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Research Article

In Vitro Micropropagation and Alkaloid Production of *Galanthus transcaucasicus* Fomin

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ABSTRACT

Background: In this study we report the production and identification of alkaloid compounds from tissue culture derived from bulb scales of *Galanthus transcaucasicus* Fomin (Amaryllidaceae), a medicinally important plant. ***Methods:*** Explants were prepared from bulb scales of *G. transcaucasicus in vitro*. The alkaloid compounds were extracted and analyzed by GC/MS. ***Results:*** Isolation of the alkaloid fraction of the produced bulblets and its GC/MS analysis led to the identification of an Amaryllidaceae alkaloid homolycorin. Moreover, galantamine was not detected in the alkaloid fraction. ***Conclusion:*** At the present study we report the first micropropagation work on *G. transcaucasicus* together with the isolation of alkaloid homolycorine from *in vitro* produced bulblets. The results indicated that *G. transcaucasicus* bulblets produce Amaryllidaceae alkaloids and could be a new source of bioactive compounds for possible pharmaceutical applications. Also, described method could be used for micropropagation of plantlets from *G. transcaucasicus.*

Introduction

Medicinal plants used in traditional medicine of various nations have the potential to provide biologically active substances which could be useful for the treatment of many disorders.[1-3] This could be obtained by taking advantage of information available from traditional medicine and also ethnobotanical knowledge.[4-7]

Amaryllidaceae family has about 85 bulbous genera and more than 1000 species which are found in relatively warm regions and tropical areas.[8] These plants are well-known for their ornamental worth and traditional uses. Moreover, these plants are famous for their bioactive alkaloids called Amaryllidaceae alkaloids.[9]

The genus *Galanthus* belonging to Amaryllidaceae, produces pharmacologically active alkaloids compounds.[10] These alkaloids have significant biological effects such as antiviral, antiprotozoal, antitumor and anti-Alzheimer.[11-13] Alzheimer's disease (AD) is related with progressive degeneration of memory and cognitive function which affects millions of people all around the world.[14] Galantamine is one of the Amaryllidaceae alkaloids which is used in treatment of AD.[15] Just a few species of *Galanthus* species have been phytochemically studied. In addition, this genus is a source of structurally novel alkaloid metabolites.

There are thousands of plant species which are used in phytomedicine.[16-18] Many of them are harvested from natural environment. At present time, the share of the cultivated medicinal plants which are used in the pharmaceutical industries is very low.[19] In this case, biotechnology offers possibilities for faster cloning and conservation of the genotype of the plants and also for modification of their gene information, regulation, and expression for preparation of bioactive natural compounds with better properties and in higher amounts.[20] In recent scientific researches, the preparation of secondary metabolites from plants using *in vitro* culture is a challenging field. Nowadays, pharmaceutical and food industries need in phytochemicals is being increased steadily. Accordingly, the establishment of *in vitro* plant protocols has to be monitored by phytochemical investigation of their selected extracts in order to supply standardized raw material.

In this work, as a part of our continuing phytochemical studies on Iranian medicinal plants, we investigated the alkaloids from bulbs callus of *Galanthus transcaucasicus* Fomin for the first time.

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G. transcaucasicus is a native species distributed in the Caucasia, Azerbaijan and Alborz mountains. It is called Gole-barfi (Snow flower) in Iran. There are several reports on phytoconstituents of the genus *Galanthus* but we could not find any study on *G. transcaucasicus* tissue culturing and phytochemical investigation of callus derived from this plant. Several classes of Amaryllidaceae alkaloids have been identified in different parts of *Galanthus* species including Galantamine, lycorin and homolycorine. In the present study, we report the identification of alkaloid compound homolycorin from the callus derived from bulbs of *G. transcaucasicus* Fomin for the first time. Homolycorine has been evaluated for anticancer efficacy both *in vivo* and *in vitro*
using murine sarcoma S180 cells, indicating that their anticancer effects, at least in part, are the result of inducing cancer cell apoptosis.[21]

Materials and Methods

Chemicals

Chloroform, diethyl ether, sodium sulfate, methanol, sulfuric acid, ammonia solution 25% and other chemicals were purchased from Merck (Germany).

Plant material

Galanthus transcaucasicus Fomin was collected in April 2015 from Khalkhal, Ardabil province, Northwestern of Iran. The plant was identified in the herbarium of the department of pharmacognosy, faculty of pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. A voucher specimen was deposited for the plant (Tbz-FPh 761).

