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HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF LYCORINE IN FOUR *GALANTHUS* SPECIES GROWING IN TURKEY

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Abstract

The detection and quantification of Lycorine in the total alkaloidal extracts prepared from aerial and underground parts of Galanthus nivalis ssp. cilicicus, G. gracilis, G. elwesii and G. plicatus ssp. byzantinus, collected during two different vegetation periods, were carried out using high performance liquid chromatography (HPLC). An isocratic system with chloroform/methanol mobile phase was used for the detection and quantitative determination of lycorine. External standard calibration was used to quantify lycorine.

Key Words*: *Galanthus, Amaryllidaceae, Lycorine, HPLC

Türkiye'de Yetişen Dört *Galanthus* Türünde Lycorine'in Yüksek Basınçlı Sıvı Kromatografisi Yöntemi ile Analizi

Vejetasyonunun iki farklı zamanında toplanmış olan Galanthus nivalis ssp. cilicicus, G. gracilis, G. elwesii ve G. plicatus ssp. byzantinus'un topraküstü ve toprakaltı kısımlarından hazırlanan total alkaloit ekstrelerinde Lycorine'in tespiti ve miktar tayini yüksek basınçlı sıvı kromatografisi (YBSK) kullanılarak gerçekleştirilmiştir. Lycorine'in tespiti ve miktar tayini için kloroform/metanol mobil fazlı bir izokratik sistemden yararlanılmıştır. Lycorine miktarının tayininde harici standart kalibrasyon yöntemi kullanılmıştır.

Anahtar Kelimeler*: *Galanthus, Amaryllidaceae, Lycorine, YBSK

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Introduction

Galanthus L. (Amaryllidaceae) is a genus of bulbous, petaloid monocotyledons, which are distributed throughout Europe, Asia Minor and the Near East (1). In folk medicine, the herbs are reported to have cardiotonic, stomachic and emmenagogue properties, whereas the poultice prepared from the fresh underground parts has external use in abcess maturation (2). The bulbs of *Galanthus* species are exported, therefore, have also of economical importance (2,3). Of the fourteeen *Galanthus* species growing wild in Turkey (4,5), *G. elwesii* Hook. and *G. ikariae* Baker are exported, the former being exported in large quantities (6).

Galanthus species are of interest due to their content of lectins and alkaloids. The latter are called Amaryllidaceae alkaloids, since they represent a diverse class of bases occurring exclusively in different species of the family Amaryllidaceae (7). Some of these alkaloids have been shown to possess a wide spectrum of biological activities. The most well-known and amply investigated alkaloid of this group, galanthamine, is a long acting, selective, reversible and competitive acetylcholinesterase inhibitor (8, 9) and is marketed as a hydrobromide salt under the name Reminyl® for the treatment of Alzheimer's Disease (10). However, lycorine (**LYC**), also a common alkaloid in this family, has been proven to have antiviral, cytotoxic, antimalarial and antiinflammatory activities (11-14). Moreover, there have been some recent reports which reveal the interaction of **LYC** with DNA and tRNA (15,16). It has, therefore, been to the interest of phytochemists to determine the content of this alkaloid in Amaryllidaceaous plants.

Different analytical techniques have been described for the qualitative and quantitative determination of **LYC** in various parts of different Amaryllidaceae plants (17-21). However, there have been only a limited number of reports regarding the quantitative determination of **LYC** in *Galanthus* species (22, 23).

In this study, specimens prepared from the aerial and underground parts of the plants collected at flowering and fruiting seasons were assayed with respect to the occurrence and content of **LYC**, by using HPLC coupled with a UV detector, with the aim of establishing criteria for the most desirable preparation of a high-quality, alkaloid-rich drug.

Experimental

Plant Material

Galanthus nivalis L. ssp. *cilicicus* (Baker) Gottlieb-Tannenhain: Bayramiç, Çanakkale, March 1999 (flowering), May 1999 (fruiting). *G. gracilis* Celak.: Mount Nif, Kemalpaşa, İzmir, March 2000 (flowering), May 2000 (fruiting). *G. elwesii* Hook.: Yamanlar Mountain, Karagöl, İzmir, March 2000 (flowering), May 2000 (fruiting). *G. plicatus* Bieb. ssp. *byzantinus* (Baker) D. A. Webb: around Lake Abant, Bolu, April 2002 (flowering), May 2002 (fruiting). The plants were identified by M. A. Önür (Ege University, Faculty of Pharmacy, Department of Pharmacognosy). Voucher samples of *G. nivalis* ssp. *cilicicus* (No's 1233, 1234, 1240), *G. gracilis* (No's 1244, 1248), *G. elwesii* (No's 1243, 1247) and *G. plicatus* ssp. *byzantinus* (No's 1281, 1285) are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Chemicals

A standard sample of **LYC** used in this study was previously isolated from several Amaryllidaceae species in our laboratory and authenticated by means of spectral analyses (UV, IR, ^{1}H and ^{13}C NMR, MS). Petroleum ether (40-60 °C) (8115) and chloroform (24216), used for the extraction of the alkaloids, were purchased from J. T. Baker and Riedel-de-Haen AG, respectively. Methanol (C2517) and chloroform (C2507E) supplied by Lab Scan were used for the HPLC analysis of **LYC**. Other chemicals were of analytical grade.

