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Original Article

Quantitative analysis of lycorine in *Galanthus alpinus* Sosn. var. *alpinus* by HPLC-DAD

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ABSTRACT

In this study, a reversed-phase, high pressure liquid chromatography (RP-HPLC) method was applied in the quantitative analysis of lycorine in the aerial parts and bulbs of *Galanthus alpinus* Sosn. var. *alpinus* collected from Rize during the flowering and fruiting seasons. A simple method to extract lycorine from the plant specimens was performed, using pre-packed columns with diatomaceous earth (Extrelut®). The chromatographic separation was achieved employing an isocratic system with a mobile phase including trifluoroacetic acid-water-acetonitrile (0.01:95:5) measured at a flow rate of 1 mL/min using a diode array detector (DAD). The lycorine content in the bulbs during the flowering and fruiting seasons was detected as 0.01576 and 0.02351 %, respectively. Also, validation studies showed that the method was specific, accurate and precise.

Keywords: *Galanthus alpinus* Sosn. var. alpinus, Amaryllidaceae, lycorine, HPLC-DAD

INTRODUCTION

Galanthus alpinus var. *alpinus* is an Amaryllidaceous plant distributed throughout the Caucasus/Transcaucasus region (Russia, Georgia, Armenia) and North-East Turkey. It is only found in the province of Rize in our country (Bishop et al. 2006). The *Galanthus* species, belonging to the Amaryllidaceae family, include diverse types of alkaloids with a wide range of biological and pharmacological activities (Unver 2007; Berkov et al. 2012). Among these alkaloids, galanthamine is a long-acting, reversible, selective and competitive acetylcholinesterase inhibitor used clinically for the treatment of mild to moderate Alzheimer's disease (Heinrich and Teoh 2004). Another important metabolite - lycorine (the main phenanthridine Amaryllidaceae alkaloid) - has been proven to possess several biological activities such as antiinflammatory (Saltan Çitoğlu et al. 2012), antiviral (Li et al. 2005; Oluyemisi et al. 2015), antimalarial (Şener et al. 2003), antifungal (Shen et al. 2014; Locarek et al. 2015), antiparasitic (Giordani et al. 2011; Giordani et al. 2012) and hepatoprotective (Ilavenil et al. 2012) effects. In particular, lycorine showed significant cytotoxic activity over different cancer cell lines due to its chemical structure (Nair and van Staden 2014; Doskočil et al. 2015; Nair et al. 2016) as shown in Figure 1.

Recently, herbal medicines have been used much more globally. Consequently their quality-control processes have been called into question in therapeutic utilization. With this in mind, in this study, plant extracts obtained from *G. alpinus* var. *alpinus* were investigated for their content of galanthamine and lycorine.

MATERIALS AND METHODS

Plant Material

Specimens of *G. alpinus* var. *alpinus* were collected from Çamlıhemşin, Rize on March 29, 2012 and April 5, 2013 during the flowering and fruiting periods. The plants were identified by Prof. Dr. M. Ali ONUR and voucher samples of *G. alpinus* var. *alpinus* (No:1513, 1526) have been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

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Chemicals and Solvents

Lycorine was isolated from *G. alpinus* var. *alpinus* and verified by multifarious spectroscopic methods (^{1}H and ^{13}C NMR, MS). HPLC grade acetonitrile (LabScan Analytical Sciences, LC 1005), and chromatographic grade double-distilled water, TFA (trifluoroacetic acid) (Merck, 108178) were used for the HPLC analysis of analytes and standards.

Sample Preparation

The dried and powdered aerial parts and bulbs of the plant (200 mg) were macerated with 5 mL of 2% hydrochloric acid for 5 h in an ultrasonic bath at 40 °C, 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. Following centrifugation at 5000 rpm for 10 min, aliquots of 3.0 mL were applied to the Extrelut® columns. After 10 min, the alkaloids were eluted with (5 mL × 3) chloroform. The organic solvent was distilled under pressure to afford the alkaloidal extract. The extract was dissolved in 1 mL 0.1% TFA, passed through a 0.45-µm filter (Grace Davison, USA), and 20 µL of the filtrate was injected into the HPLC column for analysis.

