

## Application of HPLC-DAD for the quantification of Lycorine in *Galanthus elwesii* Hook

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In the present study, a reversed-phase high-performance liquid chromatographic method has been used for the quantitative determination of lycorine in the aerial parts and bulbs of *G. elwesii* Hook. A simple method for the extraction of lycorine in low mass plant samples was employed utilizing pre-packed columns with diatomaceous earth (Extrelut®). The chromatographic separation was performed using an isocratic system with a mobile phase of trifluoroacetic acid-water-acetonitrile (0.01:92.5:7.5, v/v/v) applied at a flow rate 1 mL min<sup>-1</sup> using diode array detector. The content of lycorine in the bulbs and aerial parts of *G. elwesii* collected from Demirci (Manisa) was found as 0.130 and 0.162 %, respectively. Additionally, in the bulbs of the specimens collected from Sogucak (Balikesir), lycorine was quantified as 0.055 %, whereas in the aerial parts, it was determined as 0.006 %. The method was validated partially with respect to system specificity, linearity, accuracy, precision, limits of detection (LOD) and quantitation (LOQ). Validation procedures displayed that the method was specific, accurate and precise.

**Uniterms:** Alkaloids. Amaryllidaceae. Plant extracts. High Performance Liquid Chromatography/method validation. High Performance Liquid Chromatography/quantitative analysis.

### INTRODUCTION

*Galanthus elwesii* Hook. (Amaryllidaceae) is an easily recognized species with broad glaucous leaves, large flowers and bold markings on the inner segments. It has a relatively wide distribution and can be found in eastern parts of the former Yugoslavia, northern Greece, the eastern Aegean Islands, southern Ukraine, Bulgaria and Turkey. Within Turkey, this species has the widest distribution among others and naturally grows in northwestern, western and southern Anatolia (Bishop, Davis, Grimshaw, 2006; Yuzbasioglu, 2012).

Plants of the Amaryllidaceae are known to produce alkaloids with a wide range of pharmacological activities (Unver, 2007; Hoshino, 1998). Among the Amaryllidaceae alkaloids, galanthamine, is used for the treatment of mild and moderate cases of Alzheimer's disease (AD) (Howes, Perry, 2011). Lycorine, a major alkaloid found in many Amaryllidaceae species, has been shown to have

a wide variety of biological activities such as antitumoral (Wang *et al.*, 2014), antimalarial (Cedron *et al.*, 2010), hepatoprotective (Ilavenil, Kaleeswaran, Ravikumar, 2012), antiviral (He *et al.*, 2013), antifungal (Shen *et al.*, 2014) and antiparasitic (Giordani *et al.*, 2012) activities. Due to its diverse biological properties, it has been to the interest of phytochemists to determine the content of this alkaloid using various analytical techniques in Amaryllidaceous plants (Abou-Donia *et al.*, 2007; Petruczynik *et al.*, 2016).

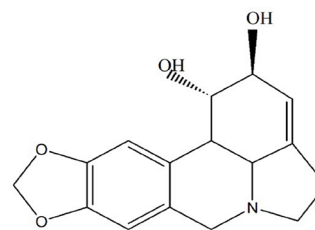


FIGURE 1 - Chemical structure of lycorine.

In the present study, during the course of our ongoing phytochemical studies on Turkish *Galanthus* species, aerial parts and bulbs of *G. elwesii*, collected

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from two different localities in Western Turkey, were quantitatively analyzed for their lycorine content by using high performance liquid chromatography (HPLC) coupled with a diode array detector (DAD). In the context of validation procedures, linearity, precision, limits of detection and quantification, accuracy, and specificity of the method were also displayed. In addition, the specimens were analyzed for their galanthamine content.

## MATERIAL AND METHODS

### Material

Standard sample of lycorine was previously isolated in our laboratory and authenticated by detailed spectral analysis (UV, IR, NMR, MS) (Kaya *et al.*, 2004a). The chemical structure of lycorine was given in Figure 1. TFA (trifluoroacetic acid) (Merck), HPLC grade acetonitrile (Lab-Scan Analytical Sciences) and chromatographic grade double-distilled water were used for the HPLC analysis. Other chemicals were of analytical grade.