Preparation of explants from G. transcaucasicus bulb scales

At first healthy bulbs were selected. Then, roots, leaves, and discolored or brown signs of outer scales were removed from the plant bulbs. Also, A thin layer of basal bulb tissues were cut away to obtain white and healthy tissues. Afterward, the bulbs were washed using detergent before sterilization. Sterilization was then applied in two steps. 1) The bulbs were soaked consecutively in 70% ethanol (1 min) and 5% sodium hypochlorite (20 min). 2) Then they were halved vertically and put in 2.5% sodium hypochlorite for (10 min). Finally, the samples were rinsed by sterile distilled water three times. Bulbs of *G. transcaucasicus* with 2 cm diameter were vertically cut into 4 equal parts. Explants were prepared with the separation of these parts in single scales, twin scales and tri-scales.

In vitro culture of G. transcaucasicus

A modified MS medium was designated (MMS) including 30 g/l sucrose and 8 g/l agar. Six levels of 6-benzylaminopurine (BAP) (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) combined with six levels of α-naphthalene acetic acid (NAA) (0, 0.1, 0.2. 0.3, 0.4 and 0.5 mg/l) were used to evaluate the effects of plant growth regulators (PGRs) on bulblet production. Explants were inoculated in jam glasses (80×120 mm) containing 50 ml of MMS (the basal plate tissue inserted into the media). The cultures were stored in a growth chamber at 25 ºC with a 16h photoperiod provided by LED lamps. All of the treatment were carried out in triplicate and each replication had 4 explants. Number of bulblets per explants, bulblet diameter and fresh weight of bulblets were recorded after 5 months from the first inoculation.

Preparation of alkaloid fraction

The *in vitro* produced bulblets were dried at room temperature and then ground to fine powder. 300 mg of the powder was solvent extracted by methanol (5 ml, three times) during 24 hours. The solvent of the resulted extract was removed using a rotatory evaporator. The crude extract was dissolved in 4ml of 3% sulfuric acid. Neutral compounds were removed by liquid-liquid extraction using diethyl ether (5 ml, three times in 30 min). The aqueous fraction was basified with ammonia solution (25%). Alkaloid metabolites were extracted using chloroform (3 ml, three times in 30 min). These alkaloid extracts were combined and were then dried over anhydrous sodium sulfate and evaporated to dryness. The obtained alkaloid fraction was dissolved in methanol and stored in -20 ºC until analysis.

Gas chromatography–mass spectrometry (GC/MS) analyses of alkaloid extract

The composition of alkaloid extract was identified using a gas chromatograph-mass spectrometer (Shimadzu, QP-5050 A) equipped with DB-1 capillary column (60 m, 0.25 mm i,d., 0.25 µm thickness). EI ionization system, with ionization energy of 70 eV and solvent delay of 5.0 min was employed for detection of components. Helium (99.9%) was the carrier gas with flow rate of 1.3 ml/min and split ratio was 1:10. The column temperature program: the initial oven temperature was kept at 50 °C for 2 min. Afterward, temperature was raised to 310 ºC using program ramp rate of 2.5 °C/min. The ultimate temperature was 310 ºC and maintained for 25 min. Injector temperature was 280° C. Identification of the alkaloid compound was carried out using Wiley and Nist Libraries search and by comparison of the fragmentation pattern of the mass spectra with those reported in the literature.[22-24]

Figure 1. Bulblets cultivated in jam glasses containing MMS basal medium supplemented with 30 g/l sucrose, 8 g/l agar, 2 mg/l BAP and 0.2 mg/l NAA.

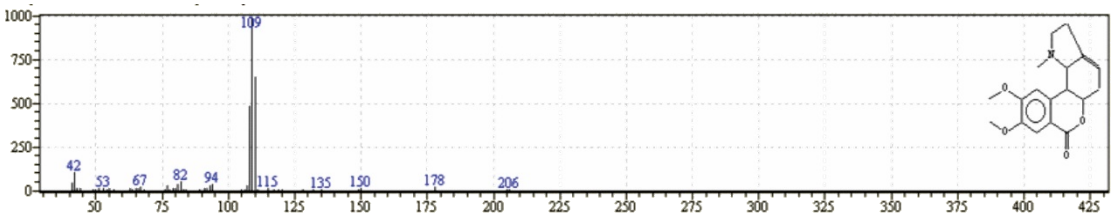

Figure 2. GC-MS results of homolycorine.

Results and Discussion

Generally, a plant could produce two or three bulblets per year.[25] In this context, micropropagation is a reliable method for the fast propagation of the bulbous plants. In this work micropropagation of *G. transcaucasicus* enhanced by direct bulblet regeneration (Figure 1). The effect of different levels of NAA and BAP on bulblet production of *G. transcaucasicus* was determined. The highest number of bulblets and the largest ones were produced on MMS medium supplemented with 0.2 mg/l NAA and 2 mg/l BAP after 5 months (Figure 1). Numerous bulblets were obtained when explants were prepared as tri scales and transferred onto medium containing 0.2 mg/l NAA and 2 mg/l BAP.