Chromatography

The analysis of the samples and validation experiments were carried out using a liquid chromatografic system (Agilent 1100 series) equipped with a quaternary pump, a vacuum degasser, a thermostatted column compartment, a manual injector with 20 µL loop (Rheodyne 7725i), a diode array detector (DAD) (Agilent 1200 series) and software Agilent ChemStation. The chromatographic resolution was performed with an isocratic mobile phase including TFA-water-acetonitrile (0.01:95:5) on a Hichrom C18 column (5 µm particle size, 250 mm, 4.6 mm) at a flow rate of 1 mL/min and λ_{max} 290 nm at 25 °C. The injection volume was 20 µL. The chromatographic run time was 45 min. All the calculations regarding the quantitative determinations were carried out by an external standard method based on peak areas.

Method Validation

Assessment of linearity, accuracy and precision studies were carried out according to the ICH validation guidelines (ICH 2005).

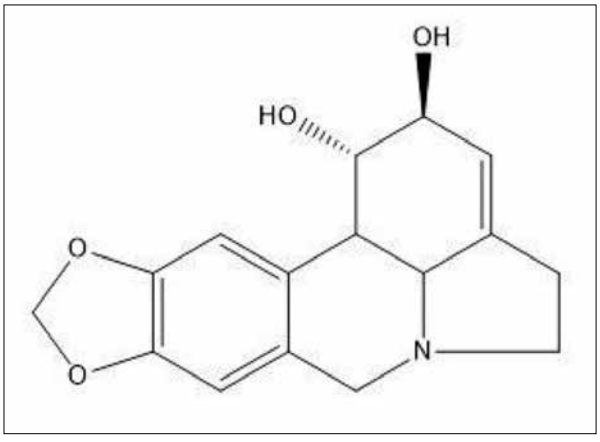

Figure 1. Chemical Structure of lycorine

Linearity

Standard stock solutions of lycorine were prepared by dissolving 2 mg in 5 mL 0.1 % TFA. Afterwards this solution was diluted with 0.1 % TFA to attain solutions at 2.5, 5.0, 7.5, 10.0, 15.0 and 20.0 µg mL^{-1}. 20 µL of standard solution was injected into the column in triplicate and then a calibration curve was constructed by plotting the peak areas of lycorine against its respective concentrations.

Precision

Intra-day and inter-day precisions were used to study the repeatability and reproducibility of the method. For the intra-day variability test, six different concentrations of standard lycorine were applied in triplicate on the same day while for the inter-day test, the standard lycorine was analysed by performing the same procedure on two different days.

Limits of Detection (LOD) and Quantification (LOQ)

Limit of detection and limit of quantification were determined by injecting the standard solution until the signal to noise ratio (S/N), was about 3 for LOD and 10 for LOQ. The LOD and LOQ were experimentally verified by ten injections of lycorine.

Recovery

Accuracy of the method was checked via recovery studies performed by a standard addition method. Mixed standard solutions with three different concentration levels were prepared and added to the plant sample and the mixtures were analyzed using the proposed method utilized in the quantitative determination of lycorine in the plant samples.

Specificity

Specificity is the ability to specifically measure the analyte in the presence of other components - typically including impurities, degradants, matrix etc. The specificity of the method was determined by the detection of lycorine in the presence of other constituents present in the extract. The selectivity of peaks of lycorine in the samples was evaluated by comparing their retention time and UV λ_{max} with the lycorine standard.

RESULTS

Sample Analysis

The results of quantitative determination of lycorine in the bulbs of *G. alpinus* var. *alpinus* are scheduled in Table 1. The chromatograms of the standard lycorine and plant extracts are shown in Figures 2-4. The identification of lycorine in plant specimens was achieved by comparison of the retention time and the UV spectrum of standard lycorine. The combination of the mobile phase was decided regarding maximum separation and selectivity. In accordance with this, the TFA-water-acetonitrile (0.01:95:5) mobile

Table 1. Lycorine content of *G. alpinus* var. *alpinus*

G. alpinus var. *alpinus* (Season)	Specimen	Content of *Lycorine* (%)
Flowering	Bulbs	0.01576±0.001686
	Aerial Parts	ND
Fruiting	Bulbs	0.02351±0.0003182
	Aerial Parts	ND

