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RESEARCH ARTICLE

Quantitative determination of Amaryllidaceae alkaloids from *Galanthus reginae-olgae* subsp. *vernalis* and *in vitro* activities relevant for neurodegenerative diseases

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Abstract

In the present work the qualitative and quantitative analysis of Amaryllidaceae-type alkaloids in the aerial parts and bulbs of *Galanthus reginae-olgae* Orph. subsp. *vernalis* Kamari is presented for the first time using GC-MS analysis. The alkaloids galanthamine, lycorine, and tazettine were identified in both extracts while crinine and neronine were found only in the bulbs. The yield of alkaloid fraction from bulbs (36.8%) is very high compared to the yield from aerial parts (9.34%). Lycorine was the major component in both fractions. The antioxidant potential was determined by three complementary methods. The preparations to reduce the stable free radical DPPH to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl with IC_{50} values of 39 and 29 μg/mL for MeOH extracts from aerial parts and bulbs, respectively. The higher activity was given by EtOAc fraction of aerial parts with IC_{50} of 10 μg/mL. This activity is probably due to the presence in EtOAc fraction of polar compounds such as polyphenols. The fraction exhibited a significant antioxidant capacity also in the β-carotene-linoleic acid test system. A higher level of antioxidant activity was observed for EtOAc fraction from bulbs with IC_{50} of 10 μg/mL after 30 min and 9 μg/mL after 60 min of incubation. In contrast, the fraction from bulbs performed poorly in the lipid peroxidation liposomes assay. Significant activity was obtained for dichloromethane fraction from aerial parts (IC_{50} of 74 μg/mL). The major abundance of alkaloid in dichloromethane fraction may be responsible of the bulbs anti-cholinesterase highest activity (38.5%) at 0.5 mg/mL.

Keywords: *Alkaloids; Alzheimer; Amaryllidaceae; antioxidant activities; cholinesterase inhibitors; Galanthus reginae-olgae Orph. subsp. vernalis Kamari*

Introduction

The Amaryllidaceae family includes more than 800 species in 89 genera distributed widely over tropical, subtropical, and Mediterranean regions (Meerow & Snijman, 1998, 2001). In the Mediterranean region three subfamilies are present with more than 120 species - Narcisseae (*Narcissus* and *Sternbergia*), Pancratieae (*Pancratium* and *Vagaria*), and Galantheae (*Acis*, *Galanthus*, and *Leucojum)* (Meerow et al., 2006). The plants belonging to this family produce a large number of structurally diverse alkaloids with a wide range of interesting physiological effects. In recent years attention has been focused on the biological activity of several Amaryllidaceae alkaloids (Lewis, 2002).

Galanthamine is the most interesting for its use in the treatment of Alzheimer's disease as a cholinesterase inhibitor (Woodruff-Pak et al., 2001; Maelicke et al., 2001). Lopéz et al. (2002) tested 23 pure Amaryllidaceae alkaloids for their acetylcholinesterase inhibitory activity using galanthamine as a reference. Only seven alkaloids, belonging to the galanthamine and lycorine skeleton

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types, exhibited such an effect, sanguinine being the most active, even more than galanthamine.

In recent years, oxidative stress has been described in the pathological changes that occur in Alzheimer's disease (AD) (Murray & Lynch, 1998; Pratico & Delanty, 2000). Acetylcholine (ACh) is found in the synapse of the cerebral cortex and a deficiency in this area is one of the major features seen in sufferers of AD (Bierer et al., 1995). Acetylcholinesterase inhibitors (AChE) such as tacrine, donepezil, and the natural products rivastigmine, and galanthamine are actually the only effective treatment for AD. These approved drugs are limited in use due their adverse side-effects such as gastrointestinal disturbance, and bioavailability problems (Schulz, 2003; Melzer, 1998).

The continuing search for novel anticholinesterases from plants as therapeutics agents for dementia and other central nervous system disorders is based on the need for agents targeted to brain areas affected, with reduced toxicity and side-effects.

