

QUANTITATIVE DETERMINATIONS ON *GALANTHUS RIZEHENSIS*

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Abstract

Galanthus rizehensis Stern (Amaryllidaceae) was collected from Maçka, Trabzon during flowering and fruiting seasons. Quality control studies and quantitative determination of lycorine were carried out on the prepared specimens. In this respect, assays for humidity, total ash, sulphated ash, hydrochloric acid-insoluble ash were performed on each drug specimen. The total alkaloidal content of *G. rizehensis* was determined by a titrimetric method. Furthermore, the identification and quantitative determination of lycorine on the aerial and underground parts of the plant collected during two different seasons were performed by using a HPLC-DAD method.

Key words: *Galanthus rizehensis*, Amaryllidaceae, Lycorine, Quality Control, HPLC-DAD.

Galanthus rizehensis Üzerinde Kantitatif Tayinler

Galanthus rizehensis Stern (Amaryllidaceae) bitkisi çiçekli ve meyveli dönemde Maçka, Trabzon'dan toplanmıştır. Hazırlanan örnekler üzerinde kalite kontrol çalışmaları ve likorin miktar tayini yapılmıştır. Bu amaçla, herbir örnek üzerinde, nem, total kül, sülfat külü ve hidroklorik asitte çözünmeyen kül deneyleri gerçekleştirilmiştir. *G. rizehensis* bitkisinin total alkaloit içeriği ise titrimetrik esaslı bir yöntemle tayin edilmiştir. Ayrıca, iki farklı zamanda toplanmış olan bitkinin toprak üstü ve toprak altı kısımlarındaki likorinin teşhisi ve miktar tayini, YBSK-DAD yöntemi kullanılarak yapılmıştır.

Anahtar kelimeler: *Galanthus rizehensis*, Amaryllidaceae, Likorin, Kalite Kontrol, YBSK-DAD.

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INTRODUCTION

G. rizehensis Stern is a small, Amaryllidaceous plant distributed in the Northeastern part of Turkey (1). The family Amaryllidaceae, consists of about 1100 species in 85 genera (2). Amaryllidaceae plants have attracted appreciable interest because of their alkaloids with interesting biological activities such as antitumor, antiviral and acetylcholinesterase inhibitory activities (3). Among these alkaloids galanthamine and lycorine have important biological properties. Galanthamine is a long acting, selective, reversible and competitive acetylcholinesterase inhibitor (4) whereas lycorine has several biological activities such as antiviral (5,6), antimalarial (7), antifungal (8), antiparasitic (9), antiinflammatory (10), antiproliferative (11) and antitumoral (12) activities.

In the present study, we performed quality control studies on the aerial parts and bulbs of *G. rizehensis* collected during flowering and fruiting periods in order to obtain data for prospective monographs on Herba and Bulbus Galanthi. European Pharmacopeia was referred to for the assays of humidity, total ash, sulphated ash, hydrochloric acid-insoluble ash, and for the determination of total alkaloids (13).

Additionally, the plant specimens were investigated for their content of lycorine by a High Pressure Liquid Chromatography-Diode Array Dedector (HPLC-DAD) method (14, 15). Pre-packed columns based on diatomaceous earth for liquid-liquid sample purification has been used for the rapid preparation of the extracts (15-17). In this study, HPLC-DAD analysis was performed using an isocratic system with a mobil phase consisting of trifluoroacetic acid:water:acetonitrile (0.01:90:10, v/v/v) (15) for the determination of lycorine in the alkaloidal extracts prepared from the aerial and underground parts of *G. rizehensis* collected during two different vegetation periods.

EXPERIMENTAL

Plant material

G. rizehensis was collected from Maçka, Trabzon on March 11, 2007 and May 2, 2007 during flowering and fruiting periods. The plant was identified by Prof. M. Ali Onur from the Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir (Turkey). Voucher specimens are deposited (No: 1371 and 1376) in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Chemicals

The standard lycorine was previously isolated from several *Galanthus* species in our laboratory and authenticated by spectral analyses (UV, IR, ¹H NMR, ¹³C NMR and MS). HPLC grade acetonitrile (Lab Scan Analytical Sciences), TFA (Trifluoroacetic acid) (Merck) and chromatographic grade double-distilled water were used for the HPLC analysis. Other chemicals were of analytical grade.

Quality Control Studies

Humidity, total ash, sulphated ash, hydrochloric acid-insoluble ash and total alkaloid content determinations

Contents of humidity, ash, sulphated ash, hydrochloric acid-insoluble ash and total alkaloids were determined according to European Pharmacopeia (13) with the aim of gathering data for prospective monographs on Herba and Bulbus Galanthi drugs.

