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Galanthamine distribution in Bulgarian *Galanthus* spp.

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Received May 28, 2003, accepted July 2, 2003

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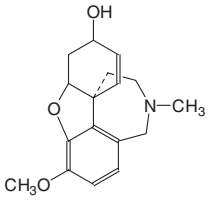
Pharmazie 58: 935–936 (2003)

Sixteen populations of *Galanthus elwesii* Hook.fil and five populations of *Galanthus nivalis* L. growing in Bulgaria were investigated for the presence of galanthamine by TLC and GC-MS. Between 3 and 11 alkaloids were detected by TLC in the total alkaloid fractions. Galanthamine was found in 2 populations of *G. elwesii*.

1. Introduction

Galanthamine, an Amaryllidaceae alkalolid, is a long acting, selective, reversible and competitive acetylcholinesterase (AChE) inhibitor [1–3], which produces beneficial effects even after drug treatment has been terminated [4]. This product is marketed as a hydrobromide salt under the name Reminyl® for the treatment of Alzheimer's disease.



Galanthamine

Galanthamine was isolated for the first time from *Galanthus woronowii* Losinsk. by Proskurina and Yakovleva [5, 6] and was later found in many other of Amaryllidaceae species. On a commercial scale, galanthamine had been produced for the first time from *Galanthus nivalis* L. in Bulgaria under the name of Nivalin® [7–8].
Bubeva-Ivanova [9] found this alkaloid in *G. nivalis* L. var. *gracilis* Celak. from populations near the towns of Yambol and Koprivshtitsa (Southeast and Central Bulgaria) in 1957. Later, it was reported that *G. nivalis* plants from this region contain higher amounts of galanthamine than plants from North and Northeast Bulgaria which contain no or trace amounts of galanthamine [10].
The taxonomical status of genus *Galanthus* in Bulgaria has been changed several times since the establishment of galanthamine in *G. nivalis*. Genus *Galanthus* had been presented by a single species – *G. nivalis* s.l. up to 1964 [11, 12]. Stojanov et al. 1966 [13] divided the genus *Galanthus* into two species in the fourth edition of "Flora of Bulgaria", namely *G. elwesii* Hook.fil, and *G. nivalis* L. with several varieties and forms. Delipavlov, 1968 [14] distinguished three species of *Galanthus* in Bulgaria – *G. nivalis* L., *G. elwesii* Hook.fil. and *G. graecus* Orph. ex Boiss. According to this author, *G. nivalis* is distributed mainly in Eastern Bulgaria [15]. Recently, *G. nivalis* L. and *G. elwesii* Hook.fil. have been accepted for Bulgaria [16–18].
In this paper we report a reinvestigation on the galanthamine distribution in Bulgarian *Galanthus* species according to the latest classifications [16–18].

2. Investigations, results and discussion

We examined 21 populations of *Galanthus* (5 of *G. nivalis* and 16 of *G. elwesii*) for their galanthamine content by TLC and GC-MS (Table). The distribution of *G. nivalis*, which we found, coincides with those reported by Delipavlov [14] for the Black Sea Coast region. Between 3 and 11 alkaloids were detected in the total alkaloid fractions by TLC. Galanthamine was found in only 2 populations of *G. elwesii* – No 13 (Levski-021) and No 20 (Karnobat-024). The occurrence of galanthamine was confirmed by parallel GC-MS with an authentic sample. Some alkaloid fractions which do not contain galanthamine according to TLC were also examined by GC-MS. The compound was not found in them even in trace amounts.
Small differences were observed between the MS of galanthamine obtained by direct insertion [19] and the GC-MS conditions, which we used. The molecular peak of galanthamine (at *m/z* 287), which is reported in the literature [19] as a base peak (100%, relative abundance) was depressed (78%) under the GC-MS conditions. The base ion appeared at *m/z* 286 (M^+-H). The other characteristic fragments of galanthamine showed similar relative abundances to those reported for the direct insertion. Previously, GC-MS has been successfully applied for identification of other Amaryllidaceae alkaloids [20, 21].
Our results on the distribution of galanthamine confirms those of Ivanova and Ivanov [10] who investigated 26 populations and found galanthamine in only a few of them. On the basis of our results and the results of Ivanova and Ivanov [10] as well as on the limited distribution of *G. nivalis*, we suppose that the first commercial scale production of Nivalin® (galanthamine hydrobromide) was mainly from *G. elwesii* according to the contemporary classification of the genus *Galanthus* in Bulgaria.

