

**BIOLOGICAL ACTIVITY OF DIFFERENT ALKALOID
CHEMOTYPES OF *GALANTHUS ELWESII* AND
*GALANTHUS NIVALIS***

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Received on January 19, 2024

Presented by D. Ivanov, Corresponding Member of BAS, on February 27, 2024

Abstract

The discovery of natural sources of biologically active compounds is crucial to medicine, organic farming, plant science, etc. Amaryllidaceae alkaloids are unique compounds of plant origin with a wide variety of biological and pharmacological properties. In the present study, six different chemotypes of *Galanthus* populations of Bulgarian origin were used for the isolation of alkaloid extracts. The alkaloid extracts were analyzed by GC–MS, and their capability to inhibit acetylcholinesterase, *Lolium perenne* seed germination and root elongation was assessed. In total, 73 alkaloids of 8 main structural types were detected. Tazettine and lycorine types were predominant in *G. nivalis* samples, whereas tyramine, lycorine, homolycorine and galanthamine types were most abundant among *G. elwesii* samples. Sample GE2110, containing 74% galanthamine type alkaloids, achieved the strongest inhibition on acetylcholinesterase ($IC_{50} = 1.32 \pm 0.09 \mu\text{g/mL}$), but also the weakest inhibition on seed germination ($8 \pm 5\%$) and root elongation ($43 \pm 17\%$). Sample GN028A, containing 51% tazettine type alkaloids, was established as the best overall extract, with a strong inhibition on acetylcholinesterase ($IC_{50} = 21.86 \pm 1.59 \mu\text{g/mL}$), *L. perenne* seed germination ($75 \pm 16\%$) and root elongation ($99 \pm 1\%$). Plant species rich in these alkaloids could be the base of a novel biocidal agent against pests and weeds.

This research was supported by the Bulgarian National Science Fund, Bulgarian Ministry of Education and Science (Grant DN 16/2, 11.12.2017).

<https://doi.org/10.7546/CRABS.2024.05.03>

Key words: acetylcholinesterase, Amaryllidaceae alkaloids, *Galanthus*, herbicidal activity

1. Introduction. The need for novel natural products in medicine and organic farming requires a continuous search for new plant species with pharmacological and biocidal properties. Weeds are one of the major problems in world agriculture because they cause major losses in crop yield. The application of plant extracts against weeds in modern organic farming is rapidly increasing [1].

Galanthus species are well known ornamental plants of the Amaryllidoideae subfamily in the Amaryllidaceae family. In Bulgaria, two species of this genus are naturally distributed – *G. elwesii* Hook f. and *G. nivalis* L. [2].

Members of the Amaryllidaceae family contain bioactive compounds referred to as Amaryllidaceae alkaloids. A great variability in the alkaloid profiles of different *G. elwesii* and *G. nivalis* populations of Bulgarian origin has been found, grouped into several major chemotypes [3].

The alkaloids found in Amaryllidaceae plants possess important pharmacological properties such as acetylcholinesterase inhibitory activity, cytotoxicity, anti-tumour, antibacterial, antifungal, anti-inflammatory activities [4, 5]. The primary medicinal application of Amaryllidaceae alkaloids is represented by galanthamine, which is commercialized as an Alzheimer's drug due to its potent and selective inhibitory activity against the enzyme acetylcholinesterase [6].

The herbicide potential of some Amaryllidaceae alkaloids has been previously studied. Narciclasine and lycorine were identified as potential allelochemicals with a strong herbicidal activity. Haemanthamine was established as more phytotoxic than the commercial herbicide glyphosate but less toxic than narciclasine [7–10].

The chemistry and biological activities of the alkaloids from *Galanthus* species have been the subject of several studies, reporting anti-acetylcholinesterase, antibacterial, antifungal, antiprotozoal, antiviral, antioxidant, antitumour activities [11]. However, no data about the usage of *Galanthus* extracts as inhibitors of seed germination and seedling growth of weeds were found.

The aim of the present study is to evaluate the inhibitory activity on AChE, seed germination and root elongation of alkaloid extracts from various *G. elwesii* and *G. nivalis* chemotypes. The obtained results will demonstrate the relationship between the above-mentioned activities and the alkaloid profiles of both species.

