



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 an effective, selective, reversible, and competitive cholinesterase inhibitor marketed under different commercial names in several countries for the treatment of Alzheimer's disease. The aim of this work was to study 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, and AChE and BuChE inhibition assays were performed by the modified Ellman method.

Results: Totally, 22 compounds with mass spectral characteristics of Amaryllidaceae alkaloids were detected in the extracts. Significant AChE and BuChE 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), is a potential source of diverse chemical structures of Amaryllidaceae alkaloids with cholinesterase inhibitory properties.

Key words: *Galanthus fosteri*, Amaryllidaceae alkaloids, anticholinesterase activity

ÖZ

Amaç: Amaryllidaceae alkaloitleri 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 alkaloit profilini incelemenin yanı sıra hem asetilkolinesteraz (AChE) hem de butiril kolinesteraz (BuChE) üzerindeki inhibitör aktivitelerini analiz etmeyi amaçlamıştır.

Gereç ve Yöntemler: Dört örneğin alkaloit profilleri gaz kromatografisi-kütle spektrometrisi ile belirlendi ve AChE ve BuChE inhibisyon analizleri modifiye Ellman yöntemi ile yapılmıştır.

Bulgular: Ekstrelerde Amaryllidaceae alkaloitlerinin kütle spektral özellikleri olan toplam yirmi iki bileşik tespit edilmiştir. Test edilen örneklerde belirgin şekilde AChE ve BuChE inhibitör aktiviteler gözlenmiştir (IC₅₀ 0,189 ile 91,23 µg/mL arasında).

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

Anahtar kelimeler: *Galanthus fosteri*, Amaryllidaceae alkaloitleri, antikolinesteraz aktivite

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Received: 09.07.2018, Accepted: 11.10.2018

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INTRODUCTION

Galanthus fosteri (*G. fosteri*) Baker (snowdrop) is a bulbous monocotyledon plant belonging to the family Amaryllidaceae occurring mainly in south- and north-central Turkey.¹ Plants of this 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 and affecting approximately 46.8 million people worldwide, is a neurodegenerative disease 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 AChE in the cholinergic system is well known, the role of BuChE is poorly understood. However, each enzyme alone is not sufficient for acetylcholine metabolism and cholinergic transmission.^{9,10} Therefore, in the treatment of AD, it is important to inhibit AChE as well as BuChE. 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 gas chromatography-mass spectrometry (GC/MS) without any previous derivatization, and they show a mass spectral fragmentation pattern very similar to those recorded.¹² In the present study, the alkaloid profile of *G. fosteri* was determined by GC/MS, using both bulbs and aerial parts of the flowering and fruiting periods. In addition, cholinesterase inhibitory activity potentials of the extracts were examined spectrophotometrically using a microplate assay modified from Ellman's *in vitro* method with a 96-well micro-plate reader.^{13,14}

MATERIALS AND METHODS

Plant material

G. fosteri was collected from Akdağ, Amasya, on March 28, 2012 and April 1, 2013 during the flowering and fruiting periods, respectively. The plants were identified by Prof. M. Ali Onur from the Department of Pharmacognosy, Faculty of Pharmacy, Ege University, İzmir, 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 were dissolved in 10 mL of 2% sulfuric acid, and the neutral compounds were removed with diethyl ether (3×10 mL). The acidic aqueous phases were basified with 25% ammonia to pH

9-10 and the alkaloids were extracted with chloroform (3×10 mL). The combined chloroform extracts were then dried over anhydrous sodium sulfate, 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 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 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 of extract in 500 μL of methanol). All injections were run in 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, USA), or applying co-chromatography with previously isolated authentic standards and in comparison with data obtained from the literature. The percentage of total ion current for each compound is given in Table 1. 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. Moreover, they can be used for a relative comparison of alkaloids.

Anticholinesterase activity

The alkaloidal extracts of the aerial parts and bulbs were tested for their AChE and BuChE inhibitory activities by 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 (GraphPad Inc., San Diego, CA, USA).