Several studies, mainly involving species and subspecies of the genus *Galanthus, Leucojum*, and *Narcissus*, have been published about the distribution of galanthamine in different populations of these plants, as well as in their different organs and during different periods of harvesting (Bastida et al., 2006; Berkov et al., 2004, 2007a, 2007b; Sidjimova et al., 2003), together with quantitative estimation and indications for sustainable use of this resource species for the pharmaceutical industry (Gussev et al., 2001, 2007). In these studies, TLC and GC-MS (Sidjimova et al., 2003; Berkov et al., 2004; Kreh et al., 1995) were successfully applied; in particular GC-MS was found to be suitable for the characterization of the alkaloids. The power of GC-MS has been exploited in these works, especially because the main Amaryllidaceae alkaloids can be easily subjected to direct GC analysis without any derivatization step.

In the present work the qualitative and quantitative analysis of Amaryllidaceae-type alkaloids in the aerial parts and bulbs of *Galanthus reginae-olgae* Orph. subsp. *vernalis* Kamari (*Galantheae* Salisbury, agg. *Galanthus*) is presented for the first time using GC-MS analysis. Furthermore, the present study was undertaken to investigate the *in vitro* antioxidant and anticholinesterase effects of *G. reginae-olgae* subsp. *vernalis*. These activities have not been investigated in the species until now. The antioxidant potential was determined by three complementary methods: DPPH radical scavenging assay that evaluates the antioxidant capacity through the hydrogen donating ability of antioxidants; bovine brain peroxidation assay that evaluates the attack of free radicals on membrane systems and the reduction of the extent of peroxidation when an antioxidant compound is incorporated in the lipid peroxidation assay reaction mixture; β-carotene bleaching test that evaluates the inhibition of the breakdown of lipid hydroperoxides. The anti-cholinesterase effect was evaluated by the *in vitro* Ellman's method.

Materials and methods

Plant material

The aerial parts and bulbs of *Galanthus reginae-olgae* Orph. subsp. *vernalis* were collected on 6 April 2006, in *Fagus sylvatica* forest; Catena Costiera Mountain - N39°19'18"; E16°06'73"; 960 m alt. - locality Crocetta, near the village of San Fili Calabria, southern Italy; a voucher specimen is deposited in the herbarium (CLU) of the Natural History Museum of Calabria and Botanic Garden, University of Calabria. The nomenclature and chorological data are after Conti et al. (2005) and the taxon was identified by D. Uzunov, of the Natural History Museum of Calabria and Botanic Garden, University of Calabria, following the identification key and description in Davis (1999, 2002).

Extraction

Aerial parts (874 g) and bulbs (179 g) were washed and cut into small pieces and extracted with MeOH (5 L) through maceration (144 h × 3 times). The resultant extracts were dried under reduced temperature and pressure using a rotary evaporator to give 52 g and 5.2 g for aerial parts and bulbs, respectively. The MeOH extract was suspended in a methanol/water (9:1) mixture and extracted with *n*-hexane, dichloromethane and ethyl acetate. In order to obtain a separation of alkaloid compounds, dichloromethane extract was acidified with HCl (pH 1) and defatted with *n*-hexane. Successively the solution was basified with NH_4OH to adjust the pH to 8, extracted with dichloromethane and evaporated in vacuum to dryness to achieve alkaloid fractions (Figure 1).

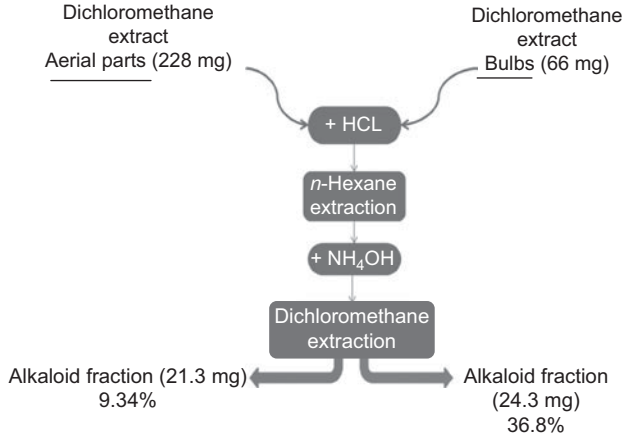

Figure 1. Isolation of alkaloid fraction from aerial parts and bulbs of *Galanthus reginae-olgae* Orph. subsp. *vernalis* Kamari.