### Apparatus

Both the analysis of the samples and the validation studies were performed on a liquid chromatographic system (Agilent 1100 series), equipped with a quaternary pump, a vacuum degasser, a thermostatted column compartment, a manual injector with 20  $\mu$ L loop (Rheodyne 7725i), a diode array detector (DAD) (Agilent 1200 series) and a Hichrom C<sub>18</sub> column (250 x 4.6 mm, i.d., particle size 5  $\mu$ m).

### Procedure

#### Samples

Specimens of *G. elwesii* were collected from Sogucak (Balıkesir) and Demirci (Manisa) in Western Turkey,

during flowering time. The plants were identified by Prof. M. Ali Onur from the Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Turkey. Voucher samples of *G. elwesii* (No's 1363 and 1401) are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

#### Preparation of samples and standard solutions

The extraction procedure of lycorine from the plant material and preparation of standard lycorine solutions were performed according to a previously used method (Kaya *et al.*, 2010).

#### HPLC determination

The extract was dissolved in 1 mL 0.1 % TFA. The injection volume was 20  $\mu$ L and the flow rate was 1 mL/min. The chromatographic run time was 50 min. Column temperature was set at 25 °C. The chromatographic separation was achieved isocratically using a mobile phase consisted of TFA-water-acetonitrile (0.01:92.5:7.5, v/v/v) on a Hichrom C<sub>18</sub> column and detection was carried out at 290 nm (Kaya *et al.*, 2010; Mustafa, Rhee, Verpoorte, 2003). Quantitative determination was carried out by an external standard method based on peak areas.

## RESULTS AND DISCUSSION

The previously proposed method (Kaya *et al.*, 2010; Mustafa, Rhee, Verpoorte, 2003) was applied for the detection of lycorine and galanthamine plus for the quantification of lycorine in *G. elwesii* specimens. Under the above-stated chromatographic conditions, lycorine was resolved within approximately 9 min. (Figure 2 and 3). Quantitative determination was carried out by the external standard method based on peak areas. The results of the mean values of three replicate injections of lycorine were reported in Table I. Significant variation in the amounts of lycorine was found in the analyzed samples.

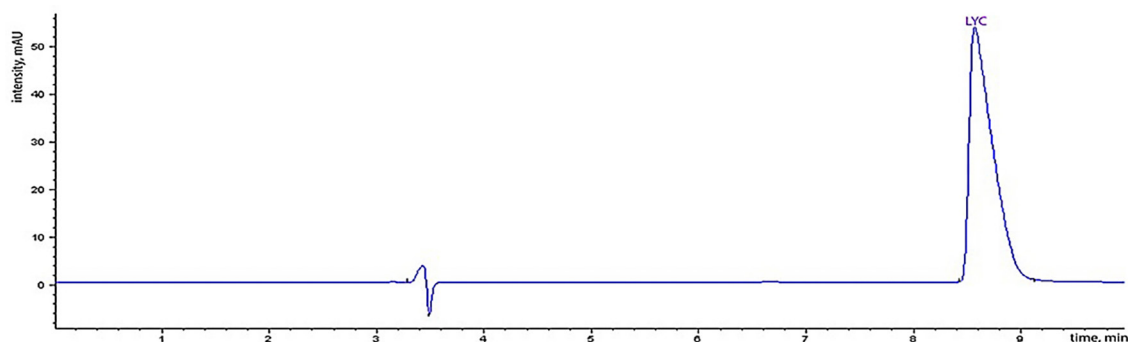
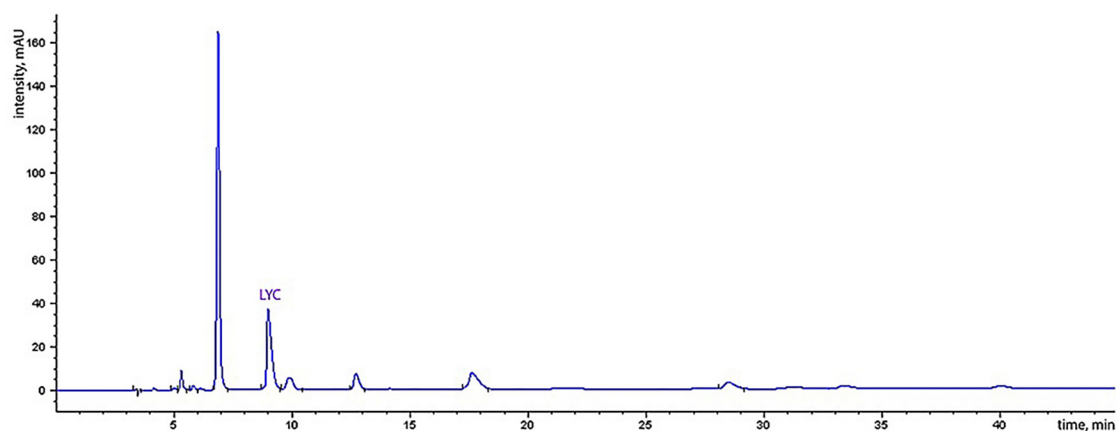