ND: Not Detected

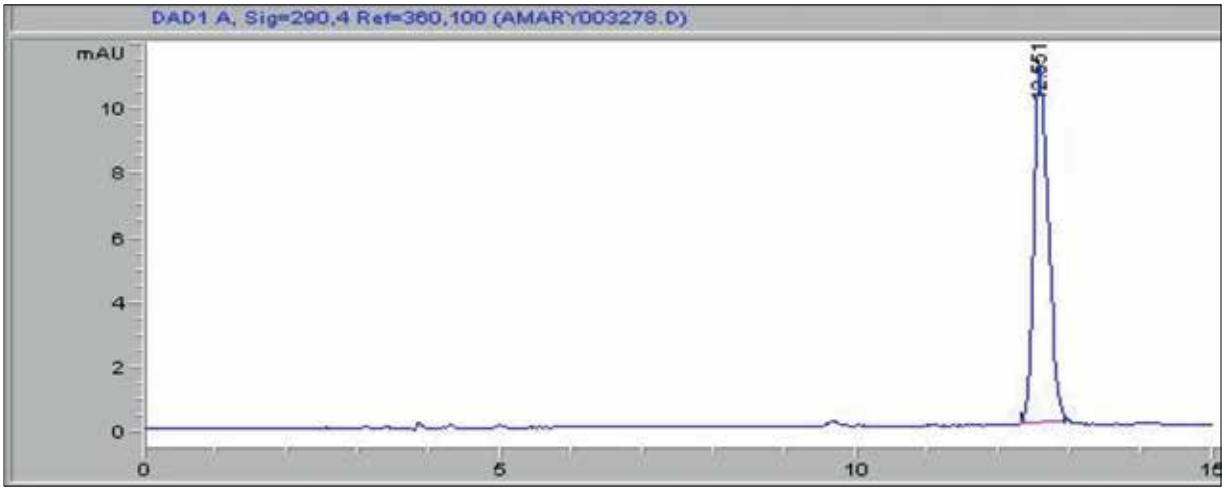

Figure 2. HPLC chromatogram of standard lycorine (Rt=12.551)

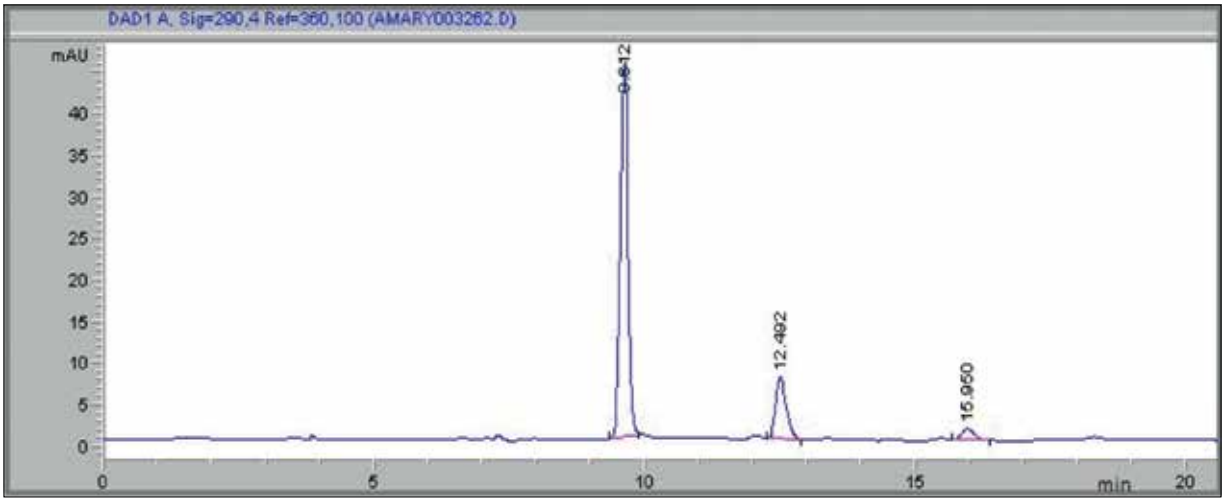

Figure 3. HPLC chromatogram of an alkaloidal extract from *G. alpinus* var. *alpinus* (Bulbs ; flowering period) (Rt=12.492)