2. Results and discussion. 2.1. Alkaloid profiles of the studied samples. A total of 73 alkaloids of 8 main structural types were determined (tyramine, galanthamine, galanthindole, narciclasine, tazettine, haemanthamine, lycorine and homolycorine), including 17 miscellaneous alkaloids, for which we do not yet know to which type they belong (Table 1). Populations of *G. nivalis* (GN028A, GN217) were found to contain alkaloids of 3 types: tazettine, lycorine and haemanthamine. Sample GN217 had 49.19% unknown alkaloids. The main alkaloids for GN028A were pretazettine (19) and didehydroanhydrolycorine (33),

T a b l e 1

Alkaloid contents of the studied *Galanthus* extracts (% of total alkaloid mixture)

Alkaloids	Studied <i>Galanthus</i> samples						
	RT	GN028A	GN217	GE213	GE215	GE028	GE2110
<i>Tyramine type</i>							
Hordenine (1)	15.09			32.10	0.61	49.75	8.32
O-Methyltyramine (2)	15.73			9.40		2.12	
Feruloyltyramine (3)	30.96					2.67	
<i>total</i>				41.5	0.6	54.5	8.3
<i>Galanthamine type</i>							
Anhydrogalanthamine (4)	29.63					0.01	0.23
Apogalanthamine (5)	30.46						0.20
Anhydrogalanthamine iso (6)	31.03						1.48
Lycoraminon (7)	32.44						2.40
Galanthamine (8)	32.51		0.03		0.01	2.62	22.79
Lycoramine (9)	32.97						8.86
N-Demethylgalanthamine (10)	33.57						3.14
Narwedine (11)	33.87						4.16
N-Formylnorgalanthamine (12)	40.90						29.51
O-Methylleucotamine (13)	42.22		0.0		0.0	2.9	0.85
<i>total</i>							73.6
<i>Galanthindole type</i>							
Galanthindol (14)	34.28	0.89					
9-O-Demethyllycosenine (15)	37.84					0.20	
<i>total</i>		0.9				0.2	
<i>Narciclasine type</i>							
Bicolorine (16)	25.92				0.10		
Trisphaeridine (17)	31.12	9.44	11.40		3.42	0.02	0.28
<i>total</i>		9.4	11.4		3.4	0.0	0.3

T a b l e 1
Continued

	Studied <i>Galanthus</i> samples						
	RT	GN028A	GN217	GE213	GE215	GE028	GE2110
Alkaloids							
<i>Tazetine type</i>							
Tazettinol (18)	33.86	13.75	5.10		1.32		
Pretazettine (19)	36.80	27.93	9.70	0.19			
Macronine (20)	39.36	0.38	5.00	0.17			
<i>epi</i> -Macronine (21)	40.55	8.63	5.80				
<i>total</i>		50.7	25.6	0.4	1.3		
<i>Haemanthamine type</i>							
HT1 (22)	36.42	0.61					
HT2 (23)	38.51		14.85				
HT3 (24)	41.44	6.94					
HT4 (25)	41.77						11.85
HT5 (26)	44.87				8.40		1.30
11,3'-O-(Dihydrobutyryl)hamayne (27)	45.51				4.21		0.07
HT6 (28)	48.47				1.07		
HT7 (29)	52.20	4.18			5.58		2.22
<i>total</i>		11.7	14.9		19.3		15.4
<i>Lycorine type</i>							
1-O-Acetylnoirpluviine (30)	33.93				0.54		
1-O-Acetylpluviine (31)	34.18				0.43		
Anhydrolycorine (32)	34.70				8.47		
Didehydroanhydrolycorine (33)	36.64	18.20	0.30		26.97		
Kirkine (34)	37.24				2.91		
Didehydrooasanine (35)	37.52				11.10		
Hippadine (36)	39.45	2.76			1.46		
LT1 (37)	42.94				0.32		
1-O-Methylpluviine (38)	43.85				0.03	0.26	
2-Methoxypratrosine (39)	43.99				1.73		