FIGURE 2 - Chromatogram of lycorine.



**FIGURE 3** - Chromatogram of an extract of *G. elwesii*.

The lycorine content ranged between 0.006-0.162%. The highest amount was found in the aerial parts of *G. elwesii* collected from Demirci (Manisa). Linearity, accuracy and precision studies were carried out according to the ICH validation guidelines on the validation of analytical procedures (ICH, 2005).

#### Specificity

Specificity is described as the ability to measure the analyte response in the presence of components such as impurities, degradants, matrix, etc. (ICH, 2005). The specificity of the method for the analysis of lycorine was evaluated in the presence of other components of the extracts. Peak purities of lycorine was evaluated by the acquisition of UV spectra with the DAD detector.

#### Linearity

Stock standard solution of lycorine was prepared by dissolving 2 mg in 10 mL 0.1 % TFA. The linearity of the method was shown by injecting seven known concentrations of the standard lycorine in the range of 2.5-200  $\mu\text{g/mL}$ . Each standard solution (20 mL) was injected into the column in triplicate and then the calibration curve of the analyte was obtained by plotting the peak area versus the concentration. The regression equation for lycorine was found as  $y = 16.17864x - 3.12657$ . Excellent linearity was obtained ( $r^2 = 0.99997$ ) exhibiting a good correlation between the alkaloid concentration and the peak area.

#### Accuracy

Accuracy may be defined as the agreement between the found value and true value of the reference material and can be presented as a percent recovery. Standard addition analysis was performed for the recovery of the method. Three known amounts of the individual standard were added to sample solutions and the mixtures were analyzed by the same method used in the analysis of lycorine in the plant samples. Recovery assay was carried out by spiking three different known concentrations of lycorine into the sample solutions prior to extraction. The mean extraction recovery of lycorine was found as in the range of 94.24 - 100.60 %. The results of the experiments are given in Table I.

#### Precision

The precision of the method was evaluated by studying intra-day and inter-day variations. Intra-day precision was determined by injecting solutions of seven different concentrations of standard lycorine (2.5, 5, 10, 25, 50, 100, 200  $\mu\text{g/mL}$ ) in triplicate on the same day. Inter-day precision was calculated by performing the same procedure on two different days. The results of the precision analysis of lycorine are summarized in Table II. RSD was found to be always less than 2.0 % when the analysis was performed at seven different concentrations.

#### Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification

**TABLE I** - Statistical data showing recovery studies of lycorine (n=3)

	in sample ( $\mu\text{g/mL}$ )	Spiked ( $\mu\text{g/mL}$ )	found ( $\mu\text{g/mL}$ )	recovery, %	RSD, %
Lycorine	0.033	0.0165	0.0249	100.60	0.725
	0.033	0.033	0.0311	94.24	0.735
	0.033	0.066	0.0498	100.60	1.737

**TABLE II** - Intra-day and inter-day precision of the method

Compound	Amount ( $\mu\text{g/mL}$ )	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)
Lycorine	2.5	1.27	1.78
	5	1.76	1.2
	10	1.54	1.34
	25	0.77	1.24
	50	1.77	1.77
	100	0.39	0.23
	200	0.17	0.34

were evaluated based on the signal-to-noise ratio (S/N) of 3 and 10, respectively. LOD and LOQ were experimentally determined by 10 injections of lycorine. The LOD and the LOQ of the analyte were calculated as  $0.0423\mu\text{g/mL}$  and  $0.14921\mu\text{g/mL}$  respectively.

