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Analysis of Amaryllidaceae alkaloids from *Zephyranthes grandiflora* by GC/MS and their cholinesterase activity

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Abstract: Amaryllidaceae are known as ornamental plants, furthermore some species of this family contain galanthamine, an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease, and other alkaloids with interesting pharmacological activity. The chemical composition of alkaloids from *Zephyranthes grandiflora* Lindl. was analyzed by GC/MS. Seven known compounds, belonging to five structural types of Amaryllidaceae alkaloids, were identified. The alkaloid extract from the bulbs showed promising cholinesterase inhibitory activities against human blood acetylcholinesterase (HuAChE; IC50 39.2±3.0 µg/mL) and human plasma butyrylcholinesterase (HuBuChE; IC50 356±9.3 µg/mL).

Introduction

Plants of the Amaryllidaceae family are well-known for their ornamental value but also for the alkaloids they produce. The chemical structures of these alkaloids are very variable as well as their pharmacological properties. Some species of this family contain galanthamine, an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease (AD) (Hostettman et al., 2006), as well as other alkaloids with interesting pharmacological activities: antimalarial, antiviral and antiproliferative (Campbell et al., 1988; Hohmann et al., 2002; Szlávik et al., 2004).

AD is the most predominant cause of dementia in the elderly. Epidemiological data indicate a potentially considerable increase in the prevalence of the disease over the next two decades (Johnson et al., 2000). In AD patients, deficit of cholinergic functions, which results in decreased levels of the neurotransmitter acetylcholine (ACh) in the cortex, is responsible for the memory impairments (Lahiri et al., 2002). The principal role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of ACh. Inhibition of AChE serves as a strategy for the treatment of AD, senile dementia, ataxia, myasthenia gravis, and Parkinson's disease (Rahman & Coudhary, 2001). In the healthy brain AChE is the most important enzyme regulating the

level of ACh while other enzyme, butyrylcholinesterase (BuChE) plays only minor role. Moreover, in late AD stages, levels of AChE have declined by up to 85% and BuChE represents the predominant cholinesterase in the brain. BuChE, primarily associated with glial cells, but also with specific neuronal pathways, cleaves ACh in a manner similar to that of AChE to terminate its physiological action (Greig et al., 2002). Galanthamine has been shown to be much less potent against BuChE than AChE activity (Fulton & Benfield, 1996). This fact has targeted BuChE as a new approach to intercede in the progression of AD and requires research of new inhibitors with dual enzymatic activity. Currently, cholinesterase inhibition is the most used therapeutic treatment for the symptoms of AD (Lleó, 2007; Recanatini & Valenti, 2004). In recent years some overviews of natural product inhibitors of AChE were reported (Barbosa-Filho et al., 2006; Hostettmann et al., 2006).

Species of *Zephyranthes* are widely used as a folk medicine in many countries. The decoction of leaves of *Z. candida* has been used in South Africa as a remedy for diabetes mellitus and *Z. parvula* appears in the history of Peru as a treatment for tumors (Pettit et al., 1984). *Z. rosea* and *Z. flava* are used for a variety of therapeutic purposes in India (Ghosal et al., 1985; Ghosal et al., 1986).

There have been few phytochemical studies of *Zephyranthes* species, and only two GC/

MS investigations. From *Z. citrina*, eight alkaloids (galanthine, haemanthamine, lycorine, lycorenine, oxomaritidine, maritidine, vittatine, and narcissidine) have been isolated (Boit et al, 1957; Herrera et al., 2001). In addition to these, zefbetaine and zeflabetaine were isolated from *Z. flava* (Ghosal et al., 1986), and pretazettine and carinatine, along with lycorine, galanthine and haemanthamine from *Z. carinata* (Kobayashi et al., 1977). Previous phytochemical investigation of *Z. grandiflora* led to the isolation of pancratistatin (Pettit et al., 1984). GC/MS analysis of the bulb extracts of *Z. concolor* and *Z. robusta* showed the domination of galanthamine-type Amaryllidaceae alkaloids and these extracts possessed promising AChE inhibitory activity (Berkov et al., 2008; Cahliková et al., 2010).