T a b l e 1
Continued

		Studied <i>Galanthus</i> samples						
		RT	GN028A	GN217	GE213	GE215	GE028	GE2110
Alkaloids		44.67	10.47		0.41	4.19	0.86	
LT2 (40)		45.75				3.55		
Oxoasoanine (41)			31.4	0.3	0.4	58.2	1.1	
<i>total</i>								
<i>Homolycorine type</i>								
Masonine (42)		38.03					0.41	
Homolycorine (43)		38.97					0.32	
HLT1 (44)		39.95					0.54	
HLT2 (45)		40.36				4.85	0.99	
2-Methoxy-8-O-demethylhomolycorine (46)		40.44			0.26	1.54	0.03	
2-Methoxyhomolycorine (47)		40.75			1.37		0.38	
8-O-Demethylhomolycorine (48)		41.33					18.54	
HLT3 (49)		42.80					0.77	
Galwesine (50)		43.31		4.70	29.11	3.94	2.99	0.87
Hippeastrine (51)		43.56			24.40		3.02	
HLT4 (52)		45.26					4.29	
9-O-Demethylgalwesine (53)		45.72			0.30			
HLT5 (54)		46.87		0.90	2.17	2.58	1.39	1.47
<i>total</i>				5.6	57.6	12.9	33.7	0.9
<i>Ismine type</i>								
Carboline derivative (55)		26.01					0.82	
Ismine (56)		30.42		2.20				
<i>total</i>				2.2			0.8	

T a b l e 1
Continued

		Studied <i>Galanthus</i> samples						
Alkaloids		RT	GN028A	GN217	GE213	GE215	GE028	GE2110
<i>Miscellaneous</i>								
UA1 (57)		29.47		1.63				
UA2 (58)		30.12		1.67				
UA3 (59)		33.73		0.29				
UA4 (60)		34.30		2.64				
UA5 (61)		35.15					1.26	
UA6 (62)		36.43		0.49				
UA7 (63)		36.66		0.33				
UA8 (64)		37.06		0.28				
UA9 (65)		37.35			0.12		3.96	
UA10 (66)		37.54		7.00				
UA11 (67)		39.99		5.50				
UA12 (68)		40.11		7.82				
UA13 (69)		40.43		0.95				
UA14 (70)		41.38		16.16				
UA15 (71)		41.54		3.60				
UA16 (72)		41.79		0.20				
UA17 (73)		42.95		0.64				
<i>total</i>				49.2	0.1		5.2	

Legend: RT – retention time; HT – haemanthamine type; LT – lycorine type;
HLT – homolycorine type; UA – unknown alkaloid

and for GN217 – UA14 (70), HT2 (23), trisphaeridine (17). *G. elwesii* samples had a different alkaloid composition. Sample GE2110 was found to be of galanthamine type, with its main alkaloids being *N*-formylnorgalanthamine (12) and galanthamine (8). Samples GE028 and GE213 consisted of alkaloids of tyramine and homolycorine types. For GE028 8-*O*-demethylhomolycorine (48) was the primary homolycorine alkaloid, and for GE213 – galwesine (50) and hippeastrine (51). The main tyramine type alkaloid in both samples was hordenine (1). Sample GE215 contained mainly lycorine and haemanthamine type alkaloids, led by didehydroanhydrolycorine (33) and didehydroassoanine (35). These results are in agreement with our previously reported data [3,12].

2.2. Inhibition on seed germination and root elongation. The results for the inhibitory activity of the studied alkaloid extracts on *L. perenne* seed germination and root elongation are presented in Table 2. The strongest inhibition on seed germination was achieved by the alkaloid extracts of *G. nivalis* (GN028A, GN217). Tazettine type alkaloids are most abundant in these two samples and are almost absent in *G. elwesii*. Extracts from GE215 (lycorine type) showed moderate inhibition on seed germination and strong inhibition on root elongation. This may be due to the wide variety of lycorine type alkaloids in this sample (some of them still unidentified), although lycorine itself is not present. Lycorine has been previously identified as a plant growth inhibitor [7,8]. Moderate to weak inhibitory activity was demonstrated by samples GE213 (homolycorine type) and GE028 (tyramine type). Sample GE2110 (galanthamine type) had the weakest inhibition of seed germination and root elongation of *L. perenne* compared to the rest of the samples, but the inhibition of root elongation may be considered moderate. Haemanthamine type alkaloids were identified in all *Galanthus* extracts, with contents between 10–20%, GE215 having the largest quantity (Table 1). The phytotoxic properties of haemanthamine have been previously studied [10].