Total alkaloid content assay

About 3 g of exactly weighed powdered plant material was macerated with 100 mL of EtOH for 24 h and then percolated until no positive reaction was observed with Dragendorff and Mayer reagents (13). The solvent was evaporated and the residue was dissolved with 1 % aqueous hydrochloric acid (250 mL in total) and filtered. Acidic filtrates were accumulated and washed with petroleum ether (3 x 100 mL) and then made alkaline with 26 % ammonium hydroxide to pH 9-10. The basic aqueous phase was extracted with 6 x 100 mL chloroform until the organic solvent displayed no reaction with Dragendorff and Mayer reagents, then the chloroform extracts were dried over anhydrous sodium sulphate and evaporated to dryness to afford the alkaloidal extract. The extract was dissolved in 0.02 N H₂SO₄ solution on water bath (50-60°C). Methyl red reagent (three drops) was added to the solution and titrated with 0.02 N NaOH. The procedure was performed in a series of three parallel experiments.

HPLC-DAD analysis of lycorine

HPLC instrument

HPLC analysis was carried out using a liquid chromatographic system (Agilent 1100 series), equipped with a DAD (Agilent 1200 series), a quaternary pump system (Agilent 1100 series G1311A), a vacuum degasser (Agilent 1100 series G1322A), a thermostatted column compartment (Agilent 1100 series G1316A), a manual injector with 20 µl loop (Agilent 1100 series G1328A Rheodyne 7725i). Data analysis was carried out with "Agilent Chem Station Software". The chromatographic assay was performed on Hichrom C₁₈ column (5µ, 250 mm, 4.6 mm) at 290.4 nm. The analysis was performed at 25°C. The mobile phase consisted of TFA:water:acetonitrile (0.01:90:10, v/v/v) applied at a flow rate of 1mL/min (14,15). The quantitative determination of lycorine was carried out by the external standard method based on peak areas.

Preparation of standard solutions

For the preparation of the calibration curve of lycorine, 2 mg of alkaloid was dissolved in 10 mL 0.1 % TFA and filtered (Sem Concept Syringe 100 x 13 mm, 0.45 µm). Nine different concentrations (0.5 µg/mL, 1.5 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 14 µg/mL, 20 µg/mL, 24 µg/mL, 30 µg/mL) were prepared by diluting the stock solution. Each standard solution was injected into the column in triplicate with a volume of 20 µl and the regression equation of the calibration curve was obtained as $y = 14.883 x + 2.22946$ ($R^2 = 0.9996$).

Extract preparation

The extraction procedure was carried out by some methods which are performed previously (15, 17). About 200 mg of powdered plant material was macerated with 5 mL of 2 % hydrochloric acid for 5 hours in an ultrasonic bath at 40°C and then the extract was made alkaline with 1 mL of 26 % ammonium hydroxide and the volume was adjusted to 10 mL in a volumetric flask with distilled water. The basic solution centrifuged at 5000 rpm for 10 min and aliquots of 3 mL were applied on the Extrelut[®] (Merck) columns. The alkaloids were eluted with chloroform (3 x 5 mL) for 10 min. The organic solvent was distilled *in vacuo* to furnish the alkaloidal extract. The extract was dissolved in 1 mL 0.1 % TFA and filtered. 20 µl was injected into the HPLC column. Each analysis was carried out in triplicate.

RESULTS AND DISCUSSION

In this study, *G. rizehensis* has been investigated by different approaches. The quality control determinations (humidity, total ash, sulphated ash, hydrochloric acid-insoluble ash and

total alkaloid quantification by a titrimetric method) on drug specimens have been carried out according to European Pharmacopoeia and the results were reported as percentage values (Table 1). Quality control results might be used for the elaboration of monographs on Herba and Bulbus Galanthi drugs. The total alkaloidal content of *G. rizehensis* ranged between 0.0111-0.1014 %. Our results revealed that, bulbs collected in fruiting period contained the highest amount of total alkaloids (sample **D**).

On the other hand, four different specimens of *G. rizehensis* were quantitatively analyzed for their content of lycorine which is a common alkaloid in the genus *Galanthus* and other Amaryllidaceae genera by HPLC-DAD (Table 2). The identification and quantitative determination of lycorine was established by the comparison of the retention time and peak area with those of standard lycorine. The HPLC chromatogram of standard lycorine is given in Figure 1. Detection by DAD increased the sensitivity of the HPLC method. The highest amount of lycorine was found in sample **D** (bulbs collected in fruiting period) (Figure 2). This result is in accordance with the total alkaloidal content results.

There are a number of records in the literature related with the quality control studies on *Galanthus* species (18-21). Concerning the quantification of lycorine by HPLC in these plants, previously, we have investigated specimens prepared from the aerial and underground parts of *G. trojanus* A.P. Davis & N. Özhatay, *G. gracilis* Čelak., *G. elwesii* Hook. and *G. plicatus* Bieb. subsp. *byzantinus* (Baker) D. A. Webb, collected during flowering and fruiting periods. As a result, lycorine was detected in all of the specimens of *G. trojanus* and the highest amount of lycorine (0.0036 %) was found in Bulbus Galanthi prepared from flowering plants of this species. Lycorine, was also found in the underground parts of *G. gracilis* collected during flowering (0.0021 %) and fruiting seasons (0.0009 %), but it was not detected in the aerial parts of this species. Moreover, it was not found to be present in any of the specimens of *G. elwesii* and *G. plicatus* subsp. *byzantinus* (22). To the best of our knowledge, quality control determinations on *G. rizehensis* and quantitative determination of lycorine in this species are reported for the first time in this study.