Alkaloids of many different skeleton types can be rapidly identified by GC/EI-MS (Wink et al., 1995; Kreh et al., 1995; Suau et al., 2002). It has been found that Amaryllidaceae alkaloids can be analyzed by GC without derivatization and, with only few exceptions, they show a mass spectral fragmentation pattern very similar to those obtained under direct insertion (Kreh et al., 1995). Several studies of the distribution of galanthamine in different subspecies and populations of mainly *Galanthus* and *Narcissus* species have been published (López et al., 2002; Berkov et al., 2004). GC/MS analysis has been successfully applied for the reliable and fast identification of Amaryllidaceae alkaloids and for comparative study of their percentage contribution in the alkaloid mixtures (Berkov et al., 2008).

Materials and Methods

Plant material

The fresh bulbs of *Zephyranthes grandiflora* Lindl., Amaryllidaceae, were obtained from Lukon Glads (Sadská, Czech Republic). The botanical identification was performed by Assoc. Prof. Lubomír Opletal, CSc. A voucher specimen is deposited in the herbarium of the Faculty of Pharmacy at Hradec Králové.

Extraction of alkaloids

Fresh bulbs (3 x 15 g) were extracted three times with EtOH (50 mL) at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue dissolved in 10 mL 2% HCl. After removing the neutral compounds with diethyl ether (3 x 15 mL), the extract was basified with 25% ammonia solution and the alkaloids extracted with EtOAc (3 x 15 mL). The organic solvent was evaporated and 10 mg of

each alkaloid extract removed for acetylcholinesterase and butyrylcholinesterase assay. The rest of the dry alkaloid fraction was dissolved in MeOH to a final concentration of 10 mg/mL for further analysis.

GC/MS analysis

The GC/MS analysis of underivatized alkaloids from *Z. grandiflora* was carried out on a gas chromatograph (Focus Thermo Scientific, USA) with a splitless injector (280 °C) and a mass detector (200 °C, GC-MS MD 800 Fisons, Manchester, UK). A DB-5MS column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies Santa Clara, CA, USA) and helium gas (constant flow 1 mL/min) were used for separation. The temperature program was: 100-180 °C at 15 °C/min, 1 min hold at 180 °C and 180-300 °C at 5 °C/min and 5 min hold at 300 °C, detection range *m/z* 40-600. The injector temperature was 280 °C. The alkaloids were identified by comparison of their MS with those in the NIST library, with those reported in the literature (Berkov et al., 2008; Gotti et al., 2006; Kreh et al., 1995; López et al., 2002), with commercially available standards (galanthamine, Changsha Organic), and with reference compounds isolated earlier in our laboratory (galanthamine, galanthine, lycoramine, haemanthamine, vittatine, and tazettine).

Preparation of red blood cells ghost

Ghosts were prepared from freshly drawn blood (taken from healthy volunteers), to which 1 mL of sodium citrate per 10 mL of blood was added, according to a slightly modified method of Steck and Kant (Steck & Kant, 1974). Briefly plasma (HuBuChE) was removed from the whole blood by centrifugation at 4000 rpm in a Boeco U-32R centrifuge with a Hettich 1611 rotor. Red blood cells were transferred to 50 mL tubes and washed three times with 5 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride (12000 rpm, Avanti J-30I, rotor JA-30.50). The washed erythrocytes were stirred with 5 mM phosphate buffer (pH 7.4) for 10 min to ensure lysis. The lysed cells were centrifuged at 20,000 rpm for 10 min and then the ghosts (HuAChE) were washed three times with phosphate buffer.

Acetylcholinesterase and butyrylcholinesterase assay

HuAChE and HuBuChE activities were determined with a modified method of Ellman et al. (Ellman et al., 1961) at concentrations 2.5, 25, and 250 µm/L using acetylthiocholine iodide and butyrylthiocholine iodide as substrates, respectively. Briefly, 25-50 µL of either ghosts or plasma, 650 µL

of DTNB and 25 μ L of either the sample or appropriate solvent, as a blank sample, were added to the semi-micro cuvette. The reaction was initiated by addition of substrate (ATChI or BuTChI). The final proportion of DTNB to substrate was 1:1. The increase of absorbance at 436 nm (ΔA) was measured for 1 min using a Shimadzu UV-1611 spectrophotometer. Each measurement was repeated three times. Galanthamine and huperzine A were used as positive standards (Cahliková et al., 2010). The inhibition (in %) was calculated according to the formula: $\%I = 100 - (\Delta A_{BL} / \Delta A_{SA}) \times 100$, where ΔA_{BL} is increase of absorbance of a blank sample and ΔA_{SA} is increase of absorbance of the measured sample.