Table: Investigated populations of *Galanthus* spp. distributed in Bulgaria

No	Floristic region	Part of Bulgaria	Voucher SOM	Species	Galanthamine	Method of analysis
Black Sea Coast						
1	Obrochiste	NE	028	*nivalis*	–	TLC/GC-MS
2	Obrochiste	NE	028a	*elwesii*	–	TLC/GC-MS
3	Tsarkva	NE	029	*nivalis*	–	TLC/GC-MS
4	Tsarkva	NE	029a	*elwesii*	–	TLC
5	Varna	NE	027	*nivalis*	–	TLC
6	Burgas	SE	025	*nivalis*	–	TLC
7	Primorsko	SE	026	*nivalis*	–	TLC/GC-MS
Northeast Bulgaria		NE				
8	Tervel (Stenata)		0216	*elwesii*	–	TLC/GC-MS
9	Tervel (Dan kula)		0218	*elwesii*	–	TLC/GC-MS
10	Tervel (Sujunlika)		0220	*elwesii*	–	TLC/GC-MS
11	Shumen		0210	*elwesii*	–	TLC/GC-MS
12	Targovoshte		0211	*elwesii*	–	TLC
Danubian plain		CN				
13	Levski		021	*elwesii*	0.1	TLC/GC-MS
Forebalkan		CN				
14	Sevlievo		0212	*elwesii*	–	TLC
15	Montana		0213	*elwesii*	–	TLC
Stara Planina Mts		Central	0224	*elwesii*	–	TLC
16	Kozja stena		0224	*elwesii*	–	TLC
17	Sliven		023	*elwesii*	–	TLC
Sofia region		SW				
18	Kokaljane		0221	*elwesii*	–	TLC
The Rhodopes		Central				
19	Bachkovo		0222	*elwesii*	–	TLC
Tundzha Hilly region		SE				
20	Karnobat		024	*elwesii*	0.6	TLC/GC-MS
21	Topolovgrad		022	*elwesii*	–	TLC

3. Experimental

3.1. Plant material

Aerial parts of *Galanthus* species at flowering stage (February–March, 2002) were collected from 21 populations distributed at different floristic regions of Bulgaria. The species were determined according to Webb [16] and Petrova [18]. Voucher specimens were deposited at the herbarium of Institute of Botany – BAS (SOM), Sofia.

3.2. Alkaloid extraction

Plants were dried at 60 °C, powdered and extracted with 3% H_2SO_4 for 2 h by shaking at room temperature. Than, the extracts were centrifuged and supernatants were separated, basified with 25% ammonia and extracted tree times with dichloroethane. The organic solvent was dried over anh. Na_2SO_4 and evaporated to dryness. The dry extracts were dissolved in known amounts of methanol for further analysis.

3.3. Alkaloid determination

Two TLC conditions were used for the development of alkaloid fractions and identification of galanthamine: (1) chloroform/methanol/25% ammonia (11 : 1 : 0.6, v/v/v), migration distance – 160 mm, and (2) chloroform/methanol/25% ammonia (12 : 1 : 0.5, v/v/v), migration distance – 80 mm. 10 µl of the alkaloid fractions were spotted and developed on Merck aluminum sheets silica gel 60 F_{254} (10 × 20 cm) together with standards. Compounds were visualized after triplicate spraying with Dragendorff's reagent. Galanthamine was quantified by TLC-densitometry. 10 min after the last spraying, the plates were scanned at 600 dpi optical resolution and images were analyzed by QuantiScan 2.1® Biosoft software. The galanthamine content of the samples was calculated from the densitogram peak areas by comparing to three standards (5, 15 and 30 µg/spot, stock solution: 1 mg/ml galanthamine hydrobromide calculated as a base) placed on the same plate.

3.4. Gas chromatography-mass spectrometry

The GC-MS was recorded on a Hewlett Packard 6890+ MSD 5973 instrument operating in EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 µm) was used. The temperature program was 80–280 °C at 10 °C min and 10 min hold at 280 °C. Injector temperature was 280 °C. The flow rate of carrier gas (He_2) was 0.8 ml · min^{-1}. Split ratio was 1 : 20.

Acknowledgement: The authors thank Sopharma Ltd. for the kind gift of a reference substance of Nivalin® (galanthamine hydrobromide). This work was supported by the Ministry of Environment and Waters, Sofia (project #3228/264).

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