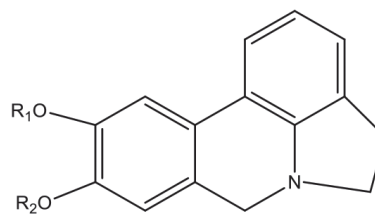
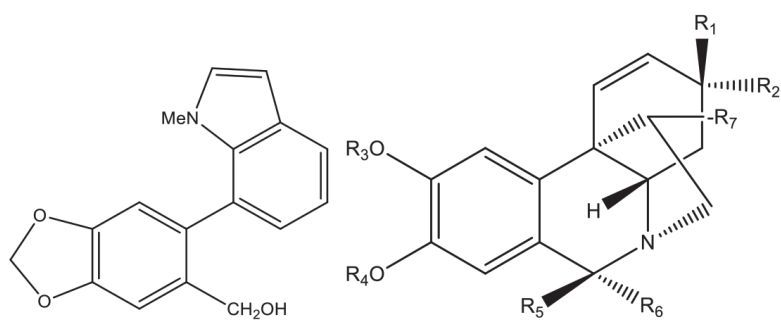
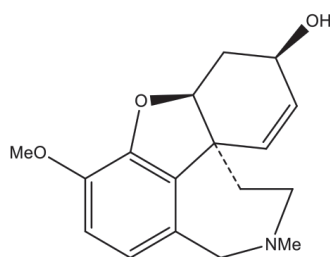
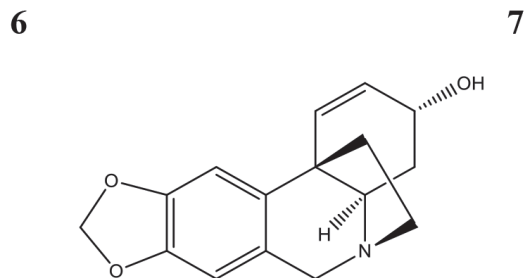
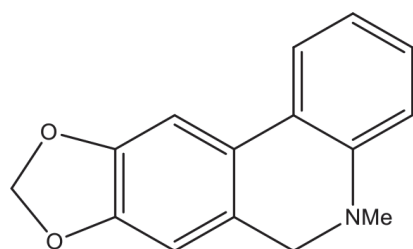
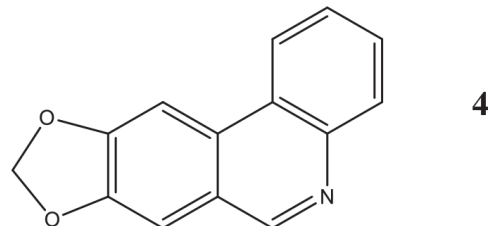
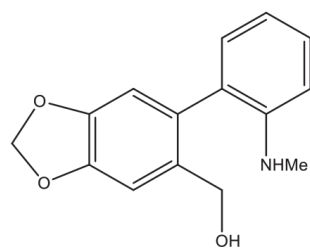
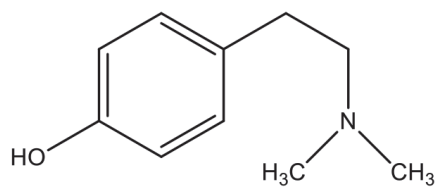
RESULTS

A great number of alkaloids present in Amaryllidaceous plant extracts have 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 are given in Figure 1. Totally, 22 compounds with mass spectral characteristics

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 ^(e) (8)	22.74	281 (100)	262 (23), 207 (26), 191 (30), 149 (20), 97 (21), 73 (26)	-	-	-	0.33	21
9-O-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
O,N-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, 26
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-O-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-O-acetyl-9-O-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-O-diacetyl-9-O-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, t: Trace <0.1, S: Standard, TIC: Total ionic concentration, *G. fosteri*: *Galanthus fosteri*