Statistical analysis

The IC₅₀ values were calculated with the use of GraphPad Prism 5.02 software.

Results and Discussion

In the present work we report the GC/MS investigation of the alkaloid extract from the bulbs of *Z. grandiflora* and the inhibitory effect of this extract on the activity of human erythrocytic AChE (HuAChE) and plasma BuChE (HuBuChE).

The ethanolic extract from *Z. grandiflora* bulbs inhibited HuAChE and HuBuChE at a concentration of 500 μ g/mL (65.5 \pm 4.0% for HuAChE and 25.0 \pm 2.2% for HuBuChE), thus indicating the presence of cholinesterase inhibitors in this plant taxon. The crude extract from the bulbs was separated into alkaloid fraction and, as expected, the alkaloid fraction possessed a promising activity with IC₅₀ value 39.2 \pm 3.0 μ g/mL for HuAChE and 356 \pm 9.3 μ g/mL for HuBuChE (Table 1).

Table 1. HuAChE and HuBuChE inhibitory activity of the alkaloid extract of *Zephyranthes grandiflora*.

Concentration (μ g/mL)	HuAChE ^a inhibition (%)	HuBuChE ^a inhibition (%)
500	90.6 \pm 5.5	57.2 \pm 3.2
250	85.7 \pm 4.7	39.9 \pm 2.9
125	75.1 \pm 3.9	23.6 \pm 1.8
25	38.1 \pm 4.4	4.4 \pm 0.4
12.5	23.6 \pm 2.8	2.1 \pm 0.2
2.5	5.01 \pm 0.5	0
0.25	0	0
IC ₅₀ (μ g/mL)	39.2 \pm 3.0	356 \pm 9.3
IC ₅₀ ^c Galanthamine ^b	6.9 \pm 0.3	156 \pm 6.9
IC ₅₀ ^c Huperzine A ^b	0.25 \pm 0.01	>1000

^a Results are the mean of three replications; ^b Reference compounds; ^c (μ M)

In order to identify the compounds in the complex alkaloid fraction of bulbs of *Z. grandiflora*, a capillary GC/MS was employed. In the bulb extract, seven alkaloids of five structural types were detected by GC (Figure 1) and identified from their retention times (RT) and mass spectra as galanthamine (**1**, galanthamine-type), lycoramine (**2**, galanthamine-type), vittatine (**3**, haemanthamine-type), nerbowdine (**4**, crinine-type), haemanthamine (**5**, haemanthamine-type), tazzetine (**6**, tazzetine-type), and galanthine (**7**, lycorine-type). The relative proportion of each alkaloid was determined as a percentage of the total ion current. The alkaloid pattern of bulbs of *Z. grandiflora* was dominated by lycoramine (**2**; 30% of TIC), haemanthamine (**5**; 23 % of TIC) and vittatine (**3**, 21 % of TIC) (Table 2). The area of the GC/MS peaks depends not only on the corresponding compounds, but also on the intensity of their MS fragmentation (response factor). Thus, the data

