solutions of the tested samples were prepared with dimethyl sulfoxide (DMSO) and the highest DMSO concentration of the medium (0.3%) did not have any significant effect on cell proliferation. After a 72-h incubation period, cytotoxicity was measured with the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay [23]. After a 4-h contact period, the precipitated formazan crystals were dissolved in DMSO, and the absorbance was read at 545 nm with an ELISA

reader. Doxorubicin and cisplatin were used as positive controls; their IC_{50} values were 0.15 mM (HeLa), 0.28 mM (MCF-7), 0.15 mM (A431) for doxorubicin, and 12.43 mM (HeLa), 9.63 mM (MCF-7), 2.84 mM (A431) for cisplatin.

Quantitative HPLC determination of physalin D

Column: LiChroCART RP18 (5 µm, 250 × 4 mm), mobile phase: acetonitrile–H_2O (7:3), flow rate 0.7 mL/min, or acetonitrile–H_2O (1:1), flow rate 0.4 mL/min; or MeOH–H_2O (4:6), flow rate 0.7 mL/min, detection at 225.4 nm. Sample preparation: extracts were prepared from 1 g exactly weighted powdered plant materials with 25 mL 50% MeOH by ultrasound assisted extraction for 15 minutes. The extracts were concentrated in vacuum to approximately ½ volumes. These extracts were purified with extraction of *n*-hexane (5 × 30 mL) in a separation funnel, followed by extraction with chloroform (5 × 30 mL). The chloroform phases were combined, evaporated and before HPLC analysis dissolved in 500 µL MeOH. Injected volume: 10 µL.

RESULTS AND DISCUSSION

Dried and ground fruits without calyces of *Ph. alkekengi* were extracted with 50% MeOH. After concentration, the extract was subjected to solvent–solvent partition, yielding *n*-hexane, $CHCl_3$ and aqueous MeOH fractions. The antiproliferative effect of the $CHCl_3$ extract was tested on HeLa and MCF-7 cell lines (Table 1). This extract was fractionated by CPC on silica gel and after TLC monitoring seven fractions (P1 – P7) were obtained.

Antiproliferative effect of fractions P1 – P7 was tested on HeLa, MCF-7 and A431 cell lines. The most active fraction was P3 on all cell lines, followed by P2 and P4 on HeLa and MCF-7 cells, and P4 on the A431 cell line (Table 2).

A white crystalline substance (**1**) was isolated from fraction P3 by crystallization from cold MeOH. The structure of compound 1 was elucidated by means of ^{1}H and ^{13}C NMR spectral analysis. The ^{13}C NMR spectrum was recorded in $CDCl_3$ and in DMSO, and showed a sterol-type molecule with 28 C atoms, suggesting physalin structure. The unambiguous NMR signal assignment was achieved with the aid of

Table 1
In vitro antiproliferative activity of $CHCl_3$ extract in MCF-7 and HeLa cells

Concentration µg/mL	MCF-7		HeLa	
	Growth inhibition %	SEM	Growth inhibition %	SEM
1	41.26	2.978	20.98	2.001
10	73.43	1.269	64.87	0.912
30	103.8	0.426	101.3	0.263

Table 2
In vitro antiproliferative activity of fractions on HeLa, MCF-7 and A431 cell lines

		Growth inhibition (%)±SEM		
		HeLa	MCF-7	A431
P1	10 µg/mL	36.95±1.22	50.33±1.67	<5*
	30 µg/mL	49.05±1.18	50.85±2.83	42.94±0.95
P2	10 µg/mL	16.60±0.40	<5	<5
	30 µg/mL	62.37±1.15	61.47±1.15	81.15±0.78
P3	10 µg/mL	88.85±0.42	87.84±1.02	79.96±1.06
	30 µg/mL	96.74±0.53	90.88±1.30	85.67±0.43
P4	10 µg/mL	25.89±1.83	29.02±2.73	29.61±1.86
	30 µg/mL	75.42±0.50	95.69±0.48	91.50±0.51
P5	10 µg/mL	31.69±1.71	26.01±2.04	28.28±2.78
	30 µg/mL	42.02±0.27	52.06±1.82	46.15±0.43
P6	10 µg/mL	16.84±1.87	8.24±0.68	<5
	30 µg/mL	19.53±1.65	32.16±3.06	7.34±0.80
P7	10 µg/mL	12.06±2.27	<5	<5
	30 µg/mL	15.76±2.53	7.84±2.44	<5