T a b l e 2

Inhibitory activity of *Galanthus* alkaloid extracts on acetylcholinesterase, *Lolium perenne* seed germination and root elongation; *pc* = positive control

Sample	Main alkaloid type	Inhibition on seed germination, %	Inhibition on root elongation, %	AChE IC ₅₀ , µg/mL
GN028A	<i>Tazettine</i>	75 ± 16	99 ± 1	21.86 ± 1.59
GN217	<i>Miscellaneous</i>	72 ± 28	93 ± 12	108.57 ± 7.32
GE213	<i>Homolycorine</i>	18 ± 8	59 ± 20	121.63 ± 12.04
GE215	<i>Lycorine</i>	50 ± 3	95 ± 9	23.15 ± 2.77
GE028	<i>Tyramine</i>	23 ± 12	67 ± 14	112.47 ± 9.68
GE2110	<i>Galanthamine</i>	8 ± 5	43 ± 17	1.32 ± 0.09
Galanthamine (<i>pc</i>)	–	–	–	0.53 ± 0.03

In the present study, we establish for the first time tazettine type alkaloids as promising sources of herbicidal activity.

2.3. Acetylcholinesterase inhibitory activity. Results for the AChE inhibitory activity are presented in Table 2. GE2110 was found to be a potent AChE inhibitor and the strongest among all tested samples. This result can be explained by the large amount of galantamine type alkaloids present in GE2110. *N*-Formylnorgalanthamine is a galanthamine derivative found in large quantities in GE2110, and to the best of our knowledge, there is no data available about its AChE inhibitory activity. Samples GE215 and GE028A presented high AChE inhibitory activity. Both contain significant amounts of lycorine type alkaloids – 58% and 31%, respectively. Lycorine type alkaloids are known to be good AChE inhibitors [13]. Samples GN217, GE028, and GE213 were established as moderate AChE inhibitors, which is in accordance with their alkaloid contents – small amounts of galanthamine and lycorine type alkaloids.

Interestingly, GN028A and GN217 contain a large percentage of tazettine type alkaloids. Tazettine and other alkaloids of this type do not inhibit AChE on their own, as high or no IC₅₀ values have been previously reported for them [14]. These alkaloids may contribute to AChE inhibition in a synergistic manner with lycorine type alkaloids, especially in GN028A.

3. Experimental. 3.1. Plant material. Aerial parts from each sampling location of *G. elwesii* and *G. nivalis* were collected at the flowering stage in February, 2023 (Table 3). The species were identified according to ANCHEV [2]. Voucher specimens have been deposited in Bulgarian Academy of Sciences herbarium (SOM). The fresh aerial parts from all samples were cut into small pieces, dried at 60 °C, and then powdered.

3.2. Phytochemical analysis. 3.2.1. Alkaloid extraction. Alkaloid extraction was prepared according to BERKOV et al. [15] with modifications. Dry plant material was macerated with methanol adjusted to pH 8 with 25% ammonia. After 2 h of extraction at room temperature assisted by an ultrasonic bath for 15 min every 30 min, the samples were centrifuged at 6000 rpm for 1 min. Then, aliquots of the methanolic extracts were concentrated using a vacuum evaporator and transferred into a separatory funnel where 2% sulphuric acid in distilled water was added. The neutral compounds were eliminated by duplicate extraction with chloroform.

The mixtures were basified with 25% ammonia to pH 10 and the alkaloids were extracted in triplicate with 15 mL chloroform for GC–MS analysis. The organic solvent was evaporated, and the dry extract was prepared for further analysis.

3.2.2. Gas chromatography–mass spectrometry. Alkaloid extracts were analyzed by gas chromatography–mass spectrometry (GC–MS). The GC–MS spectra were recorded on a Thermo Scientific Focus GC coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m ×

T a b l e 3

Population code, Voucher number, Sampling location, and Date of harvest of the studied *Galanthus elwesii* and *Galanthus nivalis* populations