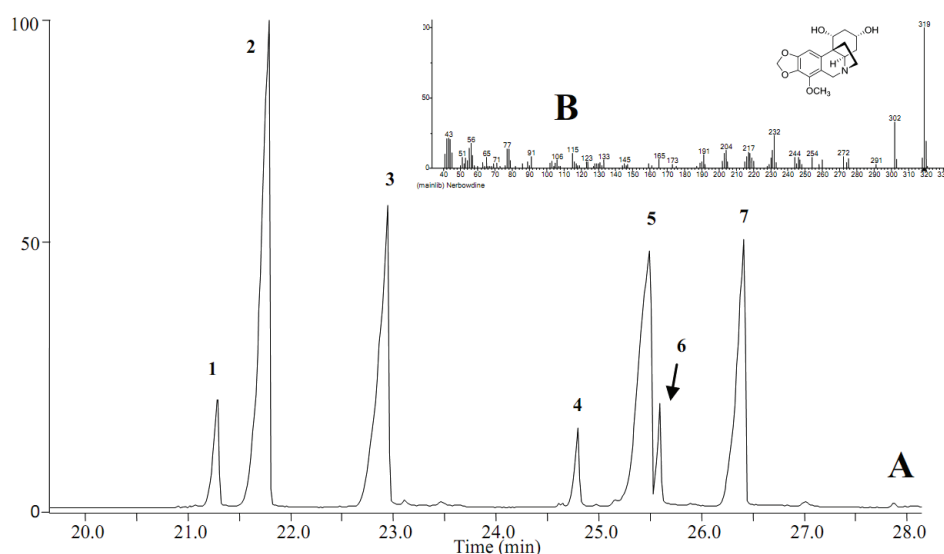


Figure 1. GC/MS of the *Zephyranthes grandiflora* alkaloid fraction (A) and MS spectrum of nerbowdine (B).

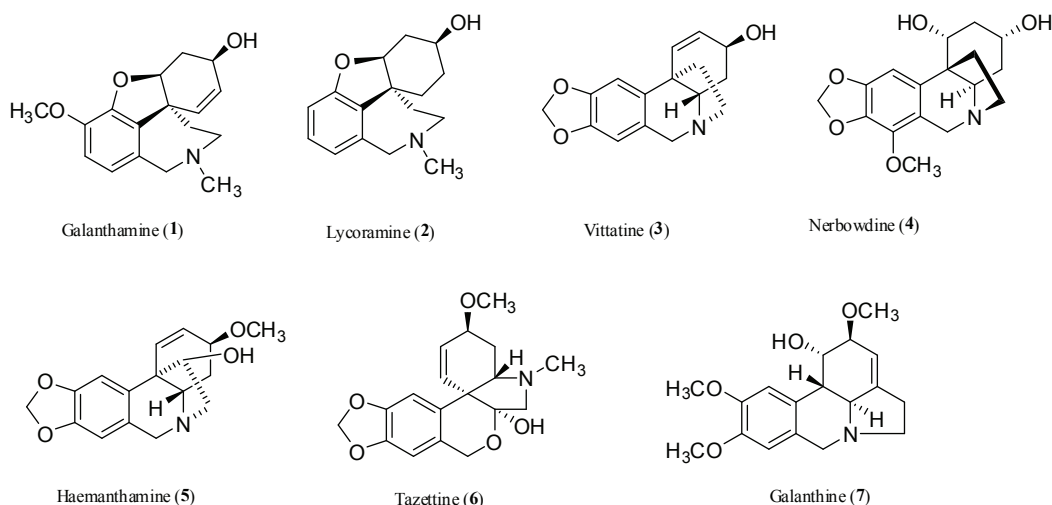


Table 2. Alkaloids identified by GC/MS in bulbs of *Zephyranthes grandiflora*.

Compound	RT ^a , (min)	[M ⁺] and characteristic ions, <i>m/z</i> (% relative intensity)	% ^b	Reference for MS data
1 Galanthamine	21.29	287(90), 286 (100), 270 (20), 244 (30), 230 (5), 216 (45)	5	NIST 08, st ^c
2 Lycoramine	21.79	289 (65), 288 (100), 274 (10), 232 (10), 202 (30)	30	NIST 08, st ^c
3 Vittatine	22.94	271 (100), 252 (30), 199 (90), 187 (95), 173 (30)	21	NIST 08, st ^c
4 Nerbowdine	24.79	319 (100), 302 (50), 272 (30), 254 (45), 232 (75)	3	NIST 08
5 Haemanthamine	25.49	301 (20), 272 (100), 240 (15), 225 (10), 211 (15)	23	NIST 08, st ^c
6 Tazettine	25.59	331 (20), 316 (20), 298 (30), 247 (100)	2	NIST 08, st ^c
7 Galanthine	26.40	317 (30), 298 (15), 284 (15), 268 (15), 243 (100), 242 (95)	16	NIST 08, st ^c

^a For GC conditions see the Materials and methods section; ^b Values are expressed as a percentage of the total ion current (TIC); ^c Standart.

given in the table are not true quantification. However, they can be used for comparison between samples.