Table 1. Quality control determination results of *G. rizehensis*.

Specimen	Humidity (%) [*]	Total Ash (%) [*]	Sulphated Ash (%) [*]	Hydrochloric Acid-Insoluble Ash (%) [*]	Total Alkaloids (%) ^{**}
A	7.3121 ± 0.0676	9.1591 ± 0.0616	12.9621 ± 0.3035	3.1275 ± 0.0264	0.0111 ± 0.0003
B	7.3757 ± 0.0822	9.3724 ± 0.1121	13.6986 ± 0.1345	3.9014 ± 0.1756	0.0835 ± 0.0019
C	6.1929 ± 0.0506	16.0719 ± 0.2202	19.4108 ± 0.1982	7.2429 ± 0.1357	0.0141 ± 0.0002
D	7.5064 ± 0.0664	11.5192 ± 0.4758	14.6252 ± 0.1732	5.6034 ± 0.1799	0.1014 ± 0.0008

*n=3, Mean Results ± Standard Deviations

**n=5, Mean Results ± Standard Deviations

A: Herba/Flowering, B: Bulbus/Flowering, C: Herba/Fruiting, D: Bulbus/Fruiting

Table 2. Content of lycorine in *G. rizehensis*.

Specimen	Lycorine (%) [*]
A	0.0081 ± 0.0008
B	0.1151 ± 0.0045
C	0.0132 ± 0.0023
D	0.2063 ± 0.0157

*n=9, Mean Results ± Standard Deviations

A: Herba/Flowering, B: Bulbus/Flowering, C: Herba/Fruiting, D: Bulbus/Fruiting

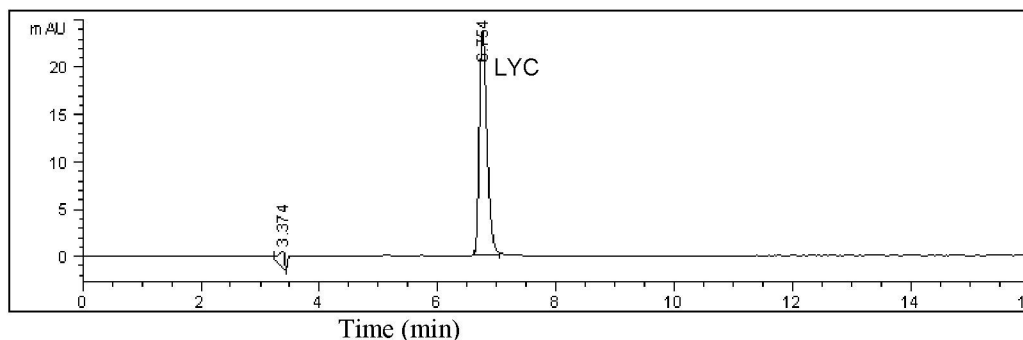


Figure 1. HPLC chromatogram of the standard lycorine LYC: Lycorine

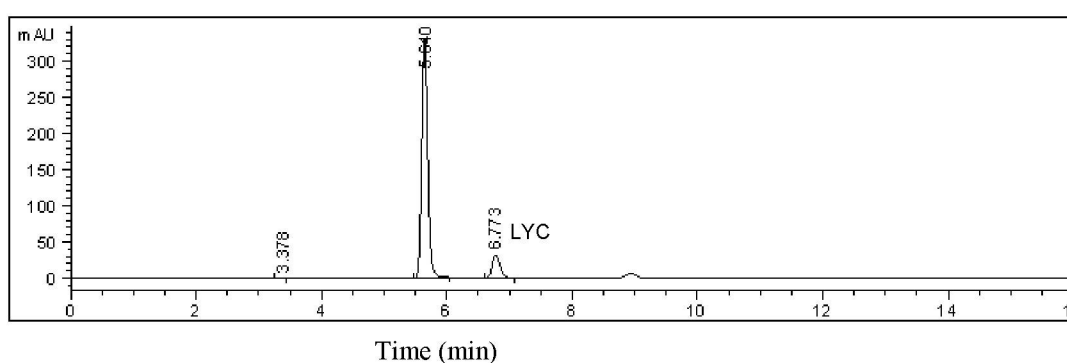


Figure 2. HPLC chromatogram of the total alkaloid extracts of *G. rizehensis* (Bulbs- Fruiting period) LYC: Lycorine

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