High Pressure Liquid Chromatography (HPLC)

HPLC analysis was carried out using a liquid chromatographic system (Agilent 1100 series), equipped with a UV variable-wavelength detector (Agilent 1100 series G1314A), 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) and a chromatographic data processing software (HP Chemstation for LC Rev. A.

06. 03 [509]). The chromatographic assay for **LYC** was performed on a Hichrom C_{18} column (300 mm x 4.0 mm i.d.; 5 μm particle size) at 290 nm. The analysis was carried out at 30°C. The mobile phase was made up of chloroform: methanol (9:1) applied at a flow rate of 1 mL/min. **LYC** (t_R= 5.0 min) was identified by its retention time under the above-mentioned conditions. Quantitative determination was carried out by the external standard method based on peak heights.

Preparation of Standard Solutions and Calibration

For the preparation of the calibration curve of **LYC**, 3.4 mg of the alkaloid was weighed accurately into a 10 mL volumetric flask, dissolved and adjusted to the final volume with the mobile phase. Three calibration levels (34 μg/mL, 68 μg/mL and 102 μg/ mL) were prepared by diluting the stock solution. 5 μl injections were performed in triplicate for each standard solution and the resulting calibration data were R^2 =0.993 and y= 3424.8x-0.5113.

Alkaloid Extraction and Sample Preparation

About 10 g of accurately weighed powdered plant material was macerated with 200 mL 96 % EtOH for 24 h, and then percolated until no positive reaction is observed with the Dragendorff and Mayer (24) reagents. After the evaporation of the solvent, the residue was dissolved in 100 mL portions of 1 % aqueous hydrochloric acid and filtered until the filtrate was no longer positive to Dragendorff and Mayer reagents. Combined acidic filtrates were washed with 3 x 100 petroleum ether (40-60 °C), made alkaline with 25 % ammonium hydroxide (pH 9-10) and extracted with chloroform until the organic solvent displayed no reaction with Dragendorff and Mayer reagents. The combined chloroform extracts were then dried over anhydrous sodium sulphate, filtered, and the organic solvent distilled *in vacuo* to furnish the total alkaloidal extract.

For the assay of **LYC** in the specimens, 5 mg of the total alkaloidal extract was dissolved in a mixture of chlorofom-methanol (9:1) and the final volume was adjusted to 2.5 mL in a volumetric flask. The solution obtained was filtered through Schleicher&Schuell (589 1 Black ribbon ashless) filter paper, prior to injection (5μL and 10μL) to HPLC.

Results

The results of our studies reveal that **LYC** is not present in any of the specimens of *G. elwesii* and *G. plicatus* ssp. *byzantinus*, but is found in all of the specimens of *G. nivalis* ssp. *cilicicus*. It has been documented that Bulbus Galanthi prepared from flowering plants (BFlw) of the latter species has the highest amount of **LYC** among the investigated samples of this plant. **LYC** is also detected in *G. gracilis*; however, it is found to be present only in the underground parts collected during flowering (BFlw) and fruiting (BFr) seasons (Table 1).

TABLE 1. Content of **LYC** in *G. nivalis* ssp. *cilicicus* and *G. gracilis*

Plant species	Specimen[a]	**LYC** % (n=3, mean±SD[b]) [c]
G. nivalis ssp. cilicicus	BFlw	0.0036±0.0006
	HFlw	0.0014±0.0002
	BFr	0.0023±0.0004
	HFr	0.0007±0.0001
G. gracilis	BFlw	0.0021±0.0002
	HFlw	Not detected
	BFr	0.0009±0.0001
	HFr	Not detected

[a] B : Bulbus; H: Herba; Flw: Flowering; Fr: Fruiting [b] SD : Standard Deviation

[c] The results were calculated on dry-weight basis

The HPLC chromatograms of the crude alkaloidal extracts obtained from the underground and aerial parts of *G. nivalis* ssp. *cilicicus* and *G. gracilis* collected during flowering and fruiting seasons are given in Figure 1. The peaks in the chromatograms were identified by comparison of their retention times with that of standard **LYC** and also by spiking the known amount of standard in sample solutions.

