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Research Article

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Production of saponarin in *in vitro* cultures of *Gypsophila* species

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ABSTRACT

Phytochemical examination of Gypsophila species revealed the presence of various groups of biological active compounds such as triterpene saponins, flavonoids, sterols and volatiles. The main component of the flavonoid fraction in G. elegans and G. trichotoma is saponarin which hepatoprotective activity that is comparable to those of silymarin. In vitro cultures are often utilized to improve both biomass and secondary metabolite production. Four factors: the homogeneity of an in vitro cell population, the large availability of material, the high rate of cell growth and the good reproducibility, make the in vitro cultured cells suitable for production of valuable compounds. Therefore the aim of our study was to induce in vitro cultures of three species – Gypsophila trichotoma, G. altissima and G. paniculata – and determine the amount of saponarin produced. For the first time we initiated and maintained shoots and callus cultures obtained from in vitro germinated seeds. All in vitro cultures of Gypsophila species produced saponarin. Analysis was performed with an optimized HPLC method. The highest amount of saponarin was determined in the shoot cultures of G. trichotoma – 2.26 %. Rapidly growing cell lines were selected to increase the efficiency of saponarin production. These results could provide a practical means for cultivation of this plant species with medical importance and for further applications.

Key words: *Gypsophila trichotoma*, *G. altissima*, *G. paniculata*, saponarin, *in vitro* cell cultures

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INTRODUCTION

Flavonoids are the largest and most important group of polyphenolic compounds synthesized by plants with many potent biological properties. Over the past decade, scientists have become increasingly interested in the potential for various dietary flavonoids to explain some of the health benefits associated with fruit- and vegetable-rich diets. Flavonoids are universally present as constituents of flowering plants, particularly of food plants. The high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of neoplasms. The use of flavonoids for prevention and cure of human diseases is already widespread. These aspects made flavonoids an interesting object for industrial production.

Phytochemical examination of genus *Gypsophila* revealed the presence of various groups of biological active compounds such as triterpene saponins, flavonoids, sterols and volatiles [1-3]. Until now only two *Gypsophila* species – *G. elegans* and *G. trichotoma* – have been reported to contain saponarin (apigenin-6-C-glucosyl-7-O-glucoside), which possesses antioxidant, hypoglycemic, antimicrobial and hepatoprotective activities [4-5].

Plants have always been a suitable source for the production of pharmaceuticals. However, the quality and quantity of active substances from wild collected and field grown plants is often fluctuating and heterogeneous depending on environmental conditions. Infestation, diseases and the application of pesticides additionally decrease the quality of the plant material. *In vitro* culture of plants can overcome these problems, since the environmental conditions

affecting plant metabolism can be strictly controlled. Working with plant cells drastically reduces the preparation time, handling and storage costs associated with the traditional whole plant approaches.

Our research was focused on the use of plant tissue cultures of *Gypsophila* plants for improvement of natural compound production. The aim of our study was to induce *in vitro* cultures of three species – *Gypsophila trichotoma, G. altissima* and *G. paniculata* – and determine the amount of produced saponarin.

EXPERIMENTAL SECTION

2.1. Plant material

Seeds from *Gypsophila trichotoma* were collected near village Balgarevo, Bulgaria (Black Sea coast, locality "Zelenka"). Seeds from *G. altissima* and *G. paniculata* were received from Botanical Garden of Medical University, Targu-Mures, Romania.

2.2. Germination of seeds, shoot cultures and callus induction

The plant material was surface-sterilized in 80% alcohol for 1 min, then in 10% commercial bleach (Domestos) for 10 min, followed by three rinses with sterile distilled water. Seeds were germinated aseptically in the dark, on Petri dishes containing 25 ml of growth-regulator-free Murashige and Skoog (MS) medium [6], supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (w/v). The pH of MS medium was adjusted to 5.6 before autoclaving. After three weeks the seedlings were transferred in flasks with 100 ml MS medium supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (w/v). When the shoots grown they were transferred in new medium every four weeks. Shoot culture parts were used for callus induction. They were grown on MS medium supplemented with 1 g/l casein. Callus proliferation was obtained on MS medium supplemented with 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l indole-3-acetic acid (IAA), and 2.0 mg/l kinetin. Subculturing was performed at intervals of three weeks. Shoot cultivation and callus induction were carried out in a growth chamber illuminated with fluorescent light.

