Isolation, *in vitro* evaluation and molecular docking of acetylcholinesterase inhibitors from South African Amaryllidaceae

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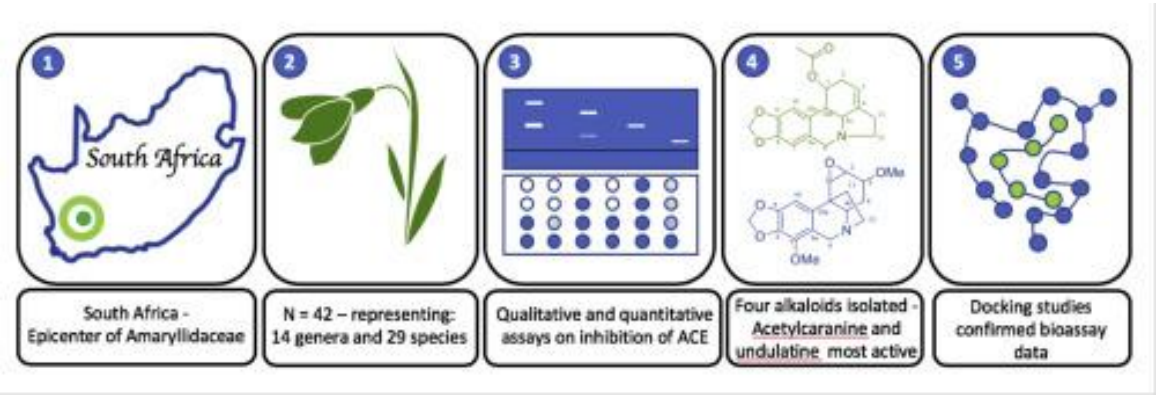
Abstract

Inhibition of acetylcholinesterase (AChE) is considered a promising strategy for the treatment of Alzheimer's disease (AD) and dementia. Members of the Amaryllidaceae family are well known for their pharmacologically active alkaloids, including galanthamine, which is used to treat AD. The aim of this study was to evaluate the potential of South African Amaryllidaceae species to inhibit AChE, to isolate the active compounds, and probe their ability to bind the enzyme using molecular docking. The AChE inhibitory activity of extracts of 41 samples, representing 14 genera and 28 species, as well as isolated compounds, were evaluated *in vitro* using a qualitative thin layer chromatography (TLC) bio-autography assay and Ellman's method in a quantitative 96-well microplate assay. Targeted isolation of compounds was achieved with the aid of preparative-high perfomance liquid chromatography-mass spectrometry. The structures of the isolates were elucidated using nuclear magnetic resonance spectrocopy, and were docked into the active site of AChE to rationalise their biological activities. The most active species were found to be *Amaryllis belladonna* L (IC_{50} 14.3 ± 2.6 μg/mL), *Nerine huttoniae* Schönland (IC_{50} 45.3 ± 0.4 μg/mL) and *Nerine undulata* (L.) Herb. (IC_{50} 52.8 ± 0.5 μg/mL), while TLC bio-autography indicated the presence of several active compounds in the methanol extracts. Four compounds, isolated from *A. belladonna*, were identified as belladine, undulatine, buphanidrine and acetylcaranine. Acetylcaranine and undulatine were previously isolated from *A. belladonna*, while belladine and buphanidrine were reported from other South African Amaryllidaceae species. Using Ellman's method, acetylcaranine was found to be the most active of the isolates towards AChE, with an IC_{50} of 11.7 ± 0.7 μM, comparable to that of galanthamine (IC_{50} = 6.19 ± 2.60 μM). Molecular docking successfully predicted the binding modes of ligands within receptor binding sites. Acetylcaranine was predicted by the docking workflow to have the highest activity, which corresponds to the *in vitro* results. Both qualitative and quantitative assays indicate that several South African Amaryllidaceae species are notable AChE inhibitors.

Graphical abstract



Keywords: Amaryllidaceae; Acetylcholinesterase inhibition; Alzheimer's disease; Galanthamine; Molecular docking

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AR	Analytical reagent
ATCI	Acetylthiocholine
BPI	Base peak ion
DTNB	*5,5*-Dithio-*bis*(2-nitrobenzoic acid)
USFDA	United States Food and Drug Administration
HPTLC	High performance thin layer chromatography
IC_{50}	Half maximum inhibitory concentration
i.d.	Internal diameter
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PDA	Photodiode array
PDB	Protein data bank
Prep-HPLC-MS	Preparative-high performance liquid chromatography-mass spectrometry
qToF-MS	Quadrupole time-of-flight-mass spectrometry
TLC	Thin layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
UPLC	Ultra performance liquid chromatography
UPLC-MS	Ultra performance liquid chromatography-mass spectrometry

1. Introduction

Alzheimer's disease (AD) is characterised by the degeneration of memory and cognitive function, caused by a loss of cholinergic neurons in the brain. The risk of developing the disorder increases with ageing [1]. Treatments that slow down the decline in cognitive

function are desirable, since the nature of AD is progressive [2]. The disease is associated with decreased levels of acetylcholine (ACh) in the synaptic area of the brain [3]. The principle role of the acetylcholinesterase (AChE) enzyme is to terminate the nerve impulse transmission at the cholinergic synapses, through the rapid hydrolysis of ACh [4]. Inhibition of AChE is considered a promising strategy for the treatment of neurological disorders, since it is key to the breakdown of ACh [5]. To date, the cure for AD remains unidentified. However, a few drugs have been developed that alleviate disease symptoms and slow down disease progression, thereby improving the quality of life of the affected individuals [4]. Donepezil, galanthamine and rivastigmine are some of the treatment drugs approved by the United States Food and Drug Administration (USFDA) [6]. The search for new lead compounds for the treatment of AD has been ongoing and many researchers have focussed on plants as sources of potential drug candidates.

South Africa harbours about a third of the global complement of 1000 Amaryllidaceae species [7]. Several species in the family are used as traditional medicine in the southern African region. For example, *Clivia miniata* (Lindl.) Bosse is used by the Zulu community to treat fever, pain and snakebite [8], and bulbs of *Crinum macowanii* Baker are used as a remedy for kidney and bladder infections, boils, acne and swelling, backache and venereal diseases [9]. A large number of secondary metabolites, including phenolic compounds and alkaloids, are produced by members of this family. *Galanthus nivalis* L., known as "snowdrop" or "common snowdrop", is widely used traditionally in Bulgaria and Turkey for the treatment of neurological disorders. Research on snowdrop led to the isolation of the bioactive compound galanthamine, which displays potent AChE inhibitory activity [10]. Galanthamine is produced by several species of Amaryllidaceae, including *Galanthus woronowii* Losinsk, *Galanthus nivalis* L. and *Narcissus* species [11,12]. The alkaloid provides complete oral bioavailability [4] and acts as a potent and selective inhibitor of AChE [13]. The commercialisation of galanthamine as a drug to mitigate symptoms associated with AD, led to a greater recognition of the Amaryllidaceae as a viable platform for chemotaxonomy-based drug discovery. The aim of this study was to screen and evaluate the potential of several South African Amaryllidaceae species, (Table 1) in search of alkaloids with AChE inhibitory activity, to isolate and identify active compounds, and to explore the enzyme-compound interactions using molecular docking experiments.

2. Experimental

2.1. Chemicals, reagents and botanical material

Acetylthiocholine iodide (ATCI), 5,5-dithio-*bis*-(2-nitrobenzoic acid) (DTNB, known as Ellman's reagent), *p*-anisaldehyde and galanthamine (94% purity) were purchased from Sigma (St. Louis, MO, USA). Solvents (dichloromethane, ethanol, ethyl acetate and methanol) and HPTLC plates (silica gel 60 F_{254}) were purchased from Merck SA (Pty) Ltd. (Johannesburg, AR grade). The following buffers and reagents were used for the AChE inhibition assay. Buffer A: 50 mM of Tris-HCl, pH 7.9, Buffer B: 50 mM of Tris-HCl containing 0.1 M NaCl and 0.02 M $MgCl_2$, pH 7.9. Acetylcholinesterase from electric eel (Type VI-S, lyophilized powder, 518 U/mg solid, 844 U/mg protein) was obtained from Sigma (St. Louis, MO, USA). The lyophilized enzyme was dissolved in Buffer A to a concentration of 6.7 U/mL enzyme stock solution, which was used to prepare 0.22 U/mL solution for the quantitative microplate assay. The enzyme stock solution was stored in a freezer. 1-Naphthyl acetate (98% purity) and Fast Blue salt (95% pure) were obtained from Sigma-Aldrich.

Table 1. Amaryllidaceae samples used with the corresponding voucher numbers and percentage inhibition of AChE by the crude methanol extracts (1 mg/mL) as determined in the microplate assay.

Species	Voucher number	% Inhibition	Species	Voucher number	% Inhibition	Species	Voucher number	% Inhibition	Species	Voucher number	% Inhibition
Amaryllis belladonna L.	A9	85.5	*Eucomis regia* (L.) Aiton	ERO1	62.1	*Haemanthus humilis* Jacq.	HHO4	27.3	*Nerine undulata* (L.) Herb	NUO1	70.1
Boophone disticha (L.f.) Herb	BDO1	37.2	*Eucomis comosa* (Houtt.) Wehrh.	ECO1	7.51	*Haemanthus lanceifolius* Jacq.	A6	34.4	*Nerine undulata* (L.) Herb	NUO2	62.6
Boophone disticha (L.f.) Herb	BDO2	50.5	*Haemanthus albiflos* Jacq.	HAO1	49.2	*Haemanthus montanus* Baker	HMO1	70.2	*Nerine undulata* (L.) Herb	NUO3	70.5
Boophone haemanthoides F.M.Leight	A1	51.9	*Haemanthus albiflos* Jacq.	HAO3	70.1	*Haemanthus nortieri* Isaac	A2	33.5	*Scadoxus puniceus* (L.) Friis & Nordal	SPO1	60.6
Brunsvigia grandiflora Lindl.	BGO1	12.1	*Haemanthus amarylloides* Jacq.	A5	65.2	*Haemanthus pauculifolius* Snijman & A.E.van Wyk	HPO1	13.4	*Scadoxus puniceus* (L.) Friis & Nordal	SPO2	61.6
Brunsvigia herrei Leight. ex W.F.Barker	BHO1	22.2	*Haemanthus barkerae* Snijman	A3	53.7	*Haemanthus pubescens* subsp. *pubescens* L.f.	A8	46.7	*Tulbaghia cernua* Fisch., C.A.Mey. & Avé-Lall.	TCEO1	39.5
Brunsvigia marginata (Jacq.) W.T.Aiton	A7	70.2	*Haemanthus carneus* Ker Gawl.	HCAO1	27.3	*Haemanthus sanguineus* Jacq.	HSO1	25.6	*Tulbaghia ludvigiana* Harv.	TLO1	26.3
Brunsvigia orientalis (L). Aiton ex Eckel.	BOO1	41.2	*Haemanthus coccineus* L.	HCOO1	33.7	*Nerine filamantosa* W.F.Barker	NFO1	65.8			
Crossyne flava (W.F.Barker ex Snijman) D.Müll.-Doblies & U. Müll.-Doblies	A4	32.8	*Haemanthus humilis* Jacq.	HHO1	52.5	*Nerine humilis* (Jacq.) Herb	NHO1	32.1	*Watsonia pillansii* L.Bolus	WPO1	12.1

Species	Voucher number	% Inhibition	Species	Voucher number	% Inhibition	Species	Voucher number	% Inhibition	Species	Voucher number	% Inhibition
Crossyne guttata D.Müll.-Doblies & U. Müll.-Doblies	CGO1	64.5	*Haemanthus humilis* Jacq.	HHO2	53.6	*Nerine humilis* (Jacq.) Herb	NHO2	25.4			
Dierama pulcherrimum (Hook.f.) Baker	DPO1	6.20	*Haemanthus humilis* Jacq.	HHO3	34.6	*Nerine huttoniae* Schönland	NHUO1	81.2			

Amaryllidaceae bulbs (n = 41), representing 14 genera and 28 species (Table 1), were supplied by African Bulbs (South Africa). Where possible, more than one sample of a species was analysed to investigate differences in activity. For example, for *Haemanthus humilis* Jacq. four bulb samples were investigated. Voucher specimens have been retained in the Department of Pharmaceutical Sciences, Faculty of Science, Tshwane University of Technology, Pretoria, South Africa. The bulbs were air-dried at room temperature and powdered using a coffee grinder (Russell Hobbs®).

2.2. Extraction of powdered bulbs

A 1.0 g portion of each powdered sample was extracted with 10.0 mL methanol for 30 min using ultrasonication. The extract was filtered using Whatman No. 1 filter paper and the residue was returned to the flask. The procedure was repeated twice, and the resulting filtrates were combined and dried under reduced pressure using a LabTech Model H50–500 rotary evaporator (LabTech, Inc., Massachusetts, USA) at 45 °C. After determining the mass of each solid obtained, it was dissolved in methanol to yield a final extract concentration of 10 mg/mL.

2.3. High performance thin layer chromatography (HPTLC)

A semi-automated CAMAG high performance thin layer chromatography (HPTLC) system (Muttenz, Switzerland), consisting of an automatic TLC sampler (ATS4), automated developing chamber (ADC2) and TLC scanner 3, was used to separate the extract constituents. The methanolic sample extracts (10 mg/mL) were applied to the HPTLC plates as 8 mm bands (50 µL). Galanthamine (1 mg/mL) was used as the reference standard. Ethyl acetate:methanol:water (100:20:15 *v/v/v*) was selected as the developing solvent after evaluating the separation achieved when using various volume ratios. After development of the plates in the twin-trough chamber of the ADC2 unit, the plates were dried on a plate heater at 100 °C. The plates were subsequently viewed under 254 nm and 366 nm radiation using the Reprostar 3 documentation device. CAMAG winCats™ 1.4.1 planar chromatography software manager was used to operate the instrument. After digital images were captured, *p*-anisaldehyde was applied as the derivatising agent. The plates were then heated as before and viewed under white light, as well as under 254 nm and 366 nm radiation.

2.4. Acetylcholinesterase inhibition bioassays

2.4.1. HPTLC bio-autography assay

The screening for acetylcholinesterase inhibition was carried out as previously described [14,15]. An aliquot (50 µL) of each extract (10 mg/mL) was applied to TLC plates, prepared in duplicate, as described. Galanthamine served as the positive control (1.0 mg/mL). After development, they were air-dried and one plate was sprayed with AChE enzyme solution (6.7 U/mL in Buffer A) until the plate was saturated. For detection of enzyme activity, the plate was sprayed with a mixture of 5 mL 1-naphthyl acetate (13.4 mM) in ethanol and 20 mL of Fast Blue salt (7.4 mM) in distilled water. White zones against a purple background indicate compounds that inhibit AChE [14]. The second TLC plate was derivatised using *p*-anisaldehyde as described, and served as the reference plate for the isolation process.

2.4.2. Microplate assay

The inhibitory activity of the extracts towards AChE was determined using a quantitative colorimetric method based on Ellman's method, with some modifications [16,17]. A 25 µL aliquot of 15 mM ATCI in water, 125 µL of 3 mM DTNB in Buffer B, 50 µL of Buffer A, and 25 µL of test sample (1.0 mg/mL in 10% aqueous methanol) were mixed in each well of a 96-well microplate (Lasec Group, South Africa), in triplicate. The absorbance of each well was measured at 405 nm, five times every 15 s, using a Spectramax190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Acetylcholinesterase (25 µL of 0.22 U/mL solution in Buffer A) was subsequently added to each well and after mixing, the plate was incubated at room temperature for 10 min. The absorbance was measured again eight times at 15 s intervals. The percentage inhibition was calculated by comparing the inhibition of the sample to that of the blank (10% aqueous methanol). To avoid an increase in absorbance arising from the hydrolysis of the substrate, the initial absorbance was subtracted from the absorbance after adding the enzyme. To determine the IC_{50} values, serial dilutions of each test substance was done by transferring appropriate volumes of the mixture to yield final test solutions with concentrations ranging from 200 µg/mL to 12.5 µg/mL. All the assays were repeated twice on different occasions.

Data were analysed using Excel for the determination of the mean and standard deviation (SD). The IC_{50} for each test substance was calculated after constructing a Prism sigmoidal dose response curve (variable slope) by plotting the percentage enzyme inhibition as a function of the concentration of the test substance [18].

2.5. Analysis of the most active extracts

2.5.1. Alkaloid extraction

Alkaloids were extracted from the powdered bulb material of *Amaryllis belladonna*, the most active extract, using an acid/base extraction protocol [19]. A 2.5 g mass of each sample was extracted with 30 mL 1.0 M H_2SO_4 by maceration and frequent agitation. After allowing the mixture to stand for 15 min, it was filtered into a separating funnel. The filtrate was basified using 20% (*v/v*) ammonia and the alkaloids subsequently partitioned into dichloromethane. This procedure was repeated twice using fresh portions of dichloromethane. The combined organic layers were filtered and concentrated using a rotary evaporator.

2.5.2. Ultra performance liquid chromatography-mass spectrometry/photo diode array analysis

The acid-base extracts (1.0 mg/mL in methanol) were analysed using a Waters Acquity Ultra Performance Liquid Chromatography system) equipped with a photodiode array detector and quadrupole time-of-flight (qToF) mass spectrometer (Waters, Milford, MA, USA). An injection volume of 1.0 µL (full-loop injection) was applied. Separation was achieved on an Acquity UPLC BEH C_{18} column (2.1 × 150 mm, i.d., 1.7 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% ammonium hydroxide (Solvent A) and 90% ultragradient acetonitrile (Romil, USA) (Solvent B), at a flow rate of 0.35 mL/min. Gradient elution was carried out as follows: initially 10% Solvent B, changed to 20% B within 2 min, then increased to 65% B, within 7 min, and finally increased to 90% B within 2 min, holding for 1 min before returning to the initial ratio within 0.5 min. The system was equilibrated for 1.5 min between analyses, bringing the total run time to 14 min.

Both the positive and negative ionisation modes were applied, but the positive mode yielded higher intensity peaks and was used for all further analyses. Nitrogen was used as the desolvation gas at a flow rate of 600 L/h. The desolvation and source temperatures were set to 450 °C and 100 °C, respectively, with a capillary voltage of 3500 V and a cone voltage of 45 V. Data were collected over the range *m/z* 100 to 1500 and processed by Masslynx 4.1 chromatographic software.

2.6. Isolation and structure determination of alkaloids

2.6.1. Preparative HPLC-MS

Target compounds, identified through their ions observed on the UPLC-MS chromatograms, were isolated from the acid/base extract of *A. belladonna* using preparative high performance liquid chromatography-mass spectrometry (prep HPLC-MS). A Waters AutoPurification system equipped with a Waters photodiode array (PDA) detector (Model 2998) and a QDa mass spectrometer (Waters, Milford, MA, USA) was used for targeted fractionation. The chromatographic conditions were optimised for maximum resolution of the target compounds using the UPLC conditions as a starting point and an injection volume of 300 µL was used. Separation was achieved on an XBridge Prep C_{18} column (250 mm × 19 mm i.d., 5 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% ammonium hydroxide (Solvent A) and acetonitrile (Solvent B) at a flow rate of 20 mL/min. Gradient elution was applied as follows: The initial ratio was 10% B, held for 1 min, changed to 20% B within 2 min, increased to 65% B within 9 min, and then to 90% B within 1 min, maintaining for 0.5 min, before returning to the initial ratio within 0.5 min over a total run time of 14 min. Data were collected by chromatographic software MassLynx 4.1 (Waters, USA). The positive ionisation mode was selected. The probe temperature was set at 600 °C, while the source temperature was maintained at 120 °C. The capillary and cone voltages were set to 800 and 10 V, respectively. Data were collected between *m/z* 100 and 700. Fractions were collected in test tubes and those containing the target compounds were selectively combined and dried in a Genevac centrifugal evaporator (EZ-2 Series). The dried fractions were dissolved in methanol and analysed by UPLC-PDA/MS as described, to monitor the purity of the four isolated compounds,

2.6.2. NMR analysis and determination of accurate mass

Spectra of the four isolated compounds, dissolved in methanol-d_4, were recorded on a Bruker 600 Avance II NMR at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR. The solvent signals were used for calibration. Accurate mass information was obtained using the UPLC-QToF-MS. The spectrometer was calibrated using leucine enkephalin as the LockSpray reference (*m/z* 554.2771).

2.7. Evaluation of AChE inhibitory activity of isolated compounds

The AChE inhibitory activities of the isolated compounds were determined using the quantitative microplate assay as described in Section 2.4.2. Galanthamine served as the positive control.

2.8. Molecular docking

The AutoDock Vina 1.1 docking program implemented in LigandScout 4.09 was used with standard settings. The Protein Data Bank (PDB) code 1qti was used for docking [20]. The validation of the docking programme and settings was performed by redocking of the co-crystallized PDB ligand galanthamine (PDB entry 1qti).

3. Results and discussion

3.1. High performance thin layer chromatography bio-autographic assay of crude extracts

Methanol extracts were prepared from the bulbs of the Amaryllidaceae samples to ensure the extraction of the alkaloids, despite the use of decoctions in traditional medicine [21]. The HPTLC separations were optimised to achieve good resolution prior to bio-autography, so that individual compounds with AChE activity could be identified on the plates. Not all of the extracts contained strong chromophores (evident in Fig. S1 in the supplementary material) thus *p*-anisaldehyde, an excellent multipurpose spray reagent that reacts with most functional groups, was applied [22]. Once acceptable separation was achieved, two identical plates were developed in tandem. Derivatisation reagent was applied to one to serve as a reference. After the application of the enzyme to the second freshly developed plate, compounds with AChE inhibitory activity became visible as white zones against a purple background. The extracts of *Brunsvigia marginata*, *Amaryllis belladonna*, *Nerine huttoniae*, *Haemanthus montanus*, *Haemanthus albiflos* and *Nerine undulata* each contained several compounds with AChE inhibitory activity (Fig. S2 in the supplementary material).

3.2. Quantitative microplate assay to determine AChE inhibitory activity of the extracts

The screening of extracts was conducted at the somewhat high concentration of 1.0 g/mL Seventeen (40%) of the samples exhibited >50% inhibition of the enzyme. The crude extracts of *H. montanus*, *B. marginata*, *N. undulata*, *N. huttoniae* and *A. belladonna* were the most active and inhibited AChE by 70.1%, 70.2%, 70.5%, 81.2% and 85.5%, respectively (Table 1). However, differences in inhibition were noted for different samples from the same species. For example, the percentage inhibition achieved for the four extracts of *N. undulata* ranged from 62.6–70.5%, while the two samples of *Haemanthus albiflos* yielded an inhibition of 49.2 and 70.1%, respectively. Extracts of *Eucomis cumosa* and *Dierama pulchemium* were unable to inhibit the enzyme.

The corresponding IC_{50} values for the crude extracts and galanthamine are provided in Table 2. *Amaryllis belladonna* was the most active plant extract of all the samples that were screened, with an IC_{50} value of 14.3 ± 2.6 μg/mL. This value compared favourably with that of galanthamine (1.78 ± 0.75 μg/mL), considering that the crude extract represented a mixture of many compounds. The AChE inhibitory activity of *A. belladonna* has not yet been reported. *Nerine huttoniae* and *N. undulata* were moderately active with similar IC_{50}'s of 45.3 ± 0.4 and 52.8 ± 0.5 μg/mL, respectively. An IC_{50} value of 14.3 μg/mL towards AChE was reported by Murray et al. [23] for *N. undulata*, which is substantially better. The lower activity obtained in the current study may be attributed to chemical differences between the plant samples, since factors such as the origin and genetic traits greatly influence the phytochemical profiles of plants [24]. The IC_{50} obtained for galanthamine (6.19 μM) was

marginally higher than the IC_{50} of 4.0 μM reported by Pejchal et al. [25] and van Rijn et al. [26] and the IC_{50} of 2.9 ± 0.29 μM reported by Bay-Smidt et al. [27].

Table 2. Acetylcholinesterase inhibition expressed as IC_{50} of the most active crude extracts compared to galanthamine, a well-known acetylcholinesterase inhibitor.

Sample name	IC_{50} (μg/mL)	Sample name	IC_{50} (μg/mL)
Amaryllis belladonna	14.3 ± 2.6	*Haemanthus montanus*	164 ± 32
Nerine huttoniae	45.3 ± 0.4	*Haemanthus albiflos*	318 ± 16
Nerine undulata	52.8 ± 0.5	Galanthamine	1.78 ± 0.75
Brunsvigia marginata	101 ± 4		(6.19 ± 2.60 μM)

3.3. Isolation of plant alkaloids

Four compounds (*m/z* = 332, *m/z* = 316, *m/z* = 314 and *m/z* = 316) were isolated from the acid/base extract of *A. belladonna*, the most active plant, using prep HPLC-MS. The acid/base extract was prepared to enrich the alkaloid fraction for easier isolation. Target compounds were selected based mainly on the largest peaks present in the UPLC-MS chromatogram of the extract. The purities of the isolated compounds were determined by both UPLC-MS (Fig. 1) and UPLC-PDA analysis. The UPLC-MS values are reported (Table 3), since they were in all cases poorer than the UPLC-PDA values.

Table 3. Relative retention time, amount isolated, purity and mass-to-charge (*m/z*) ratios for each compound isolated from *A. belladonna* as determined by UPLC-PDA analysis.

Compound	Retention time (min)	Amount isolated (mg)	Mass to charge ratio $[M-H]^+$	% Purity UPLC-MS[a]
1	6.62	6.2	332.15	90.1
2	11.01	6.6	315.15	95.6
3	8.19	8.6	316.16	82.9
4	8.75	6.1	314.14	84.0

[a] Purity determined by UPLC-PDA were in all cases higher.

3.4. Structural elucidation of isolated compounds

Compound [1] was identified as undulatine (Fig. 2), characterised by a molecular mass of 332.18 $[M + H]^+$, corresponding to a molecular formula of $C_{18}H_{21}NO_5$. The ^1H and ^{13}C NMR data of compound [1] (Table S1) corresponds to literature data [28]. Compound [2] was found to have a molecular mass of 316.18 $[M + H]^+$, corresponding to the molecular formula $C_{18}H_{21}NO_4$ and was identified as buphanidrine (Fig. 2). The ^1H and ^{13}C NMR data for compound [2] were in agreement with the reported data (Table S2). Both undulatine and buphanidrine were previously isolated and identified from *Brunsvigia josephinae* (Delile) Ker Gawl. [28]. Compound [3], characterised by a molecular mass of 316.18 $[M + H]^+$, was found to correspond to a molecular formula of $C_{19}H_{25}NO_3$. The ^1H and ^{13}C NMR data confirmed the identity of [3] as belladine (Fig. 2). The ^1H and ^{13}C NMR data (Table S3) are in agreement with Nair et al. [29], who isolated the compound from *Nerine filofolia* Baker. Compound [4], with molecular mass 313.13 $[M + H]^+$, agreed to a molecular formula of $C_{18}H_{19}NO_4$, and was identified as acetylcaranine (Fig. 2). The ^1H and ^{13}C NMR data of compound [4] (Table S4) correspond to those reported by Pettit et al. [30], who isolated the compound from *A.*

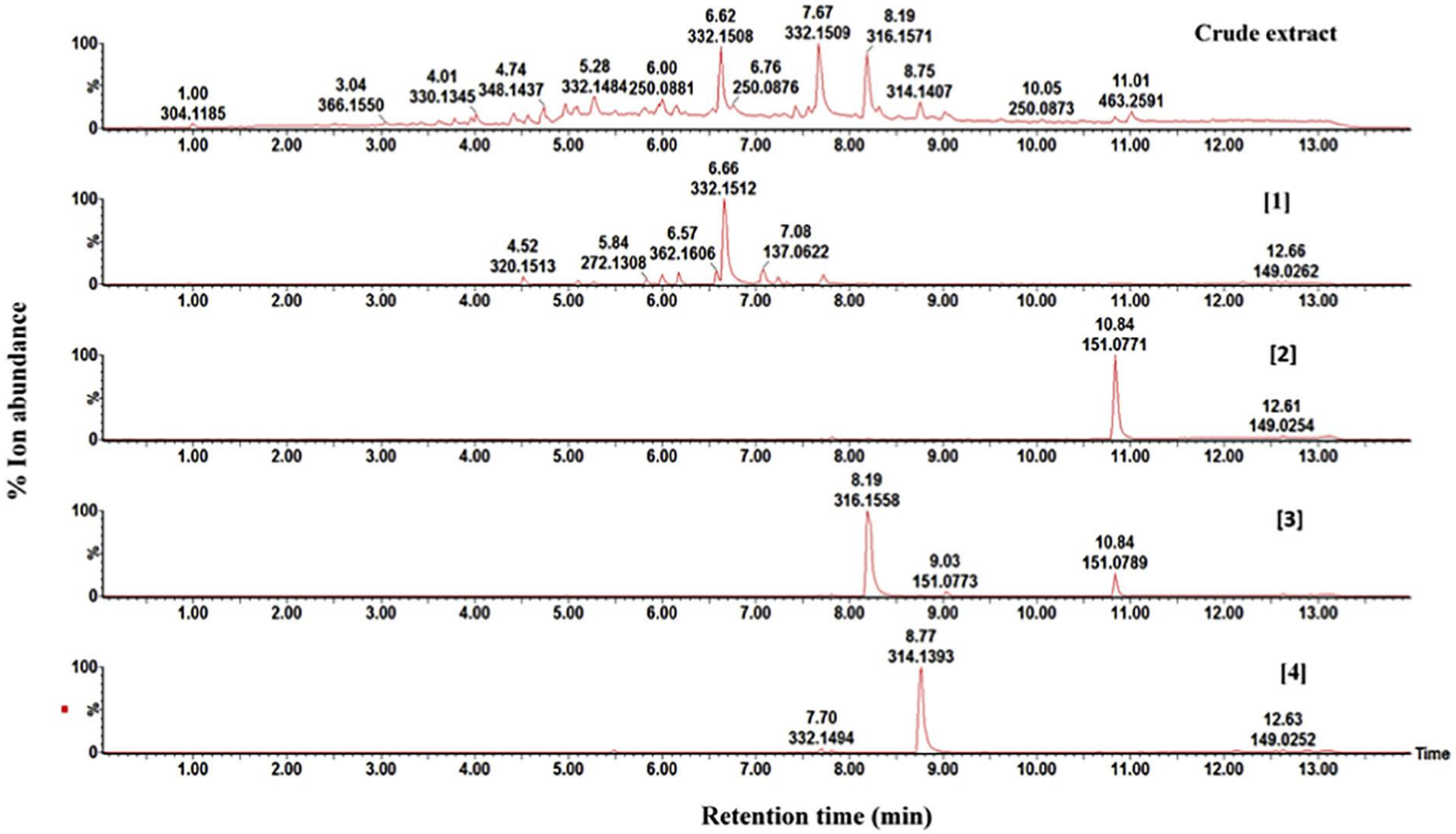


Fig. 1. UPLC-qToF-MS chromatograms of the crude acid/base extract (upper) and those of compounds [[1], [2], [3], [4]] $[M-H]^+$ isolated from A*maryllis belladonna*. (Compound [2] $[M-H]^+$ 315.15 has an abundant daughter ion at $[M-H]^+$ 151.07).

belladonna. Acetylcaranine was also isolated from the bulbs of *Ammocharis coranica* (Ker Gawl.) Herb., a member of the Amaryllidaceae family [31].

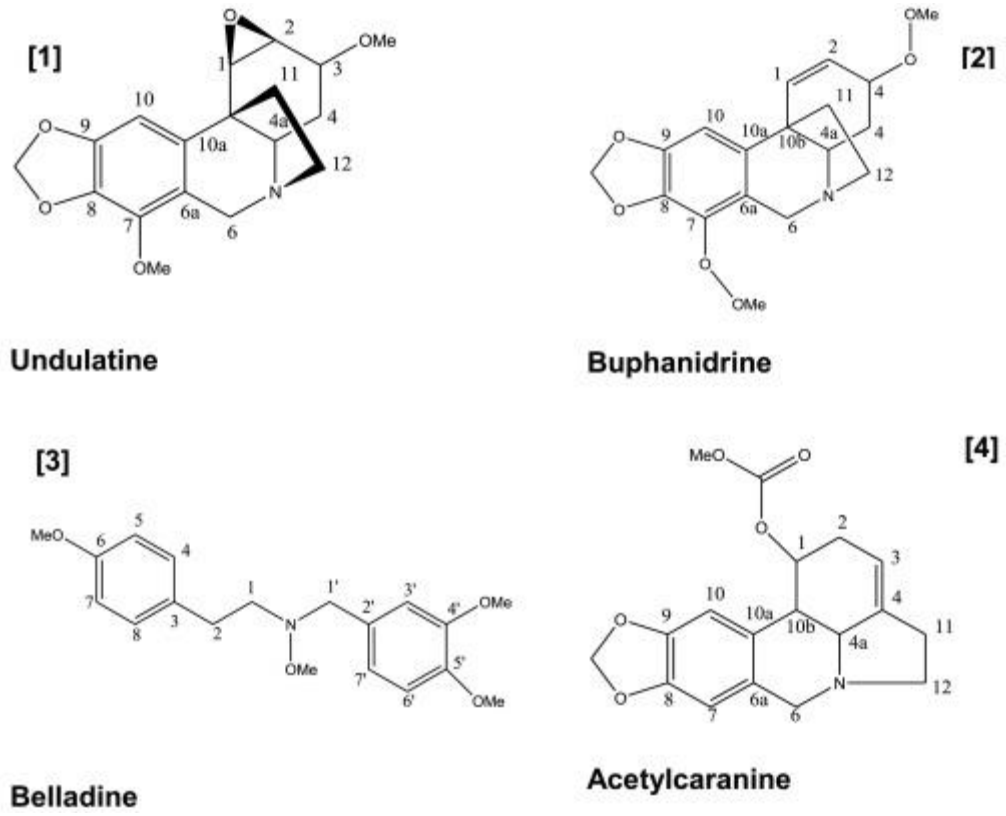


Fig. 2. Structures of the isolated compounds [1] to [4] from *A. belladonna*.

3.5. AChE inhibitory activity of isolated compounds

White bands on the purple background were indicative of AChE inhibitory activity when the four isolated compounds were analysed using TLC bio-autography (Fig. S3 in the supplementary material). This confirmed that active compounds had been targeted from the crude extract. The activity was confirmed by the IC_{50} values determined using the microplate assay as summarised in Table 4. Acetylcarinine was the most active (IC_{50} 11.7 μM) of the isolated compounds, while belladine was the least active (IC_{50} 135 μM). The activity of acetylcarinine compared favourably with that of galanthamine (IC_{50} 6.19 μM), a compound used in the treatment of AD. Cahlikova et al. [32] reported an IC_{50} of 7.4 μM for undulatine, which is considerably lower than the value obtained in this study (IC_{50} 33.9 μM).

Table 4. The IC_{50} (expressed as mean ± SD) towards AChE and calculated binding affinities of four compounds isolated from the crude extract of *Amaryllis belladonna.*

Compound	IC_{50} μM	Calculated binding affinity (kcal/mol)
Acetylcaranine	11.7 ± 0.7	−10.40
Undulatine	33.9 ± 0.4	−9.60
Buphanidrine	52.8 ± 0.4	−10.20
Belladine	135 ± 4	−8.10
Galanthamine	6.19 ± 2.60	−10.60

3.6. Molecular docking

Molecular docking is used to investigate ligand-enzyme bindings and conformations using a docking scoring function [33]. Although molecular docking studies have been carried out to determine the binding affinities of alkaloids to AChE [34], the compounds isolated in this study have not yet been docked into the active site of AChE. For this reason, the molecular architecture was retrieved from the protein data bank (PDB, code: 1qti) to explore the activities of the isolated compounds and binding interactions between ligands and the enzyme. As positive control, the well-known and co-crystallized AChE inhibitor galanthamine was used. This compound establishes interactions with AChE as indicated in Fig. 3.

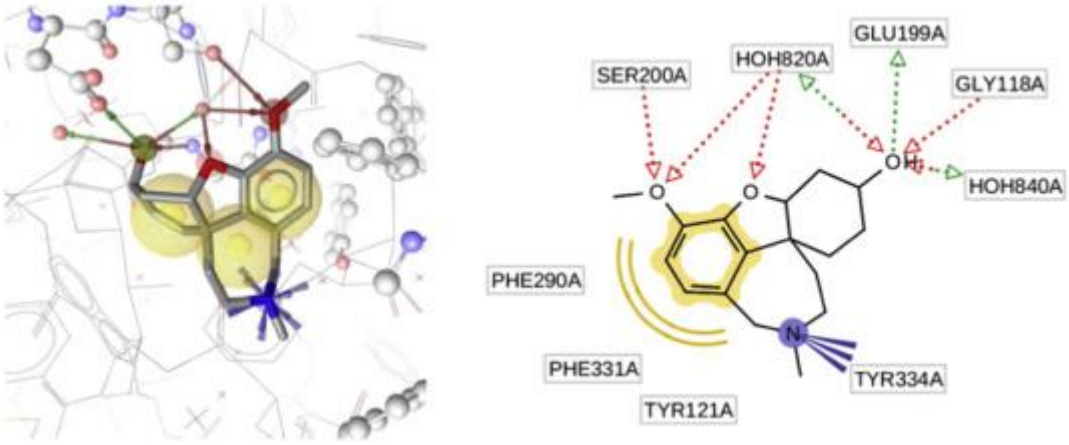


Fig. 3. Structure containing galanthamine in the binding site. Colour coding was applied to the structures by assigning the following colours to the various chemical interaction types: yellow indicating hydrophobic contact, red arrow reflecting the presence of a hydrogen bond acceptor, green arrow indicating a hydrogen bond donor and a blue star for a positively ionisable group.

The binding modes for the four compounds as identified by molecular docking, were ranked according to the information obtained using different scoring functions. Validation of the docking program and settings was achieved by redocking of the co-crystallized PDB ligand galanthamine (PDB entry 1qti). With an RMSD of <1 Å, the workflow for binding mode prediction was validated successfully. Galanthamine yielded the best docking score of all the docked compounds (Table 4).

Acetylcaranine was found to occupy part of the same ligand binding pocket area as galanthamine. However, the very rigid structure of acetylcaranine forced it to extend into an adjacent part of the active site. The best ranked position for the compound is indicated in Fig. 4A. After galanthamine, the compound yielded the second best score of the docked compounds (Table 4). This finding corresponds to the *in vitro* inhibitory activity, since the best IC_{50} value for AChE inhibition was obtained for acetylcaranine, followed by undulatine. For acetylcaranine, many typical protein contacts with AChE can be observed (Fig. 4B). Extensive interactions with the aromatic cage amino acids were established (Fig. 4C).

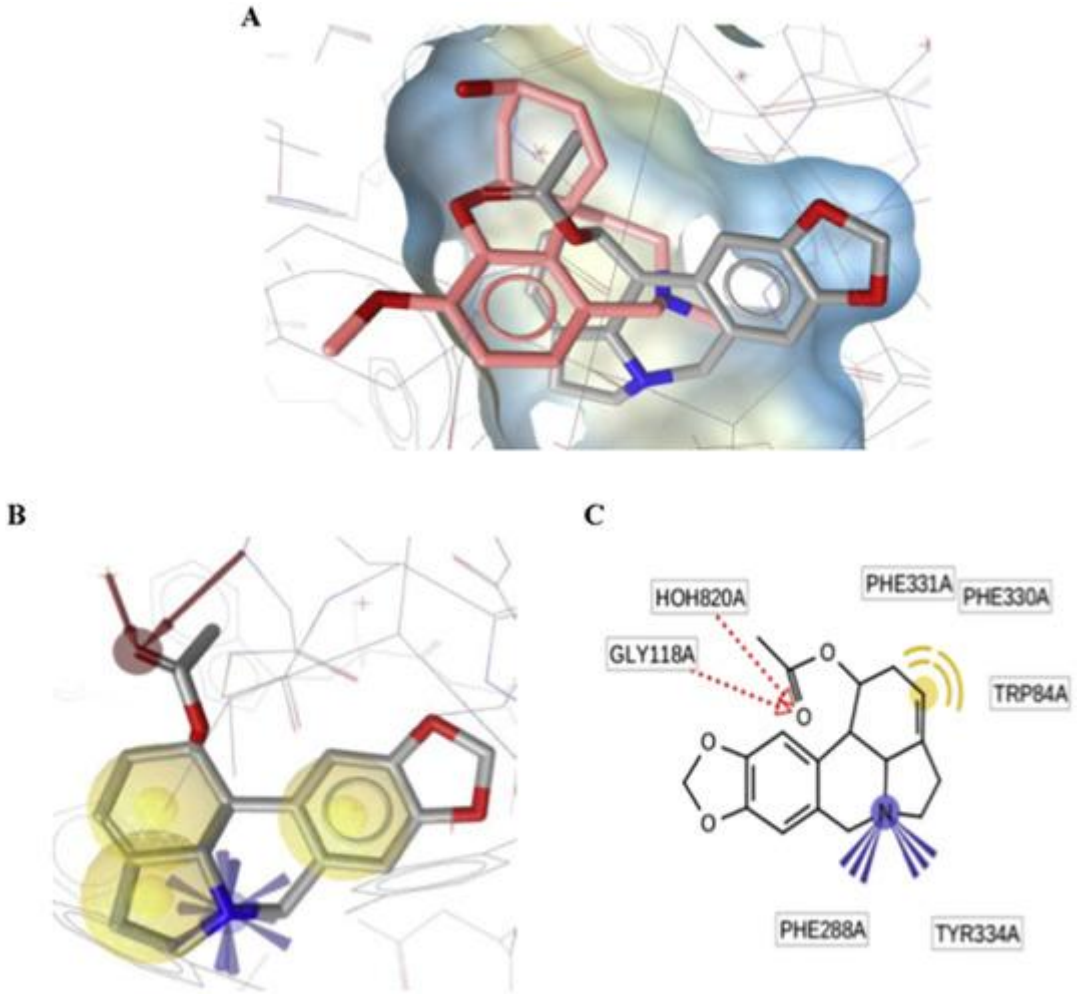


Fig. 4. Results of docking experiments using acetylcarinine, (A) Predicted best-ranked binding pose for acetylcaranine (grey) in comparison to the co-crystallized galanthaminie (rose) (B). Protein contacts of AChE with the ligand acetylcaranine (C) showing interactions with the aromatic cage amino acids.

In general, undulatine was found to bind in a similar fashion as acetylcaranine to the AChE active site. However, the methoxy-substituent on the aromatic ring alters the exact binding. The orientation in the binding site does not compare well to the very similar compound, buphanidrine. The best-ranked binding pose for undulatine is indicated in Fig. S4 (Supplementary material). The compound shows typical interactions of an AChE active site inhibitor. The interaction pattern of buphanadrine (Fig. S5; Supplementary material) resembles the interactions of many AChE inhibitors. Interactions with the aromatic cage (Trp84, Phe330 and Tyr334) are typical for this class of compounds [20]. Protein contacts for AChE for the ligand undulatine indicated interactions with the aromatic cage amino acids as indicated in Fig. S5.

Belladine is the most flexible and least active compound from this set of compounds. This compound is predicted to occupy the AChE active site in a similar way as galanthamine (Fig. S6; Supplementary material). The best docking pose of belladine had a calculated binding affinity of −8.10 kcal/mol, which was the lowest “best-ranked pose” score of this study (Table 4). Although belladine also occupies the AChE active site (Fig. S6) in this docking study, its actual activity is poor as reflected by the high IC_{50} achieved. The comparatively

high flexibility of the molecule possibly explains the poor activity. Belladine can also be considered the least active compound of all those investigated according to the docking score ranks.

When comparing the second-best ranked pose of undulatine (calculated binding affinity −9.5 kcal/mol) with the best-ranked pose of buphanidrine, a nearly identical binding orientation was observed (Fig. 5). The protein-ligand interactions were also identical, with one exception, namely that the more active undulatine formed an additional hydrogen bond with Tyr334 at the epoxy ring. As this substructure is not present in buphanidrine, there is no H-bond formation. This additional interaction may account for the higher activity of undulatine.