TLC analysis

TLC on silica gel 60 (Merck plates 0.25 mm) was performed in EtOAc–HCOOH–CH_3COOH–H_2O (100:11:11:26, v/v), *n*-hexane–diethylether (8:2, v/v), EtOAc–isopropanol–NH_4OH (65:35:20, v/v). Developed TLCs were examined under UV_{254} nm and UV_{366} nm prior to spraying. Flavonoids were detected using aminoethanol diphenylborate-PEG 400 mixture and visualized at 365 nm (Brasseur & Angenot, 1986). Dragendorff reagent was used for alkaloid detection and anisaldehyde/H_2SO_4 reagent was used to reveal the presence of terpenoids (Wagner & Bladt, 1996).

GC-MS analysis

The *n*-hexane and alkaloid fractions were analyzed by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS). Qualitative GC-MS analyses were carried out using a Hewlett-Packard 6890 gas chromatograph equipped with an SE-30 capillary column (100% dimethylpolysiloxane, 30 m length, 0.25 mm in diameter, 0.25 µm film thickness) and interfaced with a Hewlett Packard 5973 Mass Selective. Ionization of the sample components was performed in electron impact mode (EI, 70 eV). The carrier gas was helium (1 mL/min) and the analytical conditions worked with the following program: oven temperature was 5 min isothermal at 40°C, then 40°-250°C at a rate of 5°C/min; then held isothermal for 10 min. Injector and detector were maintained at 250° and 280°C, respectively. The mass range from 50 to 550 amu was scanned at a rate of 2.9 scans/s. Identification of the compounds was based on the comparison of the mass spectral data with Wiley 138 and Wiley 275 (Hewlett-Packard) and an in-house mass spectral library built from pure substances.

Quantitative GC analyses were performed on a Shimadzu GC17A gas chromatograph equipped with a flame ionization detector (FID) and controlled by Borwin Software. The samples were analyzed on a fused silica 30 m SE-30 capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm. Nitrogen was used as the gas vector at a constant flow of 1 mL/min; split ratio 1:30. Injector and detector were maintained at 250° and 280°C, respectively. Column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at 5°C/min rate and finally held for 10 min at 250°C. Stock solution of galanthamine hydrobromide was prepared in methanol at a concentration of 2 mg/mL. All determinations were performed in triplicate and averaged.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

This experimental procedure was adapted from Wang et al. (1998), modified as reported in Conforti et al. (2005). The absorbance was measured using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm against blank, which was without DPPH. All tests were run in triplicate and averaged. Ascorbic acid was used as a positive control. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as the percentage DPPH radical-scavenging that is calculated in the equation:

$$\%\text{DPPH radical} - \text{scavenging} = [1 - (\text{sample absorbance with DPPH} - \text{sample absorbance without DPPH/control absorbance})] \times 100$$

Bovine brain peroxidation assay

The *in vitro* antioxidant activity tests were carried out using the thiobarbituric acid (TBA) test described by Fernandez et al. (1997), modified as reported in Conforti et al. (2002). The TBA reaction is based on the fact that peroxidation of most membrane systems leads to formation of small amounts of free malonaldehyde (MDA). Absorbance at 532 nm was determined on a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. Extracts were tested for antioxidant activity against liposomes which were prepared from bovine brain extract (Sigma-Aldrich, Milan, Italy) in phosphate buffered saline (5 mg/mL). Propyl gallate was used as a positive control (Jacobi et al., 1999). The inhibition of lipid peroxidation in percentage terms was calculated by the following equation:

$$\%\,\text{inhibition} = [(\text{FRM} - \text{B}) - (\text{Et} - \text{B} - \text{EA})/(\text{FRM} - \text{B})] \times 100$$

where FRM is the absorbance of the control reaction, ET is the absorbance in the presence of the sample. The absorbance of liposomes only (B) and extract alone (EA) were also taken in account.