2.3. Sample preparation

0.20 g air-dried powdered *in vitro* plant material was extracted with methanol and diluted to 10 ml.

2.4. Quantitative analysis

Chromatographic system: HPLC system Shimadzu LC – 10 ADVP equipped with RP, C_{18} column, 4.6 x 250 mm, 5 μm particle size; Detector SPD 10 AVVP – UV-VIS.

Chromatographic conditions: Isocratic mobile phase Acetonitrile/1.5% Phosphoric acid solution (3:7 v/v); detection 254 nm; column temperature 25 ^{o}C; flow rate 1.5 ml/min.

Preparation of reference solution:
Reference solution of saponarin was prepared by dissolving of equivalent amount of substance Saponarin RS in the mobile phase to obtain solution with concentration 0.000005 mg/ml.

Test preparation: An aliquot of the sample solution was evaporated to dryness and the residue was dissolved with mobile phase and then injected in the HPLC system.

Statistical analysis
To determine flavonoid induction in cell cultures of three *Gypsophila* species each experiment was repeated three times. MedCalc 12.3 (MedCalc Software 2012) was used for statistical calculations. The Kruskal-Wallis one-way analysis of variance was conducted to define the statistical significance of saponarin amount. The results were expressed as mean ± SD. Probability value of $P \leq 0.05$ was used as the criteria for significance differences.

RESULTS AND DISCUSSION

The production of flavonoids *via* tissue culture techniques have been reported in both callus and cell suspension cultures. The spectrum of the produced compounds and their yields depended on the selection of plant species, explant types and culture conditions [6].

In our preliminary studies was found that darkness suppresses flavonoid biosynthesis and formation of flavonoid glycosides is light-induced [7]. Thus all cultures were cultivated in continually light (2000 Lx) conditions [8]. The chemical investigation of cell extracts led to the detection of saponarin using of HPLC (Fig. 1-6) and TLC (data not shown). Novel HPLC method for direct determination of saponarin in *Gypsophila* species was performed [9].