*Growth inhibition values of <5% are regarded as negligible and therefore not given numerically.

Table 3
^{1}H NMR spectral data of physalin D (**1**) in DMSO-d_6
(500 MHz, δ ppm. multiplicity. coupling constant J in Hz)

Protons	**1**	Protons	**1**
H-2	5.77 dd (8.6; 2.4)	H-12β	1.47 dd (16.2; 9.8)
H-3	6.62 dd (10; 2.9)	H-13	5.70 s
H-4α	1.96 m	H-16	2.77 s
H-4β	3.10 brd (19.4)	H-19	1.11 s
5-OH	4.21 s	H-21	1.81 s
H-6	3.49 m	H-22	4.56 br
6-OH	4.87 d (4.1)	H-23	1.95 d (12.7)
H-7	1.80 m	H-23	2.10 m
H-7	1.80 m	H-25	2.87 d (3.7)
H-8	2.20 dt (10.5; 4.5)	H-27	3.58 d (12.9)
H-9	3.11 m	H-27	4.25 dd (13.3; 4.4)
H-11	0.93 m	H-28	1.16 s
H-12α	2.10 m		

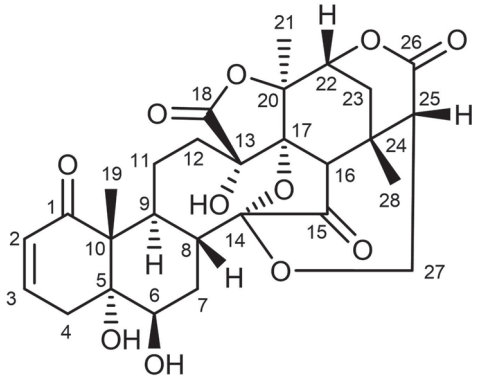

Fig. 2. Structure of physalin D (1)

2D-correlation spectroscopy (^{1}H, ^{1}H-COSY, HSQC and HMBC) (Table 3). Compound **1** was identical in all of its spectral characteristics with physalin D (Fig. 2) [7].

Strong cytotoxic activity of this compound was reported by Magalhães et al. [15] Physalin D (**1**) displayed considerable cytotoxicity against nine tumor cell lines, showing IC_{50} values in the range of 0.51–4.47 μM *in vitro*. The *in vivo* tumor growth inhibitory activity was studied in mice transplanted with Sarcoma 180 tumor model. The physalin D (**1**) treatment afforded antitumor activity associated with reversible toxic effects to the liver and kidney [15].

