Sustainable Use of Various Amaryllidaceae Plants against Alzheimer's Disease

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Abstract

Alzheimer's Disease (AD), a slowly progressive neuropsychiatric illness, principally characterized by memory deficits, has become the fourth leading cause of death in developed nations. Since the patients with AD suffer from marked reduction of cholinergic neuronal function resulting in a deficiency in acetylcholine (ACh) concentration in the brain, and these reductions are associated with impairments in memory, cholinergic enhancement strategies have been at the forefront of efforts to palliate pharmacologically the cognitive symptoms. Therefore, treatment approaches have been focused on the acetylcholinesterase inhibitors (AChEI) which are the most promising drugs demonstrating efficacy in the treatment of AD. Since, recently, galanthamine, an alkaloid isolated from different *Galanthus* species (Amaryllidaceae), has been found to be a potent and reversible acetylcholinesterase inhibitor, this development has prompted us to investigate the anticholinesterase activity in other plant species of Amaryllidaceae growing in Turkey, namely *Galanthus elwesii*, *G. ikariae*, *Narcissus tazetta* subsp. *tazetta*, *Leucojum aestivum* and *Pancratium maritimum* by Ellman method in comparison with galanthamine as the standard drug. Bioactivity-directed fractionation and isolation studies carried out on *G. ikariae* and *N. tazetta* subsp. *tazetta* extracts afforded 8 Amaryllidaceae-type alkaloids in total. We found that the activity of both of the plant extracts was due to the synergistic interaction of the alkaloids isolated.

INTRODUCTION

Alzheimer's Disease (AD) is a chronic neurodegenerative disorder characterized by a progressive decline in cognitive function, including loss of memory and disturbances in function and behavior (Adams et al., 1984; Bachman et al., 1992; Arnold et al., 1993; Aisen and Davis, 1997). A deficit of central presynaptic cholinergic function has been demonstrated in AD by degeneration of cholinergic neurons in basal forebrain (Friede, 1965; Perry et al., 1978; lnan, 1999). This led to the suggestion that acetylcholinesterase inhibitors (AChEI) would preserve a putative deficit in acetylcholine levels associated with AD, and thus might reserve the memory impairments (Schneider, 2001; Guardado-Santervas, 2001; Gauthier, 2001). Therefore, a number of AChEI have been developed as candidates for the symptomatic treatment of AD, including natural compounds, such as physostigmine, huperzine A and galanthamine as well as various synthetic compounds such as tacrine, donepezile and rivastigmine (Davis and Mohs, 1982; Liu et al., 1986; Summers et al., 1986; Mutafova-Yambolieva et al., 1993; Rogers et al., 1998; Potkin et al., 2001). Among these, galanthamine, an Amaryllidaceae alkaloid isolated from the bulbs of *Galanthus* species. Amaryllidaceae alkaloids exhibit antitumor, antiviral and anticholinergic activities. Some of them have been used in the treatment of myastenia gravis, myopathy and diseases of the nervous system (Martin, 1987). The bulbs of some species of Amaryllidaceae cultivated in Turkey have been exported as ornamental plants. In the aforementioned context, we systematically study five uninvestigated Amaryllidaceae plants growing in Turkey for their potential anticholinesterase activity.

MATERIALS AND METHODS

Plant Materials

The bulbs of *Galanthus elwesii* Hooker fil., *Narcissus tazetta* subsp. *tazetta* L. and *Pancratium maritimum* L. from Antalya-Akseki, Antalya-Kumluca and Antalya-Belek located at the Mediterranean region of Turkey, respectively, *G. ikariae* L. and *Leucojum aestivum* L. from Trabzon-Sürmene and Samsun-Terme at the northeast of Turkey, respectively, were collected and identified by Prof. Dr. B. Şener. Voucher specimens are deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Extraction

Air-dried bulbs of *Galanthus elwesii*, *G. ikariae*, *Narcissus tazetta* subsp. *tazetta*, *Leucojum aestivum* and *Pancratium maritimum* (50 g for each) were powdered in a grinder mechanically and extracted three times with chloroform:methanol (1:1) at room temperature. After removal of the solvent in vacuo at 40°C until dryness, crude extract of each species was obtained. Anticholinesterase activity of the extracts was determined at 10 μg/ml concentration by Ellman method. Subsequently, the alkaloid extracts were prepared from the powdered bulbs of each species by the following procedure. The powdered materials were percolated with ethanol (96°) at room temperature, evaporated under vacuum until dryness. The crude extracts were acidified with HCl (5%), and left for 2 days in +4°. After filtration, hydro-acidic layers were made basic with NH_4OH (25%) to adjust pH to 8, extracted with chloroform and evaporated in vacuo until dryness.