Galanthus L. species (Amaryllidaceae), are known to generate Amaryllidaceae alkaloids with diverse structures and interesting biological activities.[9] A great number of alkaloids that are found in the amaryllidaceous plants has been separated effectively and identified very quickly by GC/MS.[26,27] As shown in Figure 2, the GC/MS analysis of alkaloid fraction obtained from the cultured bulblets of *G. transcaucasicus* led to the identification of an alkaloid compound homolycorine (9,10-dimethoxy-1-methyl-Lycorenan-7-one) with molecular formula of $C_{18}H_{21}NO_4$ (Figure 3). Its molecular weight was found to be 315.3 g/mol. Figure 4 shows the pattern of mass fragments from homolycorine. There is just one phytochemical report on *G. transcaucasicus* in the literature. In that work, five isoquinoline type alkaloids namely galanthamine, narwedine, lycorine, caranine and tazettine were isolated from the bulbs of *G. transcaucasicus*.[28] Our literature review revealed that homolycorin is rare in the genus *Galanthus*.

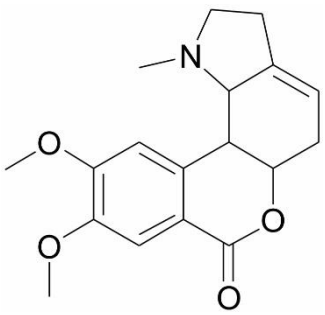

Figure 3. Chemical structure of homolycorine.

Conclusion

This is the first study on tissue culturing of *G. transcaucasicus* and evaluation of its alkaloid production. The GC-MS study of the cultured bulblets of *G. transcaucasicus* led to the identification of one alkaloid compound homolycorin.

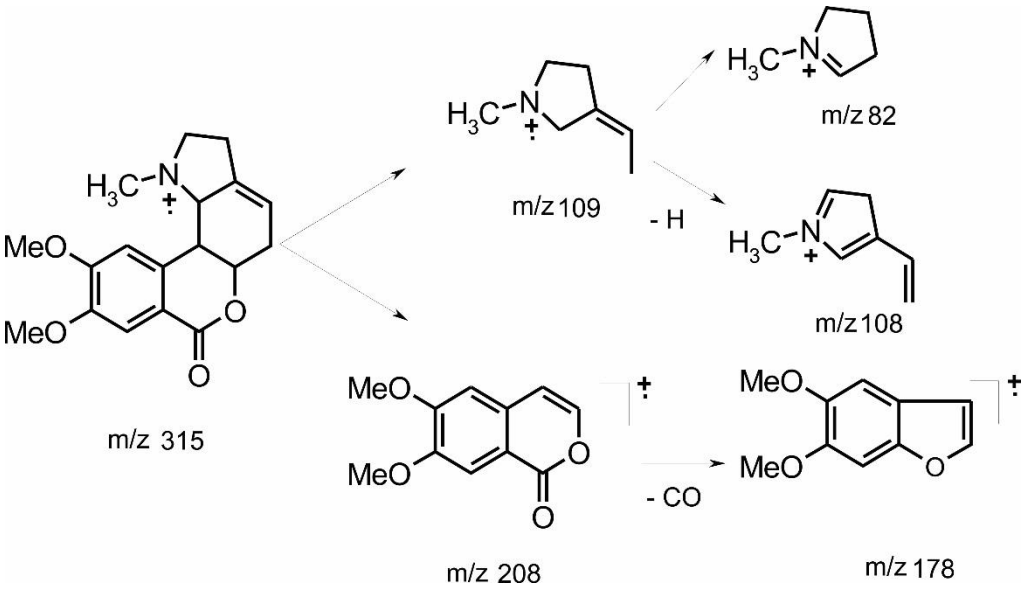

Figure 4. The pattern of homolycorine mass fragmentation.

To reach the highest number and the largest bulblets per each explant of bulblets, it is advised to use 0.2 mg/l NAA and 2 mg/l BAP. The results showed micropropagation of *G. transcaucasicus* plantlets could be utilized for production of pharmacologically important alkaloid metabolites. Accordingly, further direct and indirect tissue culture studies on various organs of *G. transcaucasicus* and other Amaryllidaceae plants for preparation of bioactive alkaloids are warranted.

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Conflict of interests

The authors claim that there is no conflict of interest.

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