Chemistry of Natural Compounds, Vol. 39, No. 4, 2003

BIOACTIVITY-DIRECTED FRACTIONATION OF ALKALOIDS FROM SOME AMARYLLIDACEAE PLANTS AND THEIR ANTICHOLINESTERASE ACTIVITY

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UDC 547.944/94

Chloroform:methanol extracts of the bulbs of five Amaryllidaceae plants, namely Galanthus elwesii *Hooker fil.*, G. ikariae *L.*, Narcissus tazetta *subsp.* tazetta *L.*, Leucojum aestivum *L., and* Pancratium maritimum *L. growing in Turkey, were evaluated for their anticholinesterase activity by the 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 eight Amaryllidaceae-type alkaloids in total. We found that the activity of both plant extracts was due to the synergistic interaction of the alkaloids isolated.*

Key words: Alzheimer's disease, Amaryllidaceae, Ellman method, galanthamine.

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 [1–4]. A deficit of central presynaptic cholinergic function has been demonstrated in AD due to degeneration of cholinergic neurons in the basal forebrain [5–7]. This led to the suggestion that acetylcholinesterase inhibitors (AChEI) would preserve the putative deficit in acetylcholine levels associated with AD and thus might control memory impairments [8–10]. 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, donepezil and rivastigmine [11–16].

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.*

The bulbs of *G. elwesii* Hooker fil., *N. tazetta* subsp. *tazetta* L., and *P. maritimum* L. from Antalya-Akseki, Antalya-Kumluca, and Antalya-Belek located in the Mediterranean region of Turkey, respectively, and *G. ikariae* L. and *L. aestivum* L. from Trabzon-Sermene and Samsun-Terme in northeast Turkey, respectively, were collected and identified by Prof. Dr. B. Sener. Voucher specimens are deposited in the Herbarium of the Faculty of Pharmacy, Gazi University, Ankara, Turkey. Their anticholinesterase activity was evaluated by the *in vitro* Ellman method in comparison with galanthamine (Reminyl®), one of the newest drugs licensed in the U.S.A., some European countries, and Turkey for the treatment of AD.

In prescreening, chloroform – methanol (1:1) extracts prepared from the bulbs of *G. elwesii*, *G. ikariae*, *N. tazetta* subsp. *tazetta*, *L. aestivum*, and *P. maritimum* were found to cause 73.18% ($p < 0.001$), 75.56% ($p < 0.001$), 46.62%, 34.39%, and 30.42% inhibition, respectively, at 10 mg/ml concentration (Table 1). Since these species are quite rich in alkaloids, we thought that the anticholinesterase activity might be due to 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 mg/ml concentration. Both of the *Galanthus* species showed high inhibitory activity compare to galanthamine. *N. tazetta* subsp. *tazetta* extract also had almost equal activity to galanthamine.

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0009-3130/03/3904-0383$25.00

TABLE 1. Anticholinesterase Activity of the Chloroform-Methanol and Alkaloid Extracts of Five Amaryllidaceae Plants Determined by the Ellman Method

Species	Inhibition rate (%)[a]
Chloroform-methanol extracts	(10μg/ml)
Galanthus elwesii	73.18 ±0.01^{+++}
G. ikariae	75.56 ±0.99^{+++}
Narcissus tazetta subs. *tazetta*	46.62 ±0.77
Leucojum aestivum	34.39 ±0.72
Pancratium maritimum	30.42 ±0.85
Alkaloid extract	(1 μg/ml)
Galanthus elwesii	76.96 ±0.30^{+++}
G. ikariae	77.23 ±0.41^{+++}
Narcissus tazetta subs. *tazetta*	46.96 ±0.08
Leucojum aestivum	39.14 ±0.33
Pancratium maritimum	27.16 ±0.49
Standard	(1 μg/ml)
Galanthamine	48.80 ±0.36

a - Values 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-Epihydroxybulbispermine	30.18 ±0.26
2-Demethoxymontanine	31.84 ±0.29
N-Norgalanthamine	34.09 ±0.25
Haemanthamine	20.8 ±0.49
Galanthamine	48.80 ±0.36

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

Following prescreening, 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 identified as lycorine (IC_{50} = 3.16 μM), tazettine, crinine, galanthamine (IC_{50} = 3.2 μM), 3-epihydroxybulbispermine, and 2-demethoxymontanine isolated from *G. ikariae*, and lycorine, tazettine, N-norgalanthamine, haemanthamine, and 3-epihydroxybulbispermine isolated from *N. tazetta* subsp. *tazetta* (Fig. 1). These alkaloids were also screened for their anticholinesterase activity at 10 μg/ml concentration and the following inhibition rates were determined: lycorine 43.69%, tazettine 36.34%, crinine 26.53%, galanthamine 48.00%, 3-epihydroxybulbispermine 30.18%, 2-demethoxymontanine 31.84%, N-norgalanthamine 34.09%, and haemanthamine 20.8% (Table 2).

