GC/MS Analysis of Alkaloids in *Galanthus fosteri* Baker and Determination of Its Anticholinesterase Activity

Galanthus fosteri Baker'da Alkaloitlerin GC/MS Analizi ve Antikolinesteraz Aktivitesinin Belirlenmesi

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Abstract

Objectives: Amaryllidaceae alkaloids are well known for their wide range of pharmacological activities. Galanthamine, an Amaryllidaceae alkaloid, is effective, selective, reversible, and competitive cholinesterase inhibitor marketed under the different commercial name in several countries for the treatment of Alzheimer's disease. This work was aimed at studying the alkaloid profiles of the aerial parts and bulbs of both flowering and fruiting periods of *Galanthus fosteri* Baker (Amaryllidaceae), as well as analyzing their inhibitory activities on both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), for the first time.

Materials and Methods: The alkaloid profiles of the four samples were determined by means of Gas Chromatography-Mass Spectrometry (GC-MS), and acetylcholinesterase and butyrylcholinesterase inhibition assays were performed by the modified Ellman method.

Results: Totally, twenty-two compounds with mass spectral characteristics of Amaryllidaceae alkaloids were detected in the extracts. Significant acetylcholinesterase and butyrylcholinesterase inhibitory activities were observed in the tested samples (IC₅₀ between 0.189 and 91.23 µg/mL).

Conclusion: This study shows that *G. fosteri*, collected from Akdağ, Amasya (Turkey), has a potential of being the source of diverse chemical structures of Amaryllidaceae alkaloids which have cholinesterase inhibitory property.

Keywords: *Galanthus fosteri*, Amaryllidaceae alkaloids, Anticholinesterase activity

ÖZ

Amaç: Amaryllidaceae alkaloidleri geniş yelpazedeki farmakolojik aktiviteleriyle iyi bilinmektedir. Bir Amaryllidaceae alkaloidi olan Galantamin, Alzheimer hastalığının tedavisi için çeşitli ülkelerde farklı ticari isimler altında pazarlanan etkili, seçici, geri dönüşümlü ve kompetitif kolinesteraz inhibitörüdür. Bu çalışma, ilk kez *Galanthus fosteri* Baker'in (Amaryllidaceae), hem çiçekli hem de meyveli dönemlerinin toprak üstü ve soğanlarının alkaloid profilini incelemenin yanı sıra hem asetilkolinesteraz (AChE) hem de butirilkolinesteraz (BuChE) üzerindeki inhibitör aktivitelerini analiz etmeyi amaçlamıştır.

Gereç ve Yöntemler: Dört örneğin alkaloid profilleri Gaz Kromatografisi-Kütle Spektrometrisi (GC-MS) ile belirlendi ve asetilkolinesteraz ve butirilkolinesteraz inhibisyon analizleri modifiye Ellman yöntemi ile yapılmıştır.

Bulgular: Ekstrelerde Amaryllidaceae alkaloidlerinin kütle spektral özellikleri olan toplam yirmi iki bileşik tespit edilmiştir. Test edilen örneklerde belirgin şekilde asetilkolinesteraz ve butirilkolinesteraz inhibitör aktiviteler gözlenmiştir (IC_{50} 0.189 ile 91.23 $\mu\text{g} / \text{mL}$ arasında).

Sonuç: Bu çalışma, Amasya Akdağ' dan (Türkiye) toplanan *G. fosteri*' nin kolinesteraz inhibitör özelliğine sahip çeşitli kimyasal yapıdaki Amaryllidaceae alkaloidlerinin kaynağı olma potansiyeline sahip olduğunu göstermiştir.

Anahtar kelimeler: *Galanthus fosteri*, Amaryllidaceae alkaloidleri, Antikolinesteraz aktivite

Introduction

Galanthus fosteri Baker (snowdrop) is a bulbous monocotyledon plant belonging to the Amaryllidaceae family, occurring mainly in the south- and north-central Turkey.¹ Plants of Amaryllidaceae family are known to possess alkaloids with diverse chemical structures and a wide spectrum of biological activities such as cholinesterase inhibitory, antimalarial, hepatoprotective, antitumoral, anti-inflammatory and antiviral.²⁻⁷

Alzheimer's disease (AD), the most common cause of dementia affecting approximately 46.8 million people worldwide, is a neurodegenerative disease that characterized by widespread loss of central cholinergic function.⁸ The human central nervous system contains two cholinesterases: acetylcholinesterase (AChE), encoded by a gene on chromosome 7, and

butyrylcholinesterase (BuChE), encoded by a gene on chromosome 3. Although the role of acetylcholinesterase in the cholinergic system is well known, the role of butyrylcholinesterase is poorly understood. However, both enzymes alone are not sufficient for acetylcholine metabolism and cholinergic transmission.⁹⁻¹⁰ Therefore, in the treatment of AD, it is important to inhibit the enzyme acetylcholinesterase as well as butyrylcholinesterase. Galanthamine, the most important alkaloid found in Amaryllidaceae plants, marketed under the commercial name of Reminyl® in Europe and elsewhere and Razadine® in the USA, is used to treat AD owing to its cholinesterase inhibitory properties.¹¹