Bioactivity-directed Isolation

Alkaloid extracts of *G. ikariae* and *N. tazetta* subsp. *tazetta* were used for bioactivity-directed fractionation and isolation. Alkaloid extract of *G. ikariae* was subjected to column chromatography (Si 60) and eluted with chloroform:methanol mixtures in increasing polarity. 50 fractions (10 ml for each) were collected in total. By monitoring with TLC, similar fractions were combined and 5 subfractions were obtained. Anticholinesterase activity of the subfractions were determined at 10 μg/ml concentration. Third and fourth subfractions displayed activity and from them, lycorine (0.09 g), tazettine (0.008 g), crinine (0.06 g), galanthamine (0.003 g), 3-epi-hydroxybulbispermine (0.074 g) and 2-demethoxymontanine (0.047 g) were isolated by preparative TLC. Same isolation procedure was also applied for the alkaloid extract of *N. tazetta* subsp. *tazetta* and lycorine (0.3 g), tazettine (0.0018 g), 3-epi-hydroxybulbispermine (0.042 g), N-nor-galanthamine (0.007 g) and haemanthamine (0.005 g) were isolated by preparative TLC from the active subfractions. The characterization of the isolated alkaloids were determined by ^{1}H-NMR and EIMS spectra in comparison with the published data (Grundon, 1985; Southon and Buckingham, 1989; Şener et al., 1993; Şener et al., 1998). Anticholinesterase activity of the pure compounds was determined by Ellman method at 10 μg/ml concentration.

Chemicals

For acetylcholinesterase inhibitory activity, electric eel acetylcholinesterase (AChE; True cholinesterase; EC 3.1.1.7., Type VI-S, EC no: 232-559, 2000 Units) was purchased from Sigma (St. Louis, MO). Sterile-apyrogen isotonic sodium chloride solution (0.9%) was obtained from Ibrahim Ethem Drug Company. The enzyme kit (Spinreact®, Spain), based on the Ellman method, was used to evaluate the acetylcholinesterase inhibitory activity. The standard drug, galanthamine, was obtained from Johnson & Johnson drug company. Organic solvents and silica gel used in the isolation were from Merck Co.

Determination of Anticholinesterase Activity

Anticholinesterase activity was determined spectrophotometrically using acetylthiocholine as substrate by modifying the method of Ellman (Ellman et al., 1961).

According to the method, the absorbance of the yellow-colored end product, 5-thio-2-nitrobenzoate, at 412 nm was determined using a spectrophotometer (Beckman DU-600 spectrophotometer, USA) in the quartz cuvettes (Starna, U.K., no:1 OG 5391). Absorbance reading was performed 6 times at every 30 seconds. All experiments were performed in triplicate and the results were analyzed by Student's *t*-test. Sterile-apyrogen isotonic sodium chloride solution (0.9%) was used as blank.

Statistical Method

The concentration of compound required for 50% enzyme inhibition (IC_{50}) was calculated according to Michaelis-Menten model by using "EZ-Fit: Enzyme Inhibition Kinetic Analysis (EZ-Fit Enzyme Kinetics MS Windows Software, Perrella Scientific, Inc.)" program. All tabulated results were expressed as means ± SEM, and were compared using Student's-*t* test. A p value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

In order to find new natural substances with acetylcholinesterase inhibitory activity, we have screened five Amaryllidaceae plants, namely *Galanthus elwesii*, *G. ikariae*, *Narcissus tazetta* subsp. *tazetta*, *Leucojum aestivum* and *Pancratium maritimum*. Their anticholinesterase activity were evaluated by in vitro Ellman method in comparison with galanthamine (Reminyl®), one of the newest drugs licensed in the USA, some European countries and Turkey in the treatment of AD.

In pre-screening, chloroform:methanol (1:1) extracts prepared from the bulbs of *Galanthus elwesii*, *G. ikariae*, *Narcissus tazetta* subsp. *tazetta*, *Leucojum aestivum* and *Pancratium maritimum* were found to cause inhibition 73.18% ($p<0.001$), 75.56% ($p<0.001$), 46.62%, 34.39% and 30.42%, respectively, at 10 μg/ml concentration (Table 1). Since these species are quite rich in alkaloids, we thought that the anticholinesterase activity might be resulting from the alkaloids. Consequently, we prepared alkaloid extracts of the bulbs of the mentioned plants and screened them for their anticholinesterase activity. The alkaloid extracts of the plants also exhibited very similar inhibition rates to their chloroform:methanol extracts which were 77.23% ($p<0.001$), 76.96% ($p<0.001$), 46.96%, 39.14% and 27.16%, respectively, at 10 μg/ml concentration. Both of the *Galanthus* species showed high inhibitory activity compared to galanthamine. *N. tazetta* subsp. *tazetta* extract also had almost equal activity to galanthamine.