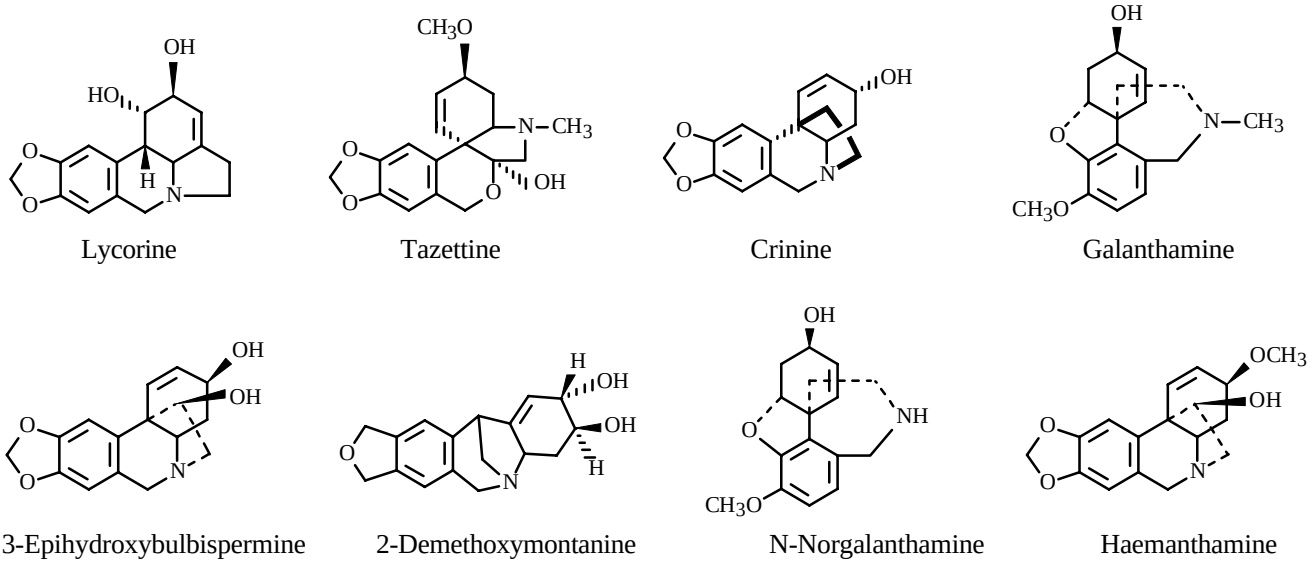

Fig. 1. Structures of the Compounds Isolated.

These results showed that a single alkaloid is not responsible for the anticholinesterase activity of *G. ikariae* and *N. tazetta* subsp. *tazetta* extracts. However, the activity may depend on the synergistic interaction between the alkaloids isolated.

EXPERIMENTAL

Air-dried bulbs of *Galanthus elwesii* Hooker fil., *G. ikariae* L., *Narcissus tazetta* subsp. *tazetta* L., *Leucojum aestivum* L., and *Pancratium maritimum* L. (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 vacuum at 40°C to dryness, a crude extract of each species was obtained. The anticholinesterase activity of the extracts was determined at 10 μg/ml concentration by the 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, and 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 vacuum until dryness.

Alkaloid extracts of *G. ikariae* and *N. tazetta* subsp. *tazetta* were used for bioactivity-directed fractionation and isolation. The alkaloid extract of *G. ikariae* was subjected to column chromatography (Si 60) and eluted with chloroform:methanol mixtures in increasing polarity. Fifty fractions (10 ml for each) were collected in total. By monitoring with TLC, similar fractions were combined and fife subfractions were obtained. The anticholinesterase activity of the subfractions was determined at 10 μg/ml concentration. The 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-epihydroxybulbispermine (0.074 g), and 2-demethoxymontanine (0.047 g) were isolated by preparative TLC. The 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-epihydroxybulbispermine (0.042 g), N-norgalanthamine (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 [17–24]. The anticholinesterase activity of the pure compounds were determined by the Ellman method at 10 μg/ml concentration.

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, Missouri). Sterileapyrogenic isotonic sodium chloride solution (0.9%) was obtained from Ibrahim Ethem. 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. Organic solvents and silica gel used in the isolation were from Merck Co.

Anticholinesterase activity was determined spectrophotometrically using acetylthiocholine as substrate by modifying the Ellman method [25, 26]. 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 quartz cuvettes (Starna, U.K., No.1 OG 5391). Absorbance reading was performed six times every 30 sec. All experiments were repeated three times and the results were analyzed by Student's *t*-test. Sterileapyrogenic isotonic sodium chloride solution (0.9%) was used as blank.

The concentration of compound required for 50% enzyme inhibition (IC_{50}) was calculated according to the Michaelis-Menten model by using the "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.

This study was supported by the Research Fund of Gazi University (Project code No. SBE, 11/2001-11).

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