β-Carotene bleaching test

Antioxidant activity was determined using the β-carotene bleaching test (Amin et al., 2004) with some modifications. Briefly 1 mL of β-carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. After evaporation of chloroform and dilution with water, aliquots of 5 mL of the emulsion were transferred into different test tubes containing 0.2 mL of samples in 70% ethanol at different concentrations. Standard propyl gallate at the same concentration as samples was used for comparison. The tubes were then gently shaken and placed at 45°C in a water bath for 60 min. The absorbance of the samples, standard and control was measured at 470 nm

using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at start time ($t = 0$) and successively at 30 and 60 min. All samples were assayed in triplicate and averaged. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation:

$$AA = \left(1 - \frac{A_0 - A_t}{A^0{}_0 - A^0{}_t}\right) \times 100$$

where A_0 and $A°_0$ are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and $A°_t$ are the absorbance values measure in the samples/standard and control respectively at $t = 30$ min and $t = 60$ min.

Bioassay for anti-cholinesterase activity

Inhibition of cholinesterases (AChE and BChE) was assessed by a modified colorimetric method of Ellman (Perry et al., 2000). Fourty μL of AChE or BChE (0.36 U/mL in buffer pH 8) and extracts at different concentrations (20 μL) were added to 2 mL of buffer pH 8 and pre-incubated in an ice bath at 4°C for 30 min. Duplicate tubes were also treated this way with 20 μL of physostigmine (0.1 mM) to allow interference of the test substances in the assay to be assessed, and to control for any hydrolysis of ACh not due to AChE activity. The reaction was started by adding DNTB solution (20 μL of 0.05 mM in buffer pH 7) and acetylthiocholine ATCI or butyrriltiocholine BTCI (20 μL 0.018 mM in buffer pH 7) and tubes were placed in a water bath for 20 min at 37°C. The reaction was halted by placing the assay solution tubes in an ice bath and adding physostigmine (20 μL 0.018 mM in buffer pH 7). The production of yellow anion was immediately recorded on the spectrophotometer at 412 nm and the percentage inhibition was calculated. Physostigmine was used as positive control. The inhibition rate (%) was calculated by the equation:

$$\text{Inhibition}\,\% = \frac{[(\text{Blank} - \text{Blank positive control}) - (\text{Experiment} - \text{Experiment control})]}{(\text{Blank} - \text{Blank positive control})}$$

Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using Student's *t*-test. Differences were considered significant at $P \leq 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated from the Prism dose response curve (Prism GraphPad, Prism version 4.0 for Windows, GraphPad Software, San Diego, CA) obtained by plotting the percentage of inhibition versus the concentrations.

Results and discussion

TLC analysis of different samples revealed the presence of flavonoids in EtOAc extract, terpenoids in *n*-hexane extracts and alkaloids in $CHCl_3$ extract. GC-MS analysis of *n*-hexane extract from aerial parts of *G. reginae-olgae* subsp. *vernalis* resulted in the identification of fatty acids, such as eicosadienoic acid, 9-octadecenoic acid, which were the major constituent. The same extract is also rich in vitamin E and different phytosterols, such as ergost-5-en-3-ol, stigmast-5-en-3-ol and stigmast-5,24-dien-3-ol. Instead, the extract from bulbs contains only fatty acids (Table 1).

Dichloromethane extracts led to identification of five Amaryllidaceae-type alkaloids in total. The alkaloids galanthamine, lycorine and tazettine were identified in both fractions while crinine and neronine were found only in the bulbs (Table 2). Quantitative determination of these alkaloids was showed. The yield of alkaloid fraction from bulbs (36.8%) is very high compared to the yield from aerial parts (9.34%). Lycorine was the major component (3.52% and 22.2% from aerial parts and bulbs, respectively) followed by tazettine (0.93% and

Table 1. Chemical composition of *n*-hexane extract from *G. reginae-olgae* subsp. *vernalis* aerial parts and bulbs.