It has been found that Amaryllidaceae alkaloids, can be analyzed by GC/MS without any previous derivatization, and they show a mass spectral fragmentation pattern very similar to those recorded.¹² In this study, alkaloid profile of *G. fosteri* was determined by GC-MS, using both bulbs and aerial parts of flowering and fruiting periods. Also, cholinesterase inhibitory activity potentials of the extracts were examined spectrophotometrically by using a microplate assay modified from *in vitro* Ellman's method with 96-well micro-plate reader.^{13,14}

Experimental

Plant material

G. fosteri was collected from Akdag, Amasya on March 28, 2012 and April 1, 2013 during flowering and fruiting periods, respectively. 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. fosteri* (No: 1516, 1525) are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Alkaloid extraction

The alkaloidal extracts were prepared from air-dried and powdered aerial parts and bulbs of *G. fosteri* Baker to be used in GC-MS analysis and in the anticholinesterase activity assay. Plant material (500 mg) was separately extracted 3 times with methanol (5 mL) at room temperature. The solvent was evaporated under reduced pressure, the residues dissolved in 10 mL 2 % sulfuric acid and the neutral compounds were removed with diethyl ether (3x10 mL). The acidic aqueous phases were basified with 25 % ammonia to pH 9-10 and the alkaloids were extracted with chloroform (3x10 mL). The combined chloroform extracts were then dried over anhydrous sodium sulphate, filtered and the organic solvent was distilled *in vacuo*

to afford the alkaloidal extract.¹⁵ The obtained extracts were used for GC-MS analysis and also screened for anticholinesterase activity.

GC-MS analysis

The GC-MS analysis was performed using Thermo GC-Trace Ultra Ver: 2.0., Thermo MS DSQ II (Thermo Fisher Scientific, San Jose, CA, USA) operating in the electron impact mode (EI, 70 eV). The oven temperature was programmed as 80 °C for 1 min, 80–250 °C (10 °C min⁻¹), 250 °C for 2 min, 250–300 °C (10 °C min⁻¹) and a 10 min hold at 300 °C. The injector temperature was 250 °C. Helium was used as a carrier gas at a flow rate of 0.8 mL min⁻¹. A TR-5 MS column (30 m × 0.25 mm × 0.25 µm) was used. The extracts were dissolved in methanol (1 mg extract in 500 µL methanol). All injections were run in the splitless mode. The spectra of co-eluting chromatographic peaks were investigated and deconvoluted by the use of Xcalibur (version 2.07; Thermo Fisher Scientific San Jose, CA, USA). The compounds were identified by comparing their mass spectral fragmentation with standard reference spectra from the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0 (2005), National Institute of Standardization and Technology, Gaithersburg, MD), or applying co-chromatography with previously isolated authentic standards and in comparison with data obtained from the literature. The percentage of the total ion current (TIC) for each compound is given in Table 2. The area of the GC-MS peaks depends both on the concentration of the corresponding compound and on the intensity of their mass spectral fragmentation. Also they can be used for a relative comparison of the alkaloids.

Anticholinesterase activity

The alkaloidal extracts of aerial parts and bulbs were tested for their AChE and BuChE inhibitory activities by using 96-well microplate assay modified from Ellman's method at the concentration range of 0.006–600 µg/ml (final concentrations in the assay 0.0015–150 µg/ml).^{13,14} Galanthamine was used as a positive control. The enzyme inhibitory activity was calculated as the percentage compared to the blank. IC₅₀ values were analyzed by the software package Prism V5.0 (Graph Pad Inc., San Diego, CA, USA).