Population code	Voucher SOM	Sampling location (District of Bulgaria)	Date of collection	Species
GN028A	001419	Northeastern Bulgaria: Above Obrochishte village, 219 m. alt., E exposition, 43.38155 N, 28.07075 E.	23.2.2023	<i>G. nivalis</i>
GN217	001417	South Black sea: Arkutino Reserve, 5 m. alt., E exposition, 42.32572 N, 27.73056 E.	22.2.2023	<i>G. nivalis</i>
GE213	001422	Northeastern Bulgaria: The Omurtag–Targovishte road, Boaza pass, 8 km before Targovishte, deciduous forest, 301 m. alt., W exposition, 43.19567 N, 26.50042 E.	21.2.2023	<i>G. elwesii</i>
GE215	001421	Northeastern Bulgaria: Between the villages of Kalinata and Vasilevo, “Chernata Gora” locality, 192 m. alt., NE exposition, 43.62410 N, 28.18748 E.	22.2.2023	<i>G. elwesii</i>
GE028	001420	Northeastern Bulgaria: The Obrochishte village, by the road to Balchik, 219 m. alt., E exposition, 43.38155 N, 28.07075 E.	23.2.2023	<i>G. elwesii</i>
GE2110	001418	East Balkan: Karnobat, “Markeli” locality, 212 m. alt., E exposition, 42.637217 N, 26.897700 E.	16.2.2023	<i>G. elwesii</i>

0.25 mm $\times$ 0.25 μ m) was used. The temperature programme was: 100–180 °C at 15 °C $\times$ min^{−1}, 180–300 °C at 5 °C $\times$ min^{−1}, and 10 min hold at 300 °C. The injector temperature was 250 °C. The flow rate of the carrier gas (Helium) was 0.8 mL $\times$ min^{−1}. The split ratio was 1:10. One microliter of solution was injected.

3.2.3. Identification of the alkaloids. The alkaloids and alkaloid types were identified by comparing mass-spectral results with data in the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0, 2005) and the Golm Metabolome Database, as well as literature data [16, 17].

3.3. Inhibitory activity on seed germination and root elongation.

A hundred seeds of *Lolium perenne* were placed in Petri dishes on filter papers moistened with the tested solutions. The alkaloid extracts were applied at a concentration of 1 mg/mL, in a water–methanol mixture (99.5:0.5). The control solution consisted only of a water–methanol mixture. The samples were incubated at room temperature for seven days. At the end of the week, the rate of germination inhibition and reduction in root growth (%) was calculated as described by ATAŞ et al. [18]. Seed germination and root elongation assays were performed in triplicate and the results are presented as means $\pm$ SD.

3.4. Assessment of acetylcholinesterase inhibitory activity. AChE inhibitory activity was measured using the microplate assay described by ELLMAN

et al. [19], with the modifications added by LÓPEZ et al. [20]. All extracts were diluted in phosphate buffer (PBS) and tested at concentrations between 1000 and 1 µg/mL. Acetylcholinesterase from *Electrophorus electricus* was used with a substrate solution containing acetylthiocholine iodide (ATCI) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, Germany).

Fifty microliters of AChE and 50 µl of the tested sample were added to the wells. Incubation of the plates was performed at room temperature for 30 min. Then, 100 µl of substrate solution were added to start the enzymatic reaction. The absorbances were read in a microplate reader (BIOBASE ELISA-EL10A, China) at 405 nm after 3 min. Enzyme activity was calculated as an inhibition percentage compared to an assay including buffer instead of an inhibitor. Galanthamine was used as a positive control. All data were analyzed with the software package Prism 3 (Graph Pad Inc., San Diego, USA). The IC₅₀ values were measured in triplicate and the results are presented as means ± SD.

4. Conclusion. To the best of our knowledge, in the present study we report for the first time data about the inhibitory activity of alkaloid extracts from *G. elwesii* and *G. nivalis* on seed germination and root elongation. For the first time tazettine chemotypes are reported to exhibit strong inhibitory activity on seed germination. Sample GE2110, containing 74% galanthamine type alkaloids, achieved the strongest inhibitory activity against AChE, but also the weakest inhibition on seed germination and root elongation. GN028A, a tazettine type sample of *G. nivalis* from Obrochishte village, was established as the best overall extract. Plant species rich in these alkaloids could be, among other applications, the base of a novel biocidal agent against pests and weeds. *Galanthus* species in nature are in a resource deficit, so alkaloids with biocidal potential should be extracted from cultivated plants. Furthermore, biotechnological approaches for the targeted accumulation of alkaloids and stability, solubility, and efficacy studies could be applied.

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