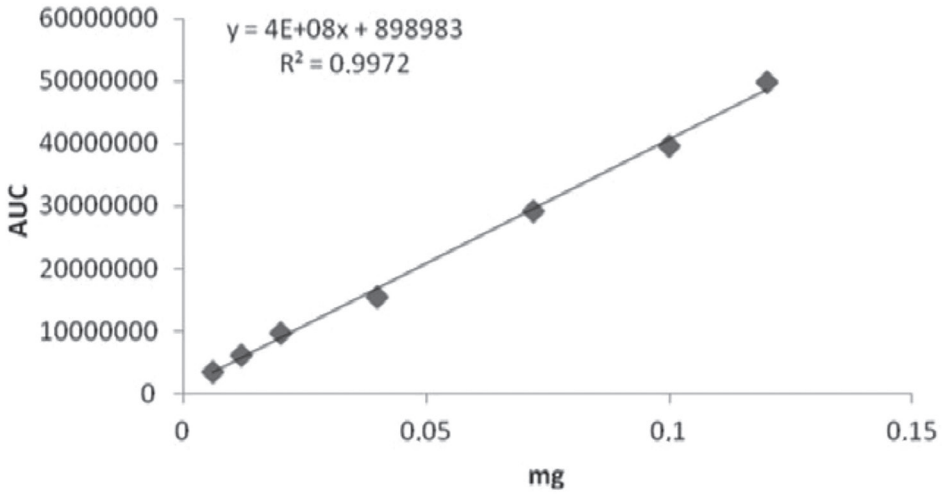

Fig. 3. Calibration curve with physalin D (**1**)

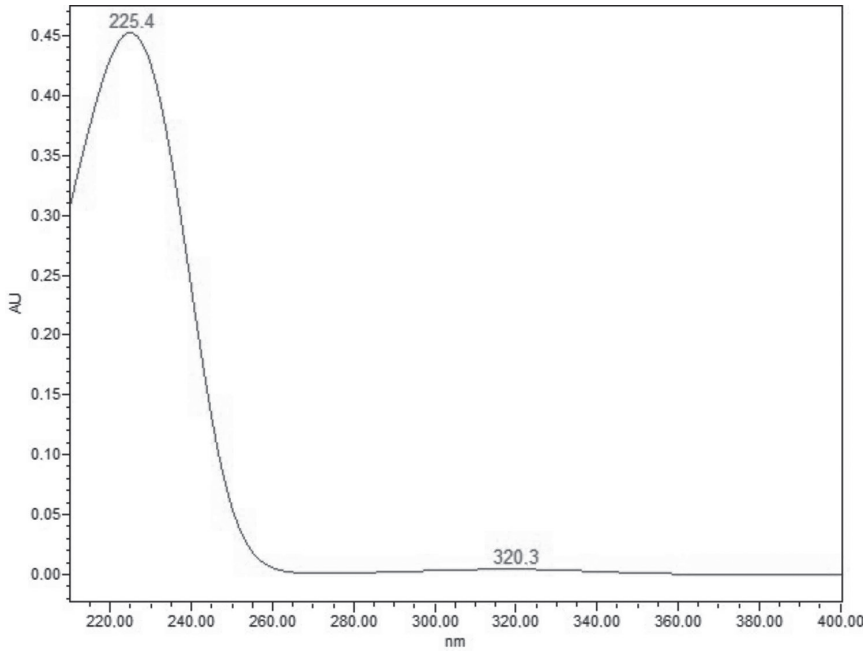

Fig. 4. UV spectrum of physalin D (**1**)