Following pre-screening, bioactivity-directed fractionation of the alkaloid extracts of *G. ikariae* and *N. tazetta* subsp. *tazetta* led to isolation of eight Amaryllidaceae-type alkaloids in total. The alkaloids were elucidated as lycorine (IC_{50} = 3.16 μM), tazettine, crinine, galanthamine (IC_{50} = 3.2 μM), 3-epi-hydroxybulbispermine and 2-demethoxy-montanine isolated from *G. ikariae*, lycorine, tazettine, N-nor-galanthamine, haemanthamine and 3-epi-hydroxybulbispermine isolated from *N. tazetta* subsp. *tazetta* (Fig. 1). These alkaloids were also screened for their anticholinesterase activity at 10 μg/ml concentration and following inhibition rates were determined: lycorine 43.69%, tazettine 36.34%, crinine 26.53%, galanthamine 48.00%, 3-epi-hydroxybulbispermine 30.18%, 2-demethoxymontanine 31.84%, N-nor-galanthamine 34.09% and haemanthamine 20.8 % (Table 2).

These findings showed that a single alkaloid is not responsible for anticholinesterase activity of *G. ikariae* and *N. tazetta* subsp. *tazetta* extracts. However, the activity depends on the synergistic interaction between the alkaloids isolated. According to these results, the bulbs of Amaryllidaceae plants can also be evaluated as a source of anticholinesterase alkaloids in addition to their ornamental properties.

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Tables

Table 1. Anticholinesterase activity of the chloroform:methanol and alkaloid extracts of five Amaryllidaceae plants by Ellman method.

Species (Chloroform:methanol extracts)	**Inhibition Rate (%)**[a] (10 µg/ml)
Galanthus elwesii	73.18±1.01^{+++}
G. ikariae	75.56±0.99^{+++}
Narcissus tazetta subsp. *tazetta*	46.62±0.77
Leucojum aestivum	34.39±0.72
Pancratium maritimum	30.42±0.85

Species (Alkaloid extracts)	**Inhibition Rate (%)**[a] (1 mg/ml)
Galanthus elwesii	76.96±0.30^{+++}
G. ikariae	77.23±0.41^{+++}
Narcissus tazetta subsp. *tazetta*	46.96±0.08
Leucojum aestivum	39.14±0.33
Pancratium maritimum	27.16±0.49

Standard	**Inhibition Rate (%)**[a] (1 mg/ml)
Galanthamine	48.80±0.36

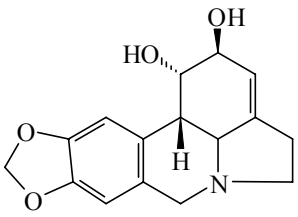
[a] Values were expressed as mean ± SEM (n=6)
p>0.05: - , p<0.05: $^{+}$, p<0.01: $^{++}$, p<0.001: $^{+++}$

Table 2. Anticholinesterase activity of the compounds isolated.

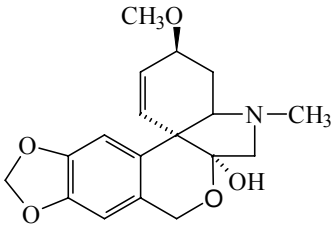
Compounds isolated	**Inhibition Rate (%)**[a] *(10 µg/ml)*
Lycorine	43.69±0.27
Tazettine	36.34±0.65
Crinine	26.53±0.66
Galanthamine	48.00±0.36
3-Epi-hydroxybulbispermine	30.18±0.26
2-Demethoxymontanine	31.84±0.29
N-nor-galanthamine	34.09±0.25
Haemanthamine	20.8 ±0.49
Galanthamine	48.80±0.36

[a] Values were expressed as mean ± SEM
p>0.05: - , p<0.05: $^{+}$, p<0.01: $^{++}$, p<0.001: $^{+++}$

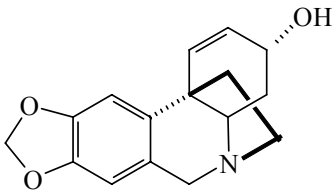
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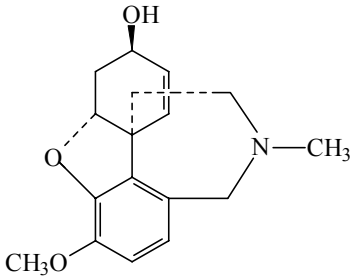
Lycorine



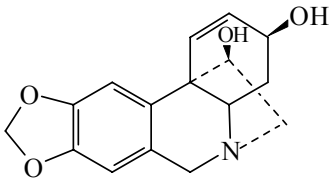
Tazettine



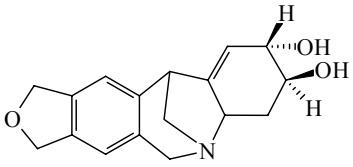
Crinine



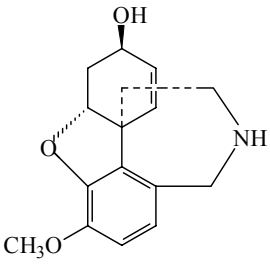
Galanthamine



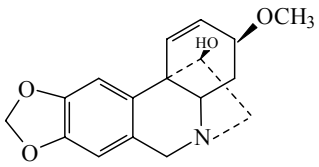
3-Epi-hydroxybulbispermine



2-Demethoxymontanine



N-nor-galanthamine



Haemanthamine

Fig. 1. Structures of the compounds isolated.