

Original Article

## Isolation and Identification of Calystegines in Root Cultures of four *Physalis* Species

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### Abstract

Calystegines were isolated from the root cultures of *Physalis divaricata*, *P. pubescens*, *P. philadelphica*, and *P. philadelphica* (solanaceae). Calystegines of *P. divaricata* were identified as calystegine A<sub>3</sub>, A<sub>5</sub>, B<sub>1</sub> and B<sub>2</sub>, with concentrations of 6.99, 4.41, 8.52, and 14.7 µg/g in fresh mass, respectively. Root cultures of *P. pubescens* contain calystegine A<sub>3</sub>, B<sub>1</sub> and B<sub>2</sub>, *P. philadelphica* only contains calystegine B<sub>2</sub> and *P. philadelphica* root cultures solely consist of calystegine A<sub>3</sub>. Isolation and identification of calystegines have been achieved by ion exchange column chromatography and gas chromatography, using authentic samples.

**Keywords:** Calystegine; polyhydroxy nortropane alkaloids; *physalis*; solanaceae; gas chromatography.

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### Introduction

Plants have been used as a drugs for centuries. The potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250000-500000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties (1, 2).

*P. divaricata* is an annual of 15 to 45 cm height, sometimes with sparse hairs on the stem. The plant bears smooth, dark-green leaves and yellowish-white flowers. The fruit is a round, yellow to orange, about the size of a cherry, containing numerous flat kidney-shaped seeds.

This plant is distributed in some parts of Iran (Lorestan and Fars provinces), Afghanistan, Pakistan and India.

The name *Physalis* derives from the Greek phusa (a bladder), for the five-cleft calyx greatly increasing in size after the corolla falls off, thus enclosing the fruit in a large, leafy bladder.

Calystegines are nortropane alkaloids with three to five hydroxyl groups in various positions. The name of these alkaloids derives from their first detection in transformed root cultures of *Calystegia sepium* (Convolvulaceae) (3). They are also found in intact plant roots of *C. sepium*, *C. arvensis* and *Atropa belladonna* (Solanaceae) (4). Tropane alkaloids and their derivatives are formed in the roots and transported to the aerial parts of the plant (5, 6).

The biosynthesis of calystegine shares

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metabolic steps and enzymes of the classical tropane alkaloid formation, which is expressed in a number of Solanaceae and some Convolvulaceae species (7). Calystegine biosynthesis via the tropane alkaloid pathway has been proven by enzymatic studies, gene isolation, NMR and GC-MS after labelled precursor feeding (4). Two enzymes and their corresponding gene sequences are known for calystegine biosynthesis (Figure 1). The genes were isolated from calystegine forming Solanaceae, namely from *Solanum tuberosum*, *Hyoscyamus niger* and *Datura stramonium*. Should tropane alkaloids be also present in *Physalis divaricata* comparison of enzymes and genes involved in their biosynthesis to those of other Solanaceae may give clues on the diversity or relatedness of tropane alkaloid metabolism within the Solanaceae. For such studies, plant tissue cultures are of great advantage. From sterile cell or tissue cultures, enzyme and mRNA encoding them could be obtained more readily.

Calystegines are strong and selective glycosidase inhibitors. They share this activity with other hydroxylated nitrogen containing bicyclic compounds (3). They also have a role in plant defense mechanism and plant-insect interactions. In usual alkaloid extraction procedures that contain a lipophilic extraction step from an aqueous alkaline medium, they remain in the aqueous phase. That may explain why they have not been found earlier in well-examined plants like *Hyoscyamus* or *Atropa* species (4). The structures of the first calystegines were published in 1990 (7).

In the course of ethnobotanic investigations of Iranian plants, we screened several Solanaceae species for cytotoxic activity. The results indicated significant cytotoxic activity of methanolic extract of *Physalis divaricata* D. Don. A particular interest in the analysis of alkaloids derived from the tropane biosynthetic

pathway resulted from previous publications reporting calystegine in the fruits of *Physalis alkekengi* was arisen (8, 9). We therefore compared *Physalis divaricata* with three *Physalis* species with respect to its' capacity to form root cultures and to synthesize calystegines in the root cultures.