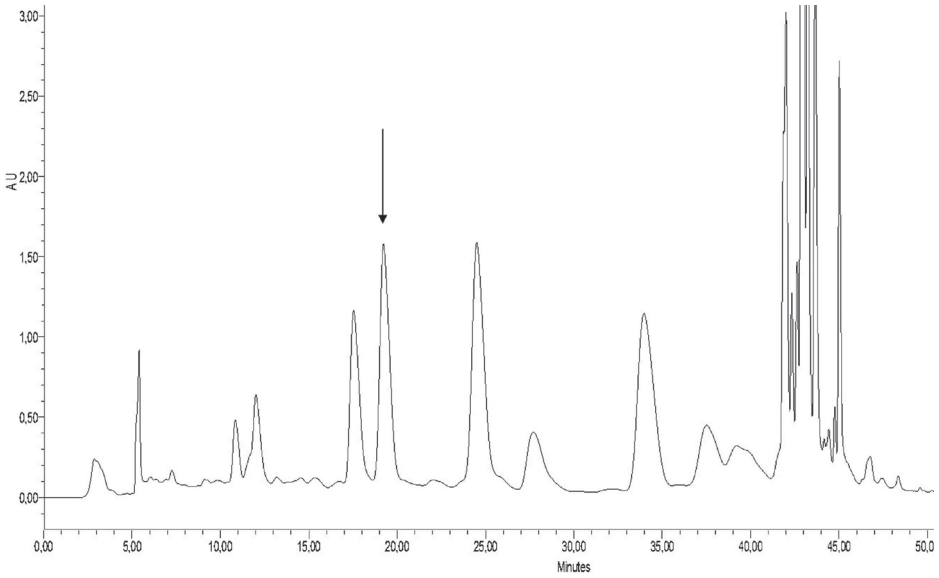

Fig. 5. HPLC chromatogram of immature winter cherry calyx extract at 225.4 nm (physalin D RT = 19.23 min)

Quantitative determination of physalin D in fruits and calyces

The quantitative determination of physalin D (1) was achieved by RP-HPLC-UV method with detection at 225.4 nm, the UV absorption maximum of the compound (Fig. 3). $CHCl_3$ extracts were prepared from the dried mature and immature fruits and calyces with solvent–solvent partition of the 50% MeOH extract. The first step of the quantitative analysis was the determination of the calibration curve. As shown in Fig. 4, good linearity was obtained with $R^2 > 0.9972$. HPLC separation was performed on LiChroCART RP18 reversed phase column with one of the acetonitrile-H_2O mixtures as mobile phases (see Materials and Methods), depending on the composition of the extract, trying to obtain an efficient separation in the area of interest. A representative chromatogram is presented in Fig. 5.

Table 4
Physalin D (**1**) content of winter cherry fruit and calyx

	Physalin D g/100 g plant material
Immature fruits	0.0992 ± 0.0083
Mature fruits	0.0259 ± 0.0021
Immature calyx	0.7880 ± 0.0612
Mature calyx	0.2028 ± 0.016

Data expressed as mean ± SD of triplicate analysis.

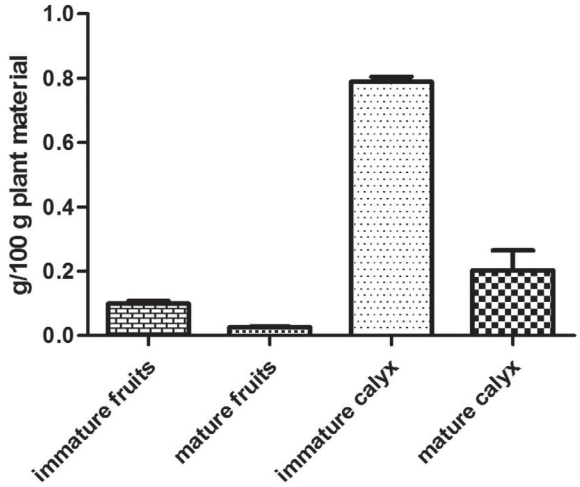

Fig. 6. Physalin D (**1**) content (%) of winter cherry fruit and calyx

Among the studied samples, immature calyx showed the highest content of physalin D (1) (0.7880 ± 0.0612 g%), while mature calyx contains 4 times less (0.2028 ± 0.016 g%). The physalin D (1) content of the fruit is much lower; immature fruits contain 0.0992 ± 0.0083 g% physalin D and mature fruits 0.0259 ± 0.0021 g% (Table 4, Fig. 6).

In conclusion, with combination of different chromatographic methods physalin D (1) was isolated from the 50% MeOH extract of *Ph. alkekengi* fruits. The structure was elucidated by ^{1}H and ^{13}C NMR spectral analysis with the aid of 2D-correlation spectroscopy (^{1}H, ^{1}H-COSY, HSQC and HMBC) and comparison with literature data. The quantity of physalin D (1) was evaluated in fruits and calyces and cytotoxic activity was tested on three cancer cell lines (HeLa, MCF-7 and A431). The occurrence and quantitative determination of physalin D in fruits of *Ph. alkekengi* is reported here for the first time.

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