## CONCLUSION

Comprising a part of our phytochemical studies on *Galanthus* species of Turkish origin, the present study was undertaken to evaluate the lycorine and galanthamine contents of two *Galanthus elwesii* specimens naturally growing in Western Turkey. As a result, lycorine was determined both in the aerial parts and bulbs of these specimens, however galanthamine was not detected in any of the plant samples. The employed method has several

advantages including simple and rapid sample preparation, requirement of a small amount of plant material, and a simple mobile phase. The method was also validated with respect to linearity, intra and inter-day precision, recovery, limits of detection and quantification. Validation studies showed that the method could be successfully used to determine lycorine in *G. elwesii* samples, which provided an efficient and reliable method for the quality control of *G. elwesii*. *G. elwesii* specimens collected from different localities in Turkey have been investigated for their content of lycorine and galanthamine in previous studies (Kaya *et al.*, 2004b; Celik-Sarier, 2002; Kaya, Gozler, 2003; Muhtar, Sener, 1996; Kaya *et al.*, 2014). The results of these studies together with the results of the present study are summarized in Table III.

The results obtained in the present study differ

**TABLE III** - Lycorine and galanthamine content of *Galanthus elwesii* from different localities

Localities	Plant parts	Lycorine, %	Galanthamine, %
Soğucak (Balıkesir) <sup>a</sup>	Bulbs	0.055 <sup>a</sup>	ND <sup>a,b</sup>
	Aerial parts	0.006 <sup>a</sup>	ND <sup>a,b</sup>
Demirci (Manisa) <sup>a</sup>	Bulbs	0.130 <sup>a</sup>	ND <sup>a,b</sup>
	Aerial parts	0.162 <sup>a</sup>	ND <sup>a,b</sup>
Yamanlar (İzmir) (Kaya <i>et al.</i> , 2004b; Celik-Sarier, 2002)	Bulbs	ND <sup>b</sup>	ND <sup>b</sup>
	Aerial parts	ND <sup>b</sup>	ND <sup>b</sup>
Akdag-Karaburun (İzmir) (Kaya, Gozler, 2003)	Bulbs	0.004-0.007	0.007-0.008
	Aerial parts	0.001-0.013	0.013-0.026
Akseki (Antalya) (Muhtar, Sener, 1996)	Bulbs	0.011	Not studied
Cimi Village (Antalya) (Kaya <i>et al.</i> , 2014)	Bulbs	ND <sup>b</sup>	0.042
	Aerial parts	ND <sup>b</sup>	0.346
Ibradi (Antalya) (Kaya <i>et al.</i> , 2014)	Bulbs	0.005	0.095
	Aerial parts	ND	0.287
Kayrak (Mersin) (Kaya <i>et al.</i> , 2014)	Bulbs	0.015	ND <sup>b</sup>
	Aerial parts	ND <sup>b</sup>	ND <sup>b</sup>

<sup>a</sup> The results of the present study; <sup>b</sup> ND: Not detected

significantly from the results of previous studies on *Galanthus elwesii* (Table III). This could be caused by endogenous and exogenous factors. Karyological investigations on Turkish *Galanthus* populations have been made by several researchers (Şenel, Ozkan, Kandemir, 2002; Zeybek, 1988; Zeybek, Sauer, 1994) and these studies have revealed a polymorphism among different karyotypes and SAT –chromosomes which were reported to be important in the variation (Kandemir, 2010). Earlier reports showed that the concentration of alkaloids vary in great amount in different Amaryllidaceae plants with the seasonal growth (Katoch *et al.*, 2012; Tram *et al.*, 2002). In addition, it has been documented that a relationship existed between the alkaloid content of an Amaryllidaceous species *Leucojum aestivum* L. and the chemical composition of the soil (Gorinova *et al.*, 1993). Therefore, the variations of the alkaloid content in *G. elwesii* specimens may be influenced by several factors including polymorphism, collection site and time.

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