R_t*	Compounds	Aerial parts	Bulbs
14.86	1-metil-4-etossi-,δ,(3)pirrolin-2-one	+	-
17.77	Neophytadiene	+	+
18.45	Exadecanoic acid, methyl ester	+	+
18.74	Exadecanoic acid	+	-
19.74	9,12-0ctadecandienoic acid, methyl ester,[E,E]	+	+
19.79	9,12,15-octadecantrienoic acid, methyl ester,[Z,Z,Z]	+	+
19.88	2-exadecen-1-ol-3,7,11,15-tetramethyl-[R-[R*,R*-(E)]]	+	-
20.09	9,12,15-octadecantrienal	+	-
23.94	9,12-octadecandienoic acid [Z,Z]-2-idrossi-1-[idrossimethyl] ethylester	+	+
24.03	2-monolinolenin	+	-
26.00	1-octadecene	+	-
29.49	9-α-fluoro-5-α-cholest-8(14)-ene-3,15-dione	+	-
30.00	Vitamin E	+	-
34.23	Ergost-5-en-3-ol,(3.β.,24 E)	+	-
34.60	Stigmast-5-en-3-ol,(3.β.,24 S)	+	-
35.06	Stigmast-5,24(38)-dien-3-ol,(3.β.,24 E)	+	-

*Compounds listed in order of elution from SE30 MS column. Retention time (min).

4.9% of aerial parts and bulbs, respectively) and galanthamine (0.28% and 1.67% from aerial parts and bulbs, respectively). In the bulbs were also determined the amount of crinine (2.6%) and neronine (1.42%).

The extracts were screened for their anticholinesterase activity at 0.5 mg/mL concentration and the following inhibition rates were determined for AChE: MeOH 15.2%, EtOAc 1.2%, *n*-hexane 1.2%, alkaloid fraction 11.8%, from aerial parts and MeOH 18.2%, EtOAc 5.0%, *n*-hexane 7.8%, alkaloid fraction 38.5%, from bulbs (Table 3). No BChE inhibition could be detected for tested extracts. The major abundance of alkaloid fraction may be responsible of the bulbs highest activity. At the same time as previously demonstrated among Amaryllidaceae identified alkaloids tazettine (IC_{50} 705 μM), crinine (IC_{50} 461 μM) and lycorine (IC_{50} 213 μM) exhibited about seven, four, and two-hundred times lower activity than galanthamine (IC_{50} 1.9 μM), respectively (Elgorashi et al., 2004). In general, the structure–activity relationship evidenced that lycorine-type alkaloids exhibited higher activity on AChE than crinine and tazettine-type alkaloids (Houghton et al., 2006). Several lycorine derivatives, such as secolycorines, showed potent inhibitory activity against acetylcholinesterase with the IC_{50} value at micromolar range and are more potent than galanthamine (Lee et al., 2007). The high lycorine-type alkaloid content shown in *G. reginae-olgae* subsp. *vernalis* bulbs should be used as a font of starting material useful for chemical transformations for the above-mentioned secolycorines. The antioxidant activities of the *G. reginae-olgae* subsp. *vernalis* extracts were carried out using different *in vitro* assays (β-carotene bleaching test and lipid peroxidation of liposomes assay), while radical scavenging activity was carried out using the DPPH test. IC_{50} values of all tests are shown in Table 4. These assays differ from each other in terms of substrates, probes, reaction conditions, and quantification methods. The activity of a plant extract, which contains different chemical compounds, is reflected in the context of specific reaction conditions such as pressure, temperature, reaction media, co-reactant, and reference point. The antioxidant activity measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay. Therefore it is appropriate to use different assays to evaluate the inhibition of different mechanisms of oxidation.