## Experimental

### Plant material

*Physalis divaricata* D. Don was collected in May 2003 from Fars (Iran), subsequently identified and the voucher specimen deposited at the Traditional Medicine and Materia Medica Research Center, Tehran, Iran. *P. divaricata*'s dried seeds were used for preparing root cultures. Seeds of *P. peruviana*, *P. philadelphica* and *P. pubescens* were obtained from the Botanical Garden, Martin-Luther-University Halle-Wittenberg, Germany.

### Root culture

Seeds were macerated first in H<sub>2</sub>O for 1 h and in EtOH 70% for 5 min subsequently. They were then transferred to a 6.5% sodium hypochlorite solution and kept for 6.5% for 5 min to improve germination. After washing with H<sub>2</sub>O (4 times), they were cultured in water-agar 0.5%. After a few days, roots of the germinated seeds were cut and cultured in 100-ml flasks containing 25-ml of B5-medium supplemented with 1µM indol-3-butyric acid and 3% sucrose on a gyratory shaker at 90 rpm in the dark. Before extraction, the roots were transferred to 300-ml flasks containing 75-ml of hormone-free medium and subcultured every two weeks.

### Extraction and derivatization

The extraction and GC measurements for calystegines were performed as described by Dräger (4). 2-5 g fresh mass of each root culture was extracted by 2×20 ml 50% (v/v) MeOH, using

**Table 1.** Concentration of calystegines in root cultures of four *Physalis* species (µg/g fresh mass).

species	Calystegine A3	Calystegine A5	Calystegine B1	Calystegine B2
<i>Physalis divaricata</i>	6.99	4.41	8.52	14.70
<i>P. peruviana</i>	0.008	-	-	-
<i>P. philadelphica</i>	-	-	-	0.006
<i>P. pubescens</i>	0.025	-	0.034	0.0008

an Ultra-Turrax homogenizer and prior to being centrifuged at 4500 g for 10 min at 4°C. MeOH was evaporated by a rotary evaporator (50°C), and the remaining crude extract was adjusted to 1 ml /g fresh mass, using distilled water. For determination of calystegine concentration, 1 ml extract was applied on a cation exchange column (Ion exchange I LAB Merck, column 1065×mm, ca 5ml ion exchange gel). The column was washed with H<sub>2</sub>O, until a neutral reaction of the elute was observed. Calystegines were eluted with 2 M NH<sub>3</sub>, and the elute concentrated in vacuo. Derivatization was necessary for GC analysis of calystegines. Further more, samples were dried by lyophilization. Trimethylsilyl ethers were formed with hexamethyldisilazane and trichlorosilane in pyridine at 80°C after 20 min.

### GC Instrumentation

Gas chromatograph HP 6890, column PHENOMENX SE52 (30×0.25 mm i.d., 0.25 µm film thickness) injection split ratio ca 1:20, detection simultaneous FID and PND, carrier gas Helium (1 ml /min), temperature program 160 °C, 2 min isotherm, 5°C/min up to 240 °C.

By comparing sample retention times with the related standard, calystegines were identified. Azobenzol was used as an internal standard in quantitative analysis, and the calystegines content were calculated by direct reference to

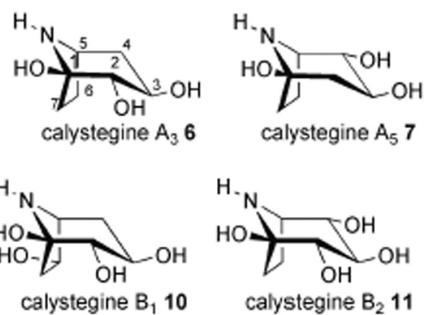


Figure 1. Structure of calystegines A3, A5, B1 and B2.

Azobenzol peak area units. Detection limits were ca. 60 pmol calystegine with FID detection and ca 10 pmol with PND detection in a sample volume of 1 µl.

### Result and discussion

Root cultures of *Physalis divaricata* contain more and greater amounts of calystegine A<sub>3</sub>, A<sub>5</sub>, B<sub>1</sub> and B<sub>2</sub> (Figure 1) with concentration of 6.99, 4.41, 8.52 and 14.70 µg/g fresh mass respectively, compared to other three investigated species (Table 1). GC chromatograms of *Physalis pubescens* with FID and PND detector are shown in Figure 2.