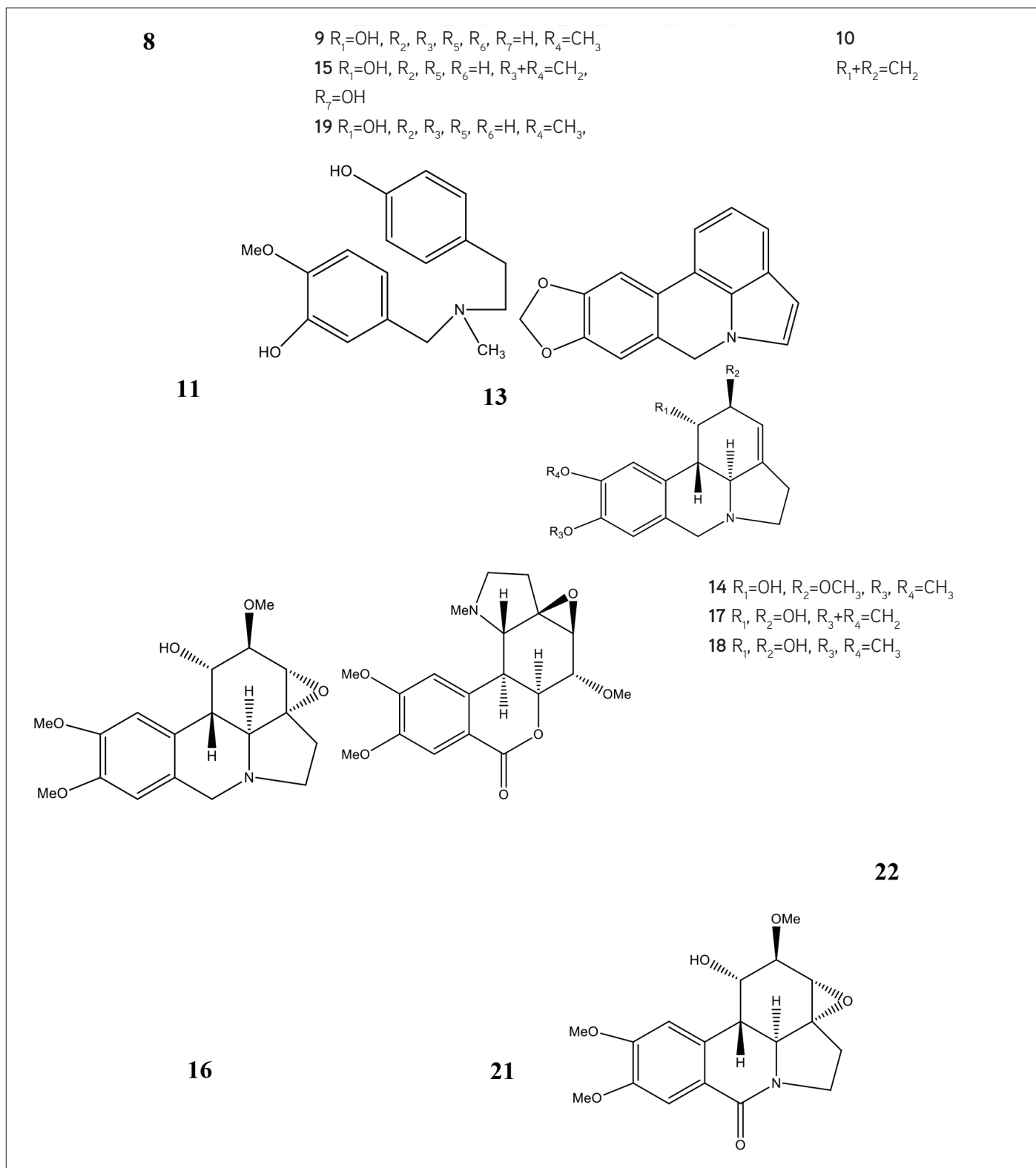


Figure 1. Structures of alkaloids identified in *Galanthus fosteri*: hordenine (1), ismine (3), trisphaeridine (4), 5,6-dihydrobicolorine (5), crinine (6), galanthamine (7), galanthindole (8), 9-*O*-demethylmaritidine (9), anhydrolycorine (10), *O,N*-dimethylnorbelladine (11), assoanine (12), 11,12-didehydroanhydrolycorine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

of Amaryllidaceae alkaloids were detected in the extracts of the aerial parts and bulbs of both the flowering and

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 another base hordenine. Hordenine is not typical for plants of the family Amaryllidaceae. 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 In addition, one compound showing mass spectral fragmentation characteristic of Amaryllidaceae alkaloids was left unidentified due to lack of reference MS spectra. Of the 22 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; IC₅₀ values (µg/mL) are shown in Table 2.

Table 2. Cholinesterase inhibitory activity of extracts

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

AChE: Acetylcholinesterase, BuChE: Butyrylcholinesterase

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 patterns of the aerial parts and bulbs were dominated by 11-*O*-acetyl-9-*O*-demethylmaritidine, which has remarkable AChE and BuChE inhibitory activities with IC₅₀ values of 6.04 µM and 29.72 µM, respectively. Furthermore, incartine is the main alkaloid in bulbs and it 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 the 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, and 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 the fruiting period, which have the highest relative amount of galantamine. Moreover, the highest BuChE inhibitory activity was detected in the bulbs of the same period, which have the highest number of lycorine type alkaloids. Compared

to the other *Galanthus* species^{31,32} in which both activities were examined, the aerial parts of the fruiting period stand out with their 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 of 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.

Conflicts of interest: No conflict of interest was declared by the authors.

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