The model of scavenging stable DPPH free radicals can be used to evaluate the antioxidant activity in a relatively short time. The scavenging effects of extracts on DPPH were examined at different concentrations (range between 5-1000 μg/mL). The preparations were able to reduce the stable free radical DPPH to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl with IC_{50} of 39 μg/mL and 29 μg/mL for MeOH extracts from aerial parts and bulbs, respectively. The higher activity was due to EtOAc extract of aerial parts with IC_{50} of 10 μg/mL (Figure 2). This activity is probably due to the presence in EtOAc extract of polar compounds such as polyphenols as previously reported for *Galanthus nivalis* (Hörhammer et al., 1967). Several studies found a correlation between the phenolic content and the antioxidant activity (Velioglu et al., 1998). Recent studies have focused on health functions of phenolics, including flavonoids from plants (You-dim et al., 2002; Qian et al., 2004).

The extracts exhibited a significant antioxidant capacity also in the β-carotene-linoleic acid test system.

Table 2. Composition of alkaloid fraction from *G. reginae-olgae* subsp. *vernalis* aerial parts and bulbs.

	% Composition	
Alkaloid	Aerial parts	Bulbs
Galanthamine	0.28	1.67
Lycorenan	3.52	22.2
Tazettine	0.93	4.9
Crinine	ND	2.6
Neronine	ND	1.42

ND, not detected.

Table 3. Anti-cholinesterase activity of extracts from*G. reginae-olgae* subsp. *vernalis* aerial parts and bulbs.

	AChE		BuChE %	
	% Inhibition (0.5 mg/mL)		Inhibition (0.5 mg/mL)	
Extract	Aerial parts	Bulbs	Aerial parts	Bulbs
MeOH	15.2 ± 0.81	18.2 ± 0.93	-	-
n-Hexane	1.2 ± 0.04	7.8 ± 0.49	-	-
Dichloromethane	11.8 ± 0.72	38.5 ± 1.08	-	-
EtOAc	1.2 ± 0.06	5.0 ± 0.42	-	-

Table 4. Antioxidant and free radical scavenging activities of extracts from G. Reginae-olgae subsp. vernalis aerial parts and bulbs.

	IC_{50} (μg/mL)			
			β-Carotene bleaching test	
Extract	DPPH	Lipid peroxidation	30 min of incubation	60 min of incubation
Aerial parts				
MeOH	39 ± 0.067	> 1000	11 ± 0.016	29 ± 0.214
n-Hexane	> 1000	> 1000	16 ± 0.045	30 ± 0.209
Dichloromethane	146 ± 0.238	74 ± 0.139	10 ± 0.011	9 ± 0.018
EtOAc	10 ± 0.020	962 ± 1.231	12 ± 0.017	23 ± 0.033
Bulbs				
MeOH	29 ± 0.051	> 1000	92 ± 0.231	> 100
n-Hexane	> 1000	> 1000	> 100	> 100
Dichloromethane	15 ± 0.031	273 ± 0.345	15 ± 0.035	35 ± 0.065
EtOAC	148 ± 0.231	> 1000	10 ± 0.019	9 ± 0.021
Propyl gallate[a]	-	7 ± 0.017	1 ± 0.009	1 ± 0.010
Ascorbic Acid[b]	2 ± 0.011	-	-	-

[a] Propyl gallate and ascorbic acid were used as positive control.