Based on tropane alkaloid biosynthesis pathway (Figure 3), it seems that tropinone reductase II (TRII), which catalyses the production of pseudotropine from tropinone in root cultures of *P. divaricata*, is much more

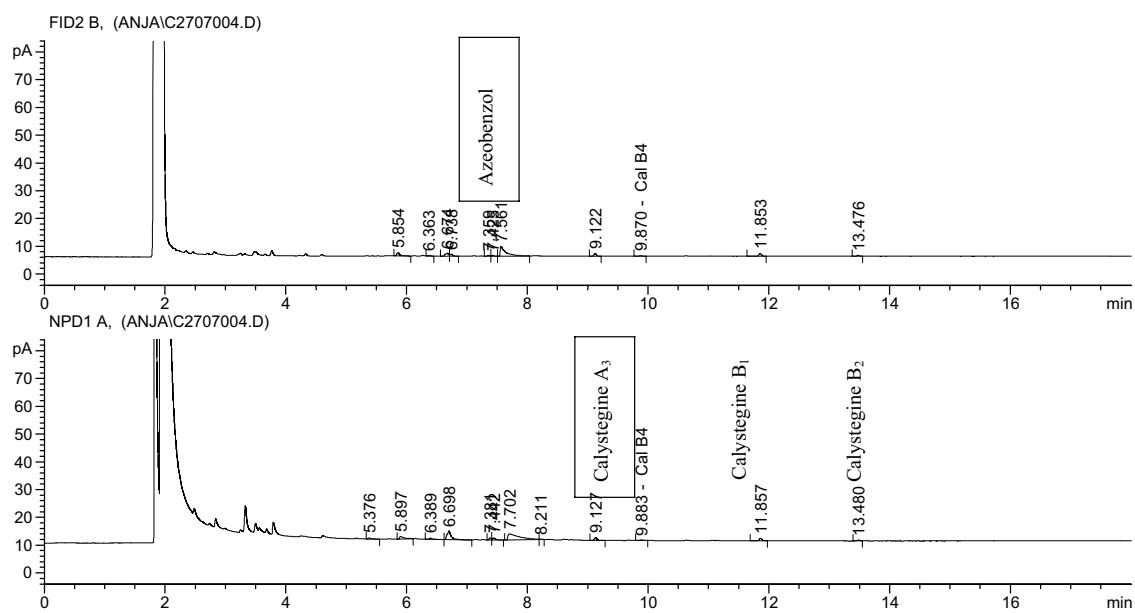
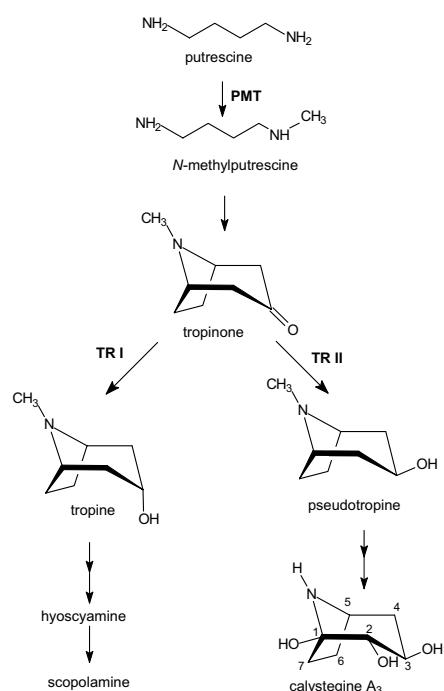


Figure 2. GC chromatogram of *Physalis pubescens* with FID and PND detector.



**Figure 3.** Tropane alkaloid biosynthesis

PMT= Putrescine Methyl Transferase

TR I = Tropinone reductase I

TR II= Tropinone reductase II

active than the other three investigated *Physalis* species. This could explain the presence small amounts of tropane alkaloids in *P. divaricata*.

The next step for further confirmation of tropane alkaloid biosynthesis pathway is to determine the amounts of tropinone, tropine and pseudotropine in these root cultures and compare these data with each other. This is being currently carried out and the results will be published soon after.

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