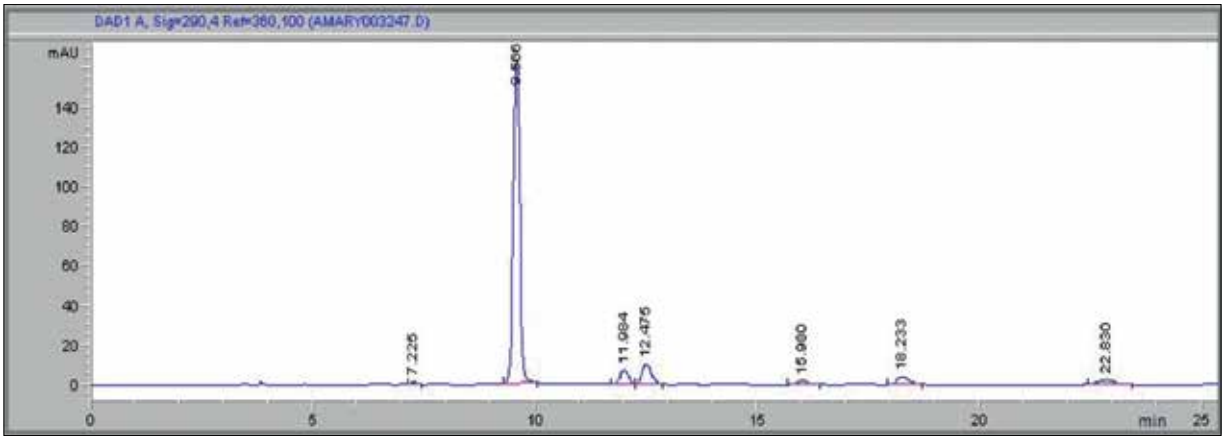

Figure 4. HPLC chromatogram of an alkaloidal extract from *G. alpinus* var. *alpinus* (Bulbs ; fruiting period) (Rt=12.475)

phase system was used. Under the described chromatographic conditions, the quantitative analysis of lycorine was performed in triplicate using the external standard method based on peak areas. As a result, the lycorine content was detected as 0.01576 and % 0.02351 % in the bulbs of plant specimens collected during flowering season and fruiting season, respectively. Lycorine was not detected in quantitative amounts in the aerial parts of this *Galanthus* species. Also, specimens of *G. alpinus* var. *alpinus* were