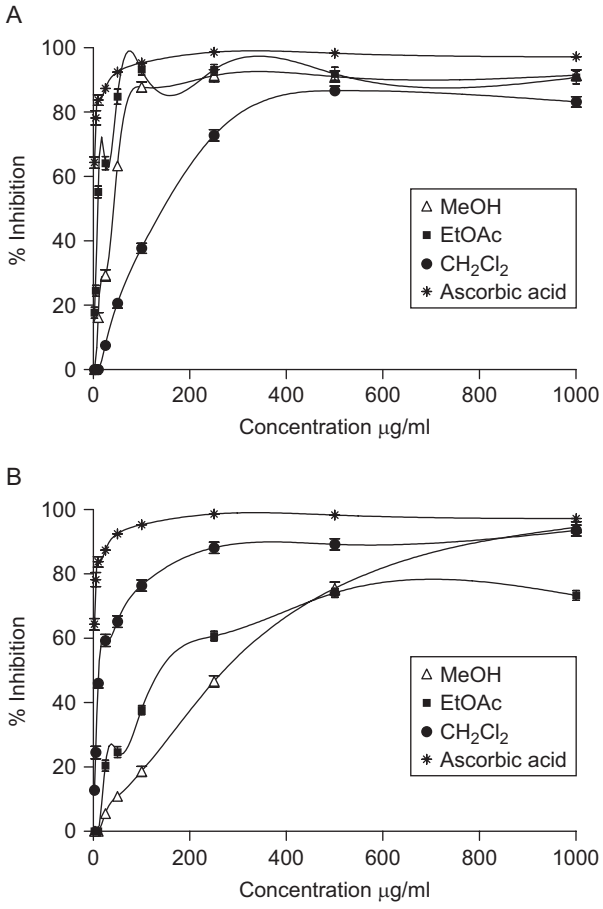

Figure 2. Radical scavenging activity of methanolic extract and fractions from (A) aerial parts and (B) bulbs of *Galanthus reginae-olgae* subsp. *vernalis* using DPPH assay. All samples were assayed in triplicate and averaged.

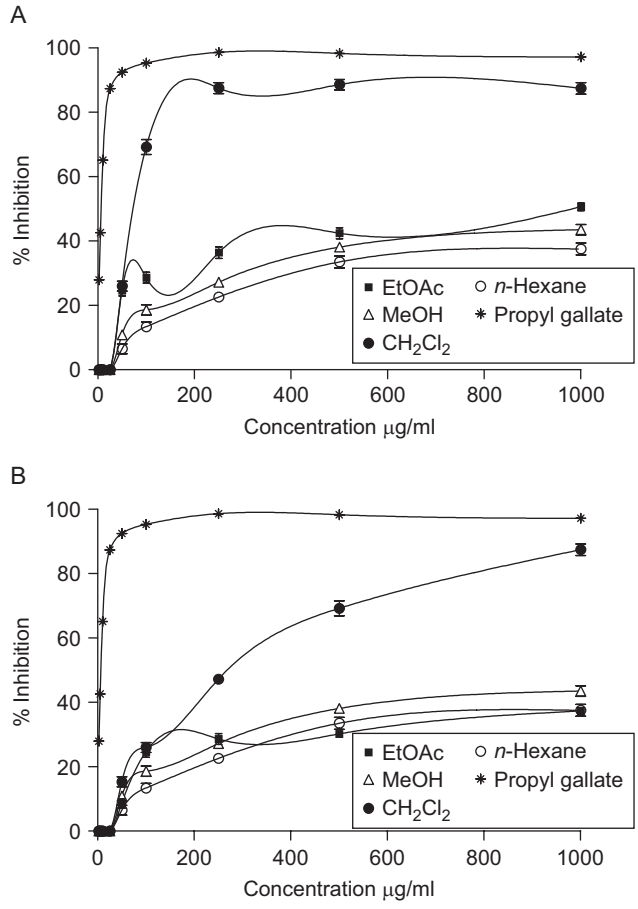

Figure 3. Antioxidant activity of methanolic extract and fractions from (A) aerial parts and (B) bulbs of *Galanthus reginae-olgae* subsp. *vernalis* using the bovine brain peroxidation assay. All samples were assayed in triplicate and averaged.

Inhibition of the breakdown of lipid hydroperoxides to unwanted volatile products allowed us to determine secondary antioxidants in related mechanisms. In the absence of antioxidants, oxidation products (lipid hydroperoxides, conjugated dienes and volatile byproducts) of linoleic acid simultaneously attack the β-carotene, resulting in bleaching of its characteristic yellow color in ethanolic solution. In the presence of the total extracts oxidation products were scavenged and bleaching was prevented. A higher level of antioxidant activity was observed for EtOAc extract from bulbs with IC_{50} of 10 μg/mL after 30 min and 9 μg/mL after 60 min of incubation, indicating that their activity was not correlated with time of heating (Figure 3). In contrast, extract from bulbs performed poorly in the lipid peroxidation liposomes assay, where its protective action against MDA formation was weak (IC_{50} > 1 mg/mL). Significant activity was obtained for dichloromethane extract from aerial parts (IC_{50} of 74 μg/mL) (Figure 4). This activity is probably due to the presence of galanthamine which previously showed antioxidant properties (Traykova et al., 2003).