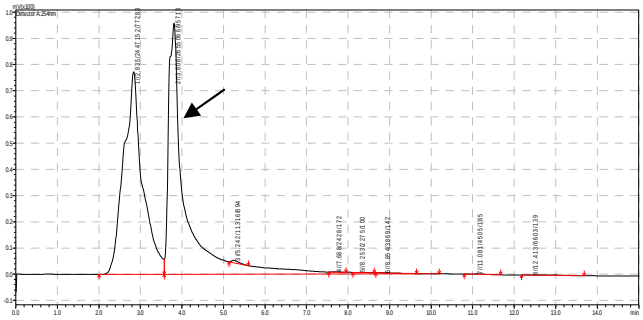

Fig. 1. Chromatogram of *G. paniculata* shoot cultures sample

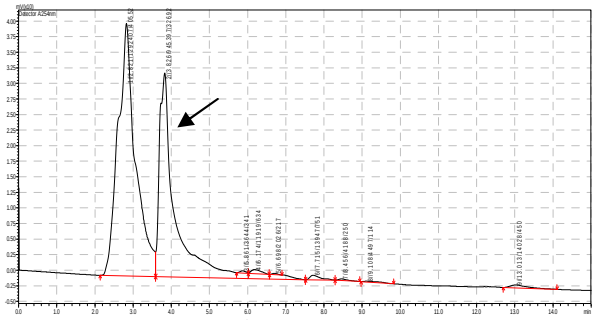

Fig. 2. Chromatogram of *G. paniculata* callus cultures sample

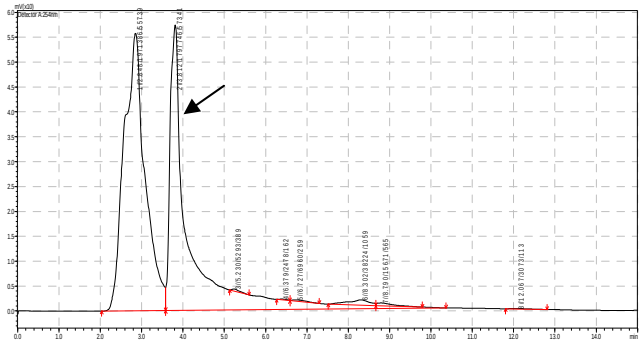

Fig. 3. Chromatogram of *G. altissima* shoot cultures sample

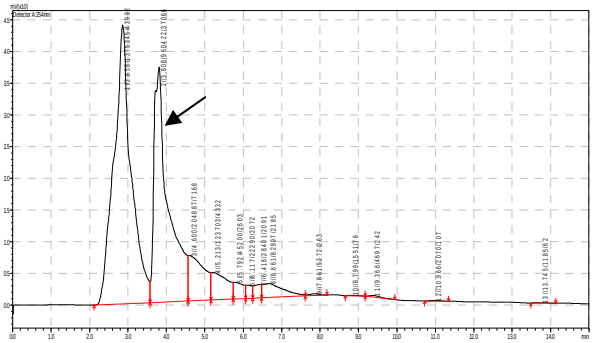

Fig. 4. Chromatogram of *G. altissima* callus cultures sample

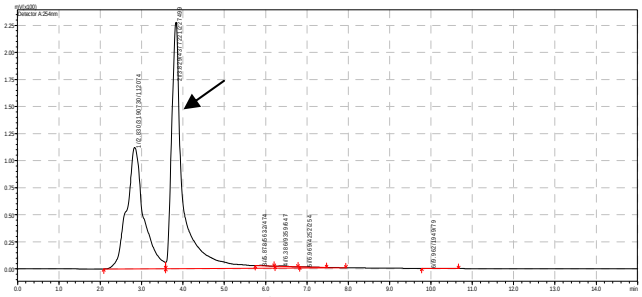

Fig. 5. Chromatogram of *G. trichotoma* shoot cultures sample

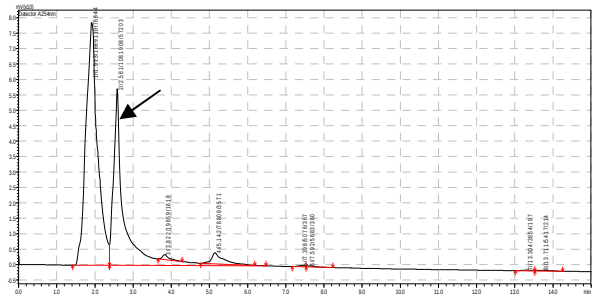

Fig. 6. Chromatogram of *G. trichotoma* callus cultures sample

All *in vitro* cultures of *Gypsophila* species produced saponarin. The highest amount was determined in the shoot cultures of *G. trichotoma* (2.26 %) of the dry weight (Tabl. 1).

Table 1. Analysis of saponarin in different *in vitro* cultures of *Gypsophila* species

Data are expressed as mean ± SD of three experiments.

Plant species	Produced saponarin [%] mean ± SD	
	shoot culture	callus culture
G. paniculata	1.42 ± 0.01	0.51 ± 0.01
G. altissima	0.97 ± 0.01	0.52 ± 0.03
G. trichotoma	2.26 ± 0.05	0.75 ± 0.02

Culture productivity is critical to the practical application of cell culture technology to production of flavonoids. All shoot cultures of *Gypsophila* species produced more saponarin than callus cultures. But no other production system as undifferentiated cells offers the potential to scalability of plant products. High-value products could be produced in sufficient amount in plant cell cultures to allow product manufacturing on a massive scale that can match global demand. Production can be more reliable, simple, and predictable. Isolation can be rapid and efficient, as compared to extraction from whole plants phytochemical complex.

Flavonoids produced in uniform plant-cell culture systems offer a novel vehicle for in-depth investigation of these compounds individually. In order to increase flavonoid production *in vitro* the effect of medium composition, plant growth regulators (auxins and cytokinins) and sucrose concentration will be optimized.

CONCLUSION

For the first time shoots and callus cultures of three *Gypsophila* species were initiated and maintained from *in vitro* germinated seeds. The composition of flavonoids was examined and it was found that saponarin has been produced from all cell cultures. It was detected for the first time in both species *G. paniculata* and *G. altissima*. Quantification of saponarin content was determined by novel HPLC method.

The development of new technologies will serve to extend and enhance the continued usefulness of higher plants as renewable sources of chemicals. We hope that a continuation and intensification efforts in this field will lead to controllable and successful biotechnological production of specific, valuable plant chemicals.

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