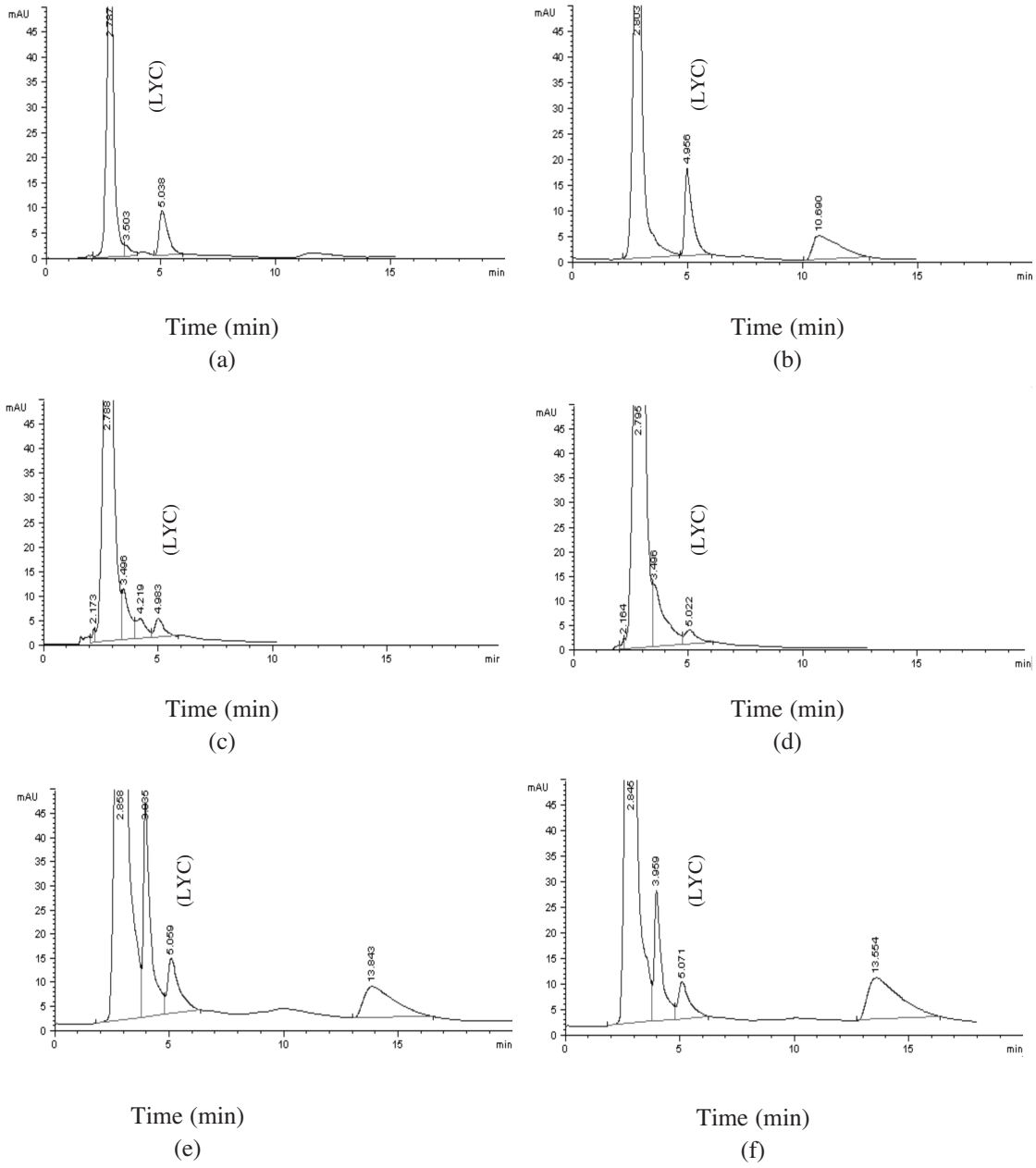

Figure 1. HPLC Chromatograms of the Total Alkaloidal Extracts

(a) Underground parts of *G. nivalis* ssp. *cilicicus* (flowering); (b) Underground parts of *G. nivalis* ssp. *cilicicus* (fruiting); (c) Aerial parts of *G. nivalis* ssp. *cilicicus* (flowering); (d) Aerial parts of *G. nivalis* ssp. *cilicicus* (fruiting); (e) Underground parts of *G. gracilis* (flowering); (f) Underground parts of *G. gracilis* (fruiting).

Discussion

The identification and quantitative determination of **LYC** was carried out by modifying the method described previously (19). The quantitative determination of **LYC** was accomplished by a comparison of the retention time and the peak area with those of standard **LYC**. To the best of our knowledge, this is the first report regarding the investigation of *G. nivalis* ssp. *cilicicus*, *G. gracilis* and *G. plicatus* ssp. *byzantinus* species for the content of **LYC**.

Recently, *Berkov* et al., have investigated the intraspecific variability in the alkaloid metabolism of Bulgarian *G. elwesii* populations by GC/MS and TLC. They have found significant differences in the presence of a particular metabolite and also in the type of the alkaloid metabolism between the populations on the relatively small geographic area of Bulgaria, and proposed that these diversities may be due to probable genetic or environmental factors regulating the biosynthetic pathways of alkaloid biosynthesis (25). In consonance with this finding, **LYC** has not been detected in *G. elwesii* specimens used in our study, although the same species collected from different localities have been proven to contain this alkaloid (22, 23, 26).

Conclusion

The results thus obtained show that, among the species and specimens tested in this study, Bulbus Galanthi prepared from the flowering plants of *G. nivalis* ssp. *cilicicus* was found to contain the highest amount of **LYC**. During the isocratic elution, employed by using chloroform:methanol (9:1) as the mobile phase, **LYC** was eluted within 5 minutes and with a good separation from the rest of the analytes.

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