Conclusion

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses. In most diseases, increased oxidant formation is a consequence of the disease activity. Potential antioxidant therapy therefore should include either natural free radical scavenging antioxidant principles or agents, which are capable of augmenting the activity of the antioxidant enzymes. ROS are capable of damaging biological macromolecules such as DNA, carbohydrates or proteins. If human disease is believed to be due to the imbalance between oxidative stress and antioxidative defense, it is possible to limit oxidative tissue damage and hence prevent disease progression

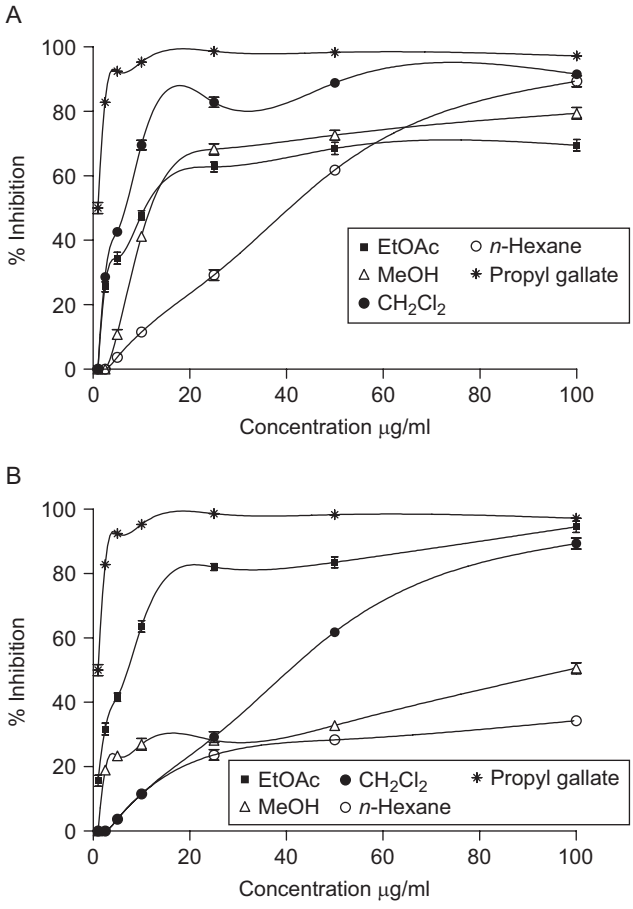

Figure 4. Antioxidant activity of methanolic extract and fractions from (A) aerial parts and (B) bulbs of *Galanthus reginae-olgae* subsp. *vernalis* using the β–carotene-linoleate system after 30 min of incubation. All samples were assayed in triplicate and averaged.

by antioxidant defense supplements. Oxidative damage may initiate and promote the progression of a number of chronic diseases, including diabetes and Alzheimer's disease. The continuing search for novel anticholinesterases from plants as therapeutic agents for dementia and other central nervous system disorders is based on the need for agents targeted to brain areas affected, with reduced toxicity and side effects (Akhondzadeh & Abbasi, 2006; Mazza et al., 2006; Maruyama et al., 2006). The present work showed for the first time the quantitative determination of Amaryllidaceae alkaloids, antioxidant potential and the *in vitro* acetylcholinesterase activity of *G. reginae-olgae* subsp. *vernalis*. This species of *Galanthus* can be an interesting source of phenolics and alkaloids with a potential use as an antioxidant in neurodegenerative diseases such as Alzheimer's disease. The most promising compound is galanthamine which exhibited about seven, four and two-hundred times higher activity than tazettine, crinine and lycorine. Moreover, the high lycorine-type alkaloid content showed bulbs should be used as a font of starting material useful for chemical transformations to obtain more active compounds.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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