Acetylcholinesterase inhibitory effects of the bulb of *Ammocharis coranica* (Amaryllidaceae) and its active constituent lycorine

I.L. Elisha a,b, E.E. Elgorashi a,1, A.A. Hussein c, G. Duncan d, J.N. Eloff a,*

a Phytomedicine Programme, Department of Paracutaneous Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa
b Drug Development Section, Biochemistry Division, National Veterinary Research Institute, Vom, Nigeria
c Department of Chemistry, University of Western Cape, Mowbray Road, Bellville 7535, South Africa
d Kirstenbosch National Botanical Garden, Private Bag X7, Claremont, Cape Town, South Africa

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A B S T R A C T

*Ammocharis coranica* (Ker-Gawl.) Herb. (Amaryllidaceae) is used in southern Africa for the treatment of mental illnesses. The ethanol extracts of the bulb of *A. coranica* and its total alkaloids rich fractions were screened for inhibition of acetylcholinesterase enzyme (AChE), which is implicated in the pathophysiology of Alzheimer’s disease. The ethanolic extracts significantly inhibited AChE with IC50 value of 14.3 ± 0.50 μg/ml. The basic ethyl acetate and butanol fractions of the crude extracts were the most active against AChE with IC50 values of 43.1 ± 1.22 and 0.05 ± 0.02 μg/ml respectively. Bioassay-guided fractionation of the basic fractions led to the isolation of lycorine and 24-methylenecycloartan-3β-ol. Lycorine which was isolated from both butanol and ethyl acetate fractions had IC50 of 29.3 ± 3.15 μg/ml, while 24-methylenecycloartan-3β-ol was not active.

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1. Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterised by dementia, behavioural abnormalities and death. It is the leading cause of dementia in elderly people and with the proportion of elderly people in the population increasing steadily, the burden of the disease, both to caregivers and national economies, is expected to become substantially greater over the next 2 to 3 decades (Francis et al., 1999; Maslow, 2008).

The brain regions associated with higher mental functions, such as the neocortex and hippocampus, are mostly affected by the characteristic pathology of AD (Cummings, 2004). These abnormal pathologic features include, extracellular deposits of β-amyloid derived from amyloid precursor protein (APP) in senile plaques, intracellular formation of neurofibrillary tangles containing hyperphosphorylated form of microtubule associated protein, tau and the loss of neuronal synapses and pyramidal neurons, as well as a decrease in levels of the neurotransmitter acetylcholine (ACh) by nearly 90% (Cummings, 2004). These changes result in the development of the typical symptoms of AD characterised by gross and progressive damage of cognitive function, often accompanied by behavioural disturbances such as memory loss, aggression, depression, and wandering. Caregivers find these features difficult to cope with, and often lead to the hospitalisation of the patient (Esiri, 1996).

The “cholinergic hypothesis of AD” was postulated on the basis of the negative cognitive effect of cholinergic antagonists and the positive effect of muscarinic agonists on memory in humans (Guillou et al., 2000), and became the neurobiological incentive for a treatment aiming at the improvement of cholinergic function in AD. Inhibitors of acetylcholinesterase (AChE), the enzyme involved in the metabolic hydrolysis of acetylcholine (ACh) at cholinergic synapses, promote increase in the concentration and duration of action of synaptic ACh (Rollinger et al., 2004), thus serving as treatment strategy for AD, senile dementia, ataxia, myasthenia gravis and Parkinson’s disease (Houghton et al., 2006). Acetylcholinesterase (AChE) inhibitors from general chemical classes such as galanthamine, an Amaryllidaceae alkaloid isolated from the plant *Galanthus woronowii* (Heinrich and Teoh, 2004) and other AChE inhibitors have been used for the symptomatic treatment of AD (Cummings, 2004). The non-selectivity of these drugs, their limited efficacy, and poor bioavailability, adverse cholinergic side effects in the periphery, such as nausea, vomiting, diarrhea, dizziness, and hepatotoxicity are among the several limitations to their therapeutic success (Burns and Iliffe, 2009) hence the continuous search for better AChE inhibitors from natural sources including plants (Hostettmann et al., 2006; Houghton et al., 2006).
2. Materials and methods

2.1. Plant material

The bulbs of *A. coranica* were collected from Bethelsdorp in the Eastern Cape region of South Africa and authenticated by Mr Graham Duncan. A voucher specimen, No. 39/69 was kept in the herbarium of the Kirstenbosch National Botanical Garden. The collected plant material was dried in an oven at 40 °C for several days and powdered before extraction.