Galanthamine (1) is a long-lasting, selective, reversible and competitive AChE inhibitor, which has been isolated or described in various genera of Amaryllidaceae plant species (*Amaryllis*, *Galanthus*, *Leucojum*, *Pancreatum*, *Zephyranthes* etc.) (Park, 2010). It is a good example of a natural product substituting synthetic drugs in the treatment of AD. Although lycoramine (2) is a galanthamine-type, its AChE inhibition activity is very low (Lopéz et al., 2002). Nerbowdine (4) is a relatively rare alkaloid, which has been isolated before from two *Crinum* sp. (*C. moorei* and *C. erubescens*), from *Boophone disticha* (Wildman et al., 1967; Lyle et al., 1960; Haut & Stauffacher, 1961), and once identified by GC/MS technique in *Zephyranthes robusta* (Cahliková et al., 2010). There are no data on biological activities of this compound, but alkaloids of the crinane-type have been shown to exhibit a range of biological activities including antimalarial and antiproliferative action as well as protein synthesis inhibition (Tram et al., 2002). Haemanthamine (5) and tazettine (6) are common alkaloids in family Amaryllidaceae. Both alkaloids were tested for their AChE

inhibition activity, but they were considered inactive (Lopéz et al., 2002). Galanthine (7) is also a common Amaryllidaceae alkaloid, but it occurs mainly as a minor component. Hitherto no data on the inhibitory effect of this compound on the AChE have been reported. However, this alkaloid, together with oxomaritine (vittatine-type), both isolated from *Zephyranthes citrina*, has shown a moderate activity *in vitro* against *Trypanosoma brucei rhodesiense* with IC₅₀ value of 3.1 and 2.8 µg/mL, respectively (Herrera et al., 2001).

Twenty-three alkaloids isolated from various Amaryllidaceae species have been previously studied for their *in vitro* AChE inhibitory activity with galanthamine as a positive control. The results of these studies showed, that the AChE inhibitory activity is associated mainly with galanthamine- and lycorine-type of alkaloids (Lopéz et al., 2002; Houghton et al., 2006; Hostettman et al., 2006). Lycorine-type compounds are less active inhibitors than the galanthamine type compounds and their activity is associated with a substitution at position in C-1 and C-2 (Houghton et al., 2006). Therefore, the presence of the above mentioned compounds could explain the AChE inhibitory activity of the extract of *Z. grandiflora*.

GC/MS analysis of bulbs of *Z. concolor* showed that galanthamine-type alkaloids represented more than 90% of the alkaloid mixture, which inhibited AChE from electric eel, using TLC bioautographic assay, by $88 \pm 0.2\%$ at a concentration of $10 \mu\text{g/mL}$. Galanthamine (64%) and chlidanthine (24%), both galanthamine-type, were identified as the main components (Berkov et al., 2008). In our study, galanthamine (**1**) was present only as a minor component (5%), and chlidanthine has not been detected. In comparison with our previous phytochemical investigation on *Z. robusta*, we identified a very similar alkaloidal profile and inhibition activity to HuAChE and HuBuChE (Cahliková, et al., 2010). Although we found the same compounds, the main differences were in the content (TIC) of galanthine (**7**) and haemanthamine (**5**). The alkaloid pattern of *Z. robusta* was dominated by galanthine (**7**), which was present only in a small amount in *Z. grandiflora*.

As mentioned, BuChE plays an important role in the late AD stages, but only a limited number of alkaloids has been tested for their BuChE inhibitory activity so far. The screening of crude and/or alkaloid extracts from plants, followed by GC/MS analysis, seems to be a good way for searching of new bioactive natural compounds with AChE/BuChE inhibition activity. In case of identification of unknown structures by GC/MS, the isolation of these compounds would be interesting due to its potential activity as cholinesterase inhibitors.

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