Results

A great number of alkaloids that are in the Amaryllidaceous plant extracts has been separated effectively and identified very quickly by GC/MS indicating that this method for chemical analysis is useful and reliable for studies on the alkaloid metabolism in this family.^{12,16} To the best of our knowledge, this is the first report of a GC/MS study on the alkaloids of *G. fosteri* growing in Turkey. The alkaloid patterns of the samples were normalized and presented as a % of individual compounds in the total alkaloidal mixture based on the deconvoluted peak area (Table 1). The structures of the alkaloids were given in Figure 1. Totally, twenty-two compounds with mass spectral characteristics of Amaryllidaceae alkaloids were detected in the extracts of aerial parts and bulbs of both flowering and fruiting periods (Table 1). The identified compounds possessed various Amaryllidaceae alkaloid skeleton types including lycorine, haemanthamine, narciclasine, homolycorine, norbelladine, galanthamine and crinine types and additionally indole alkaloid and other base hordenine. Hordenine is not typical for plants of the Amaryllidaceae family. It has been found mainly in other plant families like Poaceae (*Hordeum vulgare*), Cactaceae (in a very wide range of species), and in a few algae and fungi.²⁰ Also one compound showing mass spectral fragmentation characteristic of Amaryllidaceae alkaloids were left unidentified due to lack of reference MS spectra. Of the twenty-two alkaloids identified, only seven, hordenine, galanthamine, *O,N*-dimethylnorbelladine, galanthine, incartine, 11-*O*-acetyl-9-*O*-demethylmaritidine and oxoincartine were common in all the samples. The results obtained from the microplate assay for cholinesterase inhibitory activity of the samples ranged between 0.189 µg/ml and 91.23 µg/ml; also IC₅₀ values (µg/mL) are shown in Table 2.

Conclusion

The findings of the present study demonstrate the potential of *G. fosteri* collected in north-central Turkey, as a source of cholinesterase inhibitor compounds with diverse chemical structures of Amaryllidaceae alkaloids. The alkaloidal pattern of the aerial parts and bulbs were dominated by 11-*O*-acetyl-9-*O*-demethylmaritidine which has a remarkable AChE and BuChE inhibitory activities with IC₅₀ values of 6.04 µM and 29.72 µM, respectively. Also, incartine is the main alkaloid in bulbs which has shown low anticholinesterase activity.²⁴ The number of alkaloids in the bulbs was considerably higher in comparison with the aerial parts. Generally, lycorine and haemanthamine type alkaloids were major components in the tested samples. Thus, the products of *ortho-para*' and *para-para*' oxidative phenolic coupling dominate in samples. Both groups of alkaloids have quite pronounced biological activities.

Lycorine type alkaloids have antitumor, antiviral, antibacterial, antifungal, anticholinesterase, analgesic activities while haemanthamine type alkaloids have antitumor, antimalarial, antibacterial, analgesic and anti-inflammatory activities.²⁷⁻³⁰

The highest anticholinesterase activity was shown in the aerial parts of fruiting period, which have the highest relative amount of galantamine. Also, the highest BuChE inhibitory activity was detected in the bulbs of the same period which has the most number of lycorine type alkaloids. Compared to the other *Galanthus* species^{31,32} in which both activities were performed, the aerial parts of fruiting period is taking attention with its high AChE inhibitor activity, while BuChE inhibitor activity has similar values.

In conclusion, the present study clearly suggests that GC/MS is an appropriate method for the rapid analysis of the qualitative and relative amounts alkaloid composition in *G. fosteri* specimens which have diverse alkaloid structures and anticholinesterase activity.

Acknowledgements

We thank the Pharmaceutical Science Research Centre (FABAL) and Ege University Research Fund (2014/ECZ/20) for their financial support.

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Table 1. GC-MS data and relative composition of the alkaloids in the extracts of *G. fosteri*

Compound	R.T. (min)	[M ⁺]	m/z (relative intensity,%)	Content (percentage of TIC)				References
				Flowering period		Fruiting period		
				Aerial parts	Bulbs	Aerial parts	Bulbs	
Hordenine ^(a) (1)	11.2	165(1)	121(2), 107(4), 91(3), 77(7), 58(100)	1.86	7.40	6.08	5.85	17
M ⁺ 255 ^(a) (2)	16.91	255(22)	240((42), 181(100)153(37), 152(47), 97(27), 73(51)	-	-	-	0.11	18
Ismine ^(b) (3)	20.13	257(29)	238(100), 211(6), 196(11), 168(8)	0.16	0.22	t	1.35	19
Trisphaeridine ^(b) (4)	21.05	223(100)	222(38), 167(11), 138(27), 111(19), 69(21)	-	0.35	t	1.18	19
5,6-Dihydrobicolorine ^(b) (5)	21.05	239(36)	238(100), 180(15), 129(65), 113(18), 112(25), 111(27), 71(37)	t	-	0.16	1.51	15
Crinine ^(c) (6)	21.40	271(100)	270(35), 254(5), 228(37), 214(10), 199(26),185(31), 150(51), 122(54)	0.13	0.27	0.16	-	20
Galanthamine ^(d) (7)	21.60	287(80)	286(100), 270(14), 244(39), 230(19), 216(38)	0.21	0.94	3.18	0.33	S, NIST 05
Galanthindole ^(c) (8)	22.74	281(100)	262(23), 207(26), 191(30), 149(20), 97(21), 73(26)	-	-	-	0.33	21
9- <i>O</i> -Demethylmaritidine ^(f) (9)	22.84	273(81)	202(29), 201(100), 189(71), 175(28), 115(27)	-	9.07	-	2.62	22
Anhydrolycorine ^(g) (10)	22.98	251(41)	250(100), 224(17), 192 (16), 191(14), 96(11)	-	-	-	1.24	23
<i>O,N</i> -dimethylnorbelladine ^(h) (11)	23.22	303(9)	180(29), 137(100), 122(6), 94(5), 77(7)	1.48	2.37	1.04	1.36	24
Assoanine ^(g) (12)	23.56	267(52)	266(100), 250(31), 222(14), 207(14), 193(12), 180(15)	t	-	-	0.72	15
11,12- Didehydroanhydrolycorine ^(g) (13)	24.03	249(57)	248(100), 207(10), 191(16) 190(30), 95(23),	-	0.67	-	0.96	25