2.2. Extraction and bioassay guided fractionation

The dried, powdered plant materials (210 g) were extracted using 96% ethanol (2 × 2 l) by shaking for 48 h. The supernatant was filtered using Whatman No. 1 filter paper and concentrated to dryness under reduced pressure to yield 16 g of crude extract. The crude extract was treated with 6% acetic acid (300 ml) and then basified with ammonia to pH 9.5, after removal of acidic and neutral material with dichloromethane (200 ml × 3). The basified solution was extracted with ethyl acetate and butanol (200 ml × 3) respectively and the organic phases evaporated to dryness.

The ethyl acetate fraction (2.08 g) was subjected to column chromatography (20 × 5 cm) on silica gel 60 (200 g, Merck), eluted with 100% dichloromethane enriched gradually with 10% methanol up to 100% methanol. Nine fractions (A–I) were collected based on UV absorption, Dragendorff’s test and AChE bioautographic assays. Fraction E (tubes 68–78) was dissolved in dichloromethane:methanol (1:1) and allowed to crystallise at room temperature to give compound 1. Sub-fraction C (tubes 19–35) was dissolved in dichloromethane: methanol (1:1). This was then applied on a preparative TLC plate and eluted in hexane:ethyl acetate (2:1). The target compound was scraped off the plate and the silica gel eluted with dichloromethane to give compound 2.

The butanol fraction was subjected to column chromatography (10 × 5 cm) on 100 g silica gel 60 (Merck) eluted with 100% dichloromethane and then with dichloromethane enriched gradually with 20% methanol up to 100% methanol. Fractions 7–11 were combined and allowed to crystallise and give more of compound 1.

2.3. Acetylcholinesterase enzyme inhibitory assay

2.3.1. Microtitre plate assay for acetylcholinesterase inhibition

Inhibition of acetylcholinesterase by the crude extract and, acid–base fractions and the isolated compounds was determined using the microplate assay (Rhee et al., 2001). Acetylcholinesterase (AChE) from electric eel (type VI-s, lyophilised powder), the substrate, Acetylthiocholine iodide (ACTI), Ellman’s reagent, 5.5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) and Physostigmine, the reference compound were purchased from Sigma (South Africa). The following buffers were used, Buffer A: 50 mM Tris–HCl, pH 8; Buffer B: 50 mM Tris–HCl, pH 8, containing 0.1% bovine serum albumin (BSA); buffer C: 50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M MgCl₂.6H₂O (ATCI) in buffer A and 15 mM in Millipore water were used for TLC bioautographic and microplate assay respectively. For the TLC bioautographic assay, 1 mM of DTNB in buffer A was used, while 3 mM DTNB was used for the microplate assay. In the 96 well plates, 25 μl of 15 mM ATCI in water, 125 μl DTNB (3 mM) in buffer C, 50 μl buffer B and 25 μl of serially diluted samples (two-fold for extracts, ten-fold for pure compounds and Physostigmine) were added and the absorbance was measured, in a microplate reader, at 405 nm every 30 s three times, at 30 °C. Finally, 25 μl of 0.2 U/ml of enzyme solution was added in and the absorbance was measured again after 3, 5, and 10 min respectively. The assays were conducted in triplicate. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the average absorbance before adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated by comparing the inhibition of the sample to the blank (10% methanol in buffer A). The inhibitory concentration of 50% of AChE (IC₅₀) value was calculated from at least four different concentrations of the sample using the Microsoft Excel statistical package.