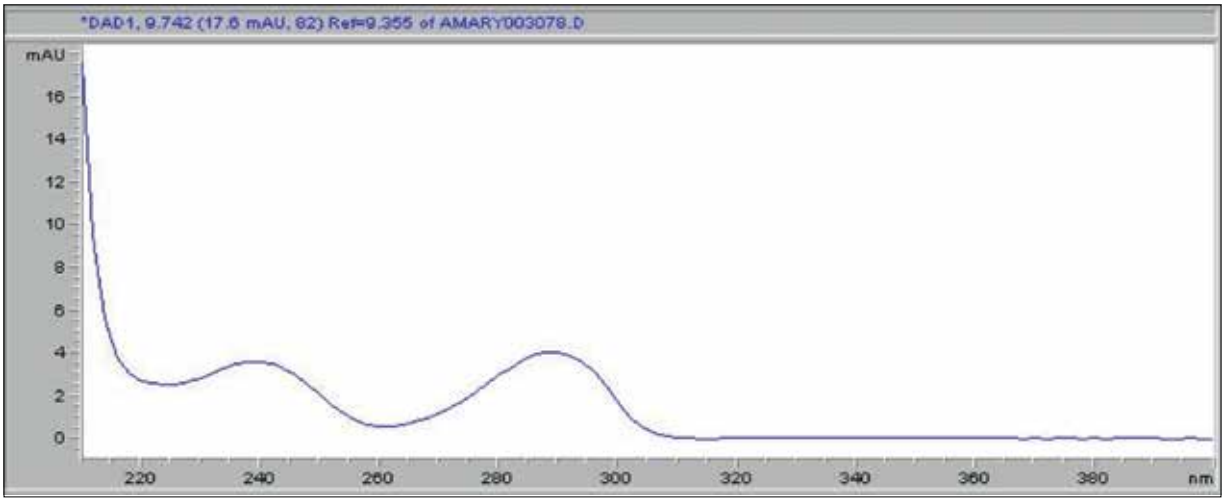

Figure 5. UV Spectrum of standart lycorine

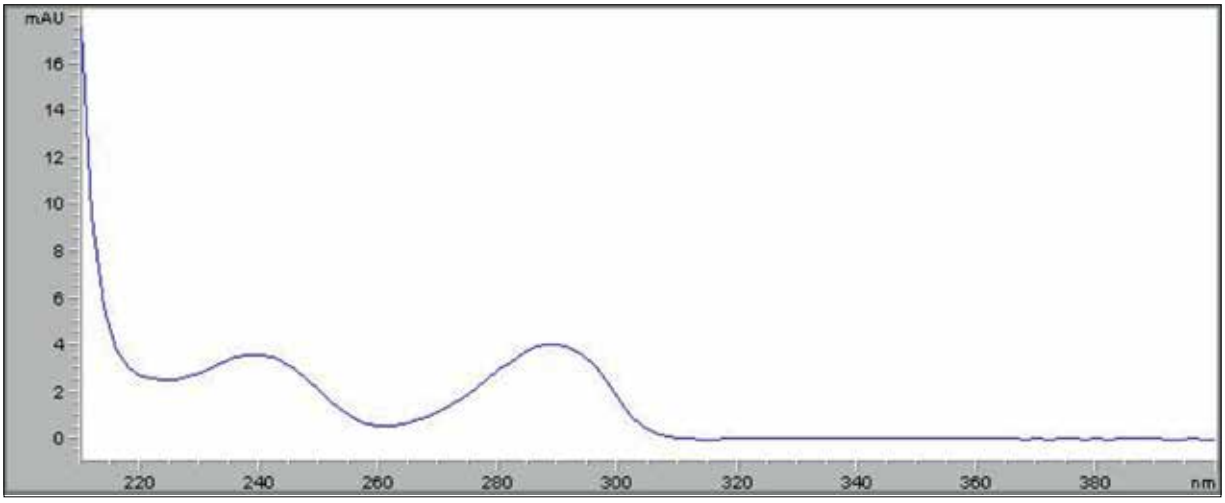

Figure 6. UV Spectrum of lycorine in extract of bulbs (flowering period)

Table 2. Intra-day and inter-day precision of lycorine for *G. alpinus* var. *alpinus*

Compound	Amount (µg/mL)	Intra-day Precision (RSD, %)	Inter-day Precision (RSD, %)
Lycorine	2.5	0.958	0.869
	5	1.252	1.276
	7.5	0.771	1.213
	10	0.870	0.743
	15	0.984	0.214
	20	0.896	1.001

ND: Not Detected

investigated for their galanthamine content, but it was detected neither in the aerial parts nor in the bulbs of this plant.

Method Validation

Linearity

The regression equation for lycorine was found as y=10.73592x-2.67864. Perfect linearity was acquired (r^2=0.99965) performing a good correlation between the alkaloid concentration and its peak area.

Precision

The results of the precision analysis of lycorine are summarized in Table 2. RSD values were found to be less than 1.5 % which indicated plausible repeatability for this method.

Limits of Detection (LOD) and Quantification (LOQ)

The LOD (signal /noise ratio of 3:1) was calculated as 0.037 µg mL^{-1} and the LOQ (signal/noise ratio of 10:1) was determined as 0.125 µg mL^{-1}. At the LOD and LOQ levels RSD % was determined to be 9.96 for lycorine.

Recovery

The mean extraction recovery of lycorine was found within the range of 94.289 – 98.533 %. The results of the experiments are given in Table 3.

Specificity

Peak purity of lycorine was evaluated by the acquisition of UV spectra with the DAD detector. The spectra of the analyzed compound is shown in Figures 5-7.