Galanthine ^(g) (14)	24.54	317(15)	316(11), 284(10), 268(16), 266(10), 244(16), 243(92), 242(100)	0.36	3.08	0.33	1.08	19
11-Hydroxyvittatine ^(f) (15)	24.89	287(9)	258(100), 242(12), 211(15), 186(20), 181(20), 153(10), 115(17)	-	8.37	2.38	13.51	S, 19
Incartine ^(g) (16)	25.10	333(30)	332(77), 259(72), 258(100), 244(18)	24.88	40.03	22.21	32.70	S, 17
Lycorine ^(g) (17)	25.28	287(19)	268(17), 250(14), 228(11), 227(67), 226(100), 147(12)	-	5.39	-	0.97	23
9- <i>O</i> -methylpseudolycorine ^(g) (18)	25.36	303(18)	302(16), 284(16), 243(72), 242(100), 240(11), 227(21), 226(30), 207(34)	-	1.34	-	0.99	S, 24
11- <i>O</i> -asetil-9- <i>O</i> - demethylmaritidine ^(f) (19)	25.8	331(100)	272(54), 271(86), 254(51), 242(58), 226(74), 181(66), 115(47)	64.51	12.57	62.79	9.12	S, 24
3,11- <i>O</i> -diacetyl-9- <i>O</i> - demethylmaritidine ^(f) (20)	26.28	373(42)	254(38), 242(48), 229(69), 227(66), 226(98), 211(90), 181(100)	5.25	-	3.22	-	S, 24
Galwesine ⁽ⁱ⁾ (21)	26.78	361(12)	207(43), 155(88), 140(100), 112(20), 96(16), 73(20)	0.21	-	-	-	20
Oxoincartine ^(g) (22)	29.52	347(40)	273(100), 258(68), 207(42), 73(16)	0.32	1.54	0.43	2.70	S, 24

a: other b: phenanthridine c: crinine d: galanthamine e: indole f: haemanthamine g: lycorine h: norbelladine i: homolycorine S: Standard t: trace < 0.1

Sample		AChE [IC ₅₀ (μg/ml)]	BuChE [IC ₅₀ (μg/ml)]
Flowering period	Aerial parts	13.15	91.23
	Bulbs	8.63	37.125
Fruiting period	Aerial parts	0.189	21.98
	Bulbs	7.12	20.65
Galanthamine		0.043	0.711

Table 2. Cholinesterase inhibitory activity of extracts.

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9 $R_1=OH$, R_2 , R_3 , R_5 , R_6 , $R_7=H$, $R_4=CH_3$

15 $R_1=OH$, R_2 , R_5 , $R_6=H$, $R_3+R_4=CH_2$, $R_7=OH$

19 $R_1=OH$, R_2 , R_3 , R_5 , $R_6=H$, $R_4=CH_3$, $R_7=OCOCH_3$

20 R_1 , $R_7=OCOCH_3$, R_2 , R_3 , R_5 , $R_6=H$, $R_4=CH_3$

10 $R_1+R_2=CH_2$

12 R_1 , $R_2=CH_3$

11

13

14 $R_1=OH$, $R_2=OCH_3$, R_3 , $R_4=CH_3$

17 R_1 , $R_2=OH$, $R_3+R_4=CH_2$

18 R_1 , $R_2=OH$, R_3 , $R_4=CH_3$

Figure 1. Structures of alkaloids identified in *G. fosteri*: Hordenine (1), ismine (3), trisphaeridine (4), 5,6-dihydrobicolorine (5), crinine (6), galanthamine (7), galanthindole (8), 9-*O*-demethylmaritidine (9), anhydrolycorine (10), *O,N*-dimethylnorbelladine (11), assoanine (12), 11,12-didehydroanhydrolycorine (13), galanthine (14), 11-hydroxyvittatine (15), incartine (16), lycorine (17), 9-*O*-methylpseudolycorine (18), 11-*O*-asetil-9-*O*-demethylmaritidine (19), 3,11-*O*-diacetyl-9-*O*-demethylmaritidine (20), Galwesine (21), Oxoincartine (22).