2.3.2. Bioautographic assay for acetylcholinesterase inhibition

The TLC bioautographic assay was carried out as adopted from Rhee et al. (2001). The dried crude extract of the bulbs of *A. coranica* was dissolved in methanol (MeOH) to a concentration of 10 mg/ml, of which 3 μl was spotted on TLC plate (Silica gel 60 F₂₅₄, Merck) and developed using chloroform:methanol (4:1) as the eluent. Physostigmine (3 μl of 0.01 M) was used as a positive control. Thereafter, enzyme inhibiting activity of the samples was detected by spraying a mixture of the substrate (1 mM ATCI) and the dye (1 mM DTNB) until the silica was saturated with the solution. The plates were allowed to dry for 3–5 min, before 3 units/ml enzyme solutions were sprayed on the plates. A yellow background appeared, while white spots for inhibiting compounds became visible after 5 min. The TLC plates were scanned or photographed within 15 min before the reaction colour disappeared.

2.3.3. Test for false positive results in the bioautographic method

The false positive reactions were eliminated using a method developed by Rhee et al. (2003). The TLC plate was prepared as described above and sprayed with 1 mM DTNB in buffer A. The plate was allowed to dry (3–5 min) and then sprayed with thiocholine, the product formed when 1 mM ATCI in 3 units/ml enzyme stock solution was incubated at 37 °C for 20 min. The appearance of a yellow background or white spot was scanned or photographed and saved.

3. Results and discussions

Table 1 shows the IC₅₀ values for AChE inhibitory activity of the ethanol extracts of *A. coranica*, the different fractions obtained using acid–base fractionation and the isolated compounds there from. The ethanol crude extracts of *A. coranica* had an IC₅₀ value of 14.3 ± 0.50 μg/ml. However, butanol and ethyl acetate fractions had the highest AChE inhibitory effect among the fractions tested with IC₅₀ values of 0.05 ± 0.02 and 43.1 ± 1.22 μg/ml respectively, which had been chosen for the isolation of compounds with AChE inhibitory activity. A number of Amaryllidaceae species have also shown AChE inhibitory activity including Narcissus species (Coelho and Birks, 2001), *Crinum macowanii*,...

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**Table 1**

Acetylcholinesterase inhibitory activity of the ethanolic crude extracts, butanol, ethyl acetate fractions and lycorine isolated from the bulbs of *Ammocharis coranica*, expressed as IC₅₀.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (μg/ml) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol crude extract</td>
<td>143.4 ± 0.50</td>
</tr>
<tr>
<td>Butanol fraction</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>43.1 ± 1.22</td>
</tr>
<tr>
<td>Lycorine</td>
<td>29.3 ± 3.15</td>
</tr>
<tr>
<td>Physostigmine (reference compound)</td>
<td>1.21</td>
</tr>
</tbody>
</table>

*Expressed as mean ± standard error mean (SEM).*
Bioassay-guided fractionation of the butanol and ethyl acetate fractions led to the isolation of two compounds lycorine (Fig. 1) and 24-methylene cycloarten-3β-ol (Fig. 1). The 1H NMR (DMSO-δ6) spectra of lycorine revealed peaks of H-8, H-11 at 6.79, 6.66, signal of a methylenedioxy group (CH2-12) at 5.94, signals of H-1, H-2, and H-3 appear at 4.90, 4.26 and 5.35 respectively; in addition to the signals of three methylene groups of H-4, H-5, and H-7 between 4.00 and 2.20. The 13C NMR (DMSO-δ6) exhibited 16 carbons, four of them were methylene groups at 100.6, 56.7, 53.3 and 28.1 of C-7, C-5, C-4 and seven methane groups at 118.5, 105.1, 71.7, 70.2, 60.8 and 40.2 of C-3, C-8, C-11c and C-11b data are in agreement with previously published data for lycorine (Evidente et al., 1983; Schultz et al., 1996; Torizuka et al., 2008) and 24-methylene cycloarten-3β-ol (Koorbanally et al., 2000; Mesquita et al., 2008; Teresa et al., 1987). The NMR (CDCl3) data of 24-methylene cycloarten-3β-ol revealed in the 1H NMR spectra signals assigned to H-3 proton at 3.19, two proton singlet of CH2-28 at 4.69, 4.64, high field signals at 0.13, 0.36 of CH2-19, in addition to unresolved seven methyl group signals around 1.00 ppm. The 13C NMR data confirmed the presence of the terminal double bond at 157.3, 106.3, and the hydroxylated C-3 at 76.5. The remaining data could not be resolved due to the compactness of the spectra.

Lycorine is the most widely distributed alkaloid in the family Amaryllidaceae and has been isolated from almost every member of the family including A. coranica (Koorbanally et al., 2000). Lycorine also had a wide range of biological activities including antifungal (Evidente et al., 2004), antiviral (Leven et al., 1982), antimalarial (Campbell et al., 1998) activities and toxic effects both in vitro and in vivo (Likhitwitzayawud et al., 1993; Lin et al., 1995; Liu et al., 2007).

In our experiment the IC50 of lycorine was 29.4 ± 3.15 μM. Lycorine isolated from other Amaryllidaceae plants has also been tested for AChE inhibitory activity. Elgorashi et al. (2004) found that the IC50 of lycorine isolated from C. macowanii was 213 μM while Houghton et al. (2004) established an IC50 of 450 μM for lycorine isolated Crinum jagus and Crinum glaucum from south-western part of Nigeria. According to López et al. (2002) and McNulty et al. (2010) lycorine was not active, however, McNulty and co-workers tested the alkaloid at a maximum concentration of 10 μM which is not expected to exhibit any activity while information on the concentration used by López et al. (2002) is lacking. The discrepancies in the IC50 of lycorine are obvious and could be attributed to differences in the method and conditions adopted in the independent experiments, and the poor solubility of lycorine in most organic solvents including methanol. Although the calculated values of the three independent IC50 of lycorine varied, the most consistent deduction from the three experiments is that lycorine has appreciable inhibitory activity against AChE. In our experiment physostigmine (positive control) is about 68 times a stronger inhibitor of AChE than lycorine. The fact that lycorine is toxic (Ghosal et al., 1985; Lin et al., 1995) and has weak inhibitory effect on AChE (Elgorashi et al., 2004; Houghton et al., 2004) makes it a less suitable candidate for the development of an AD drug.

The second compound, 24-methylene cycloarten-3β-ol, has been isolated previously from A. coranica (Koorbanally et al., 2000). The compound did not exhibit acetylcholinesterase inhibitory activity. The negative values indicate that the compound had no inhibitory activity against AChE at the concentration used in the microplate assay, although it showed zone of inhibition when screened using the thin layer chromatography bioautographic assay. This is however, the first report of the test of this compound for AChE inhibitory activity. Lycorine is the only alkaloid isolated in this study from the most active butanol fraction of A. coranica. Previous studies, however, highlighted the presence of the alkaloids lycorine, caranine, acetylcaranine, crinamine, hippadine, 6-oxhydroxypowelline, 1-O-acetyl-9-O-demethylpluviine, buphandrine, ambelline, epivittatine and 1-O-acetyllycorine in extracts from A. coranica (Koorbanally et al., 2000; Mason et al., 1955). Given the low AChE inhibitory activity of lycorine, it is possible that the potency of the butanol fraction may have resulted from some these alkaloids, especially 1-O-acetyllycorine, one of the most potent AChE inhibitors.

In conclusion, the ethanolic crude extract of the bulbs of A. coranica like other members of the Amaryllidaceae family showed AChE inhibitory activity. The preliminary thin layer chromatography bioautographic assay investigation of the ethanolic crude extract of the bulbs of A. coranica showed several white spots of inhibition, confirmed not to be false positives. Lycorine is again established to inhibit acetylcholinesterase, while 24-methylene cycloarten-3β-ol is not active. The high acetylcholinesterase inhibitory activity of the butanol fraction could be attributed to the synergistic action of its constituents and more research should be carried out on this fraction due to the promising acetylcholinesterase inhibitory activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2012.11.008.

References