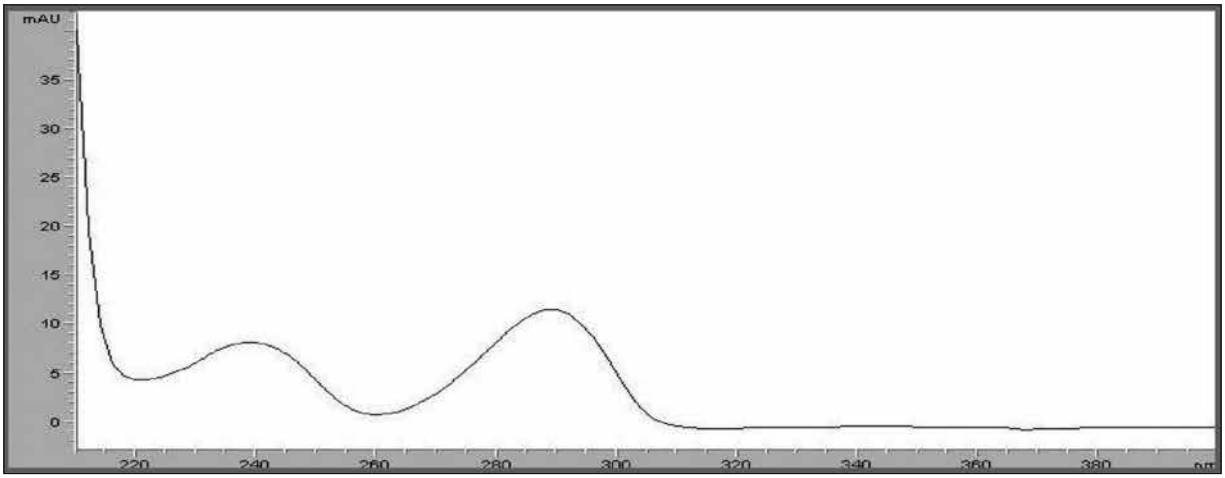

Figure 7. UV Spectrum of lycorine in extract of bulbs (fruiting period)

Table 3. Statistical data for recovery studies of lycorine

Compound	Concentration in Sample (mg/mL)	Amount Spiked (mg/mL)	Mean Amount Found in Mixture (mg/mL)	Mean Recovery (%)±SD	RSD (%)
Lycorine	0.01	0.005	0.0074	98.533±0.14	0.143
	0.01	0.01	0.0094	94.289±1.158	1.228
	0.01	0.02	0.0144	96.257±0.439	0.456

DISCUSSION

Quantitative analysis of lycorine in the *Galanthus* species was accomplished using a chromatographic method with different separation selectivity and a high system efficiency. Additionally, simple and rapid sample preparation, small plant samples and basic composition of the mobile phase are counted among the other benefits of the method. The method was validated in accordance with linearity, precision, recovery and limits of detection and quantification. This simple, rapid and reliable HPLC method is suitable for the quantitative analysis of lycorine, which is a biologically important Amaryllidaceae alkaloid. Comparisons between the samples from the flowering and fruiting periods showed a higher lycorine content during the fruiting period of *G. alpinus* var. *alpinus*. . Moreover, significant differences in the content of this alkaloid have been observed in the aerial parts and bulbs. In previous studies, several *Galanthus* species were reported to contain only lycorine - not galanthamine -as in the results of this study. Finally, lycorine content was calculated to be higher in bulbs than aerial parts in accordance with relevant literature (Kaya et al. 2004; Sarıkaya et al. 2012; Unver Somer et al. 2013; Kaya et al. 2014; Emir et al. 2017). The fact that the diversity of these alkaloids, their distribution and amounts are different in species and populations, may be explained by infraspecific variations which are influenced by several factors such as polymorphisms in genotype as well as stage of maturity, soil composition and the different climate and drying conditions (Larsen et al. 2010). To the best of our knowledge, this is the first report on the quantification of lycorine in *G. alpinus* var. *alpinus* of Turkish origin.

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Conflict of Interest: The authors have no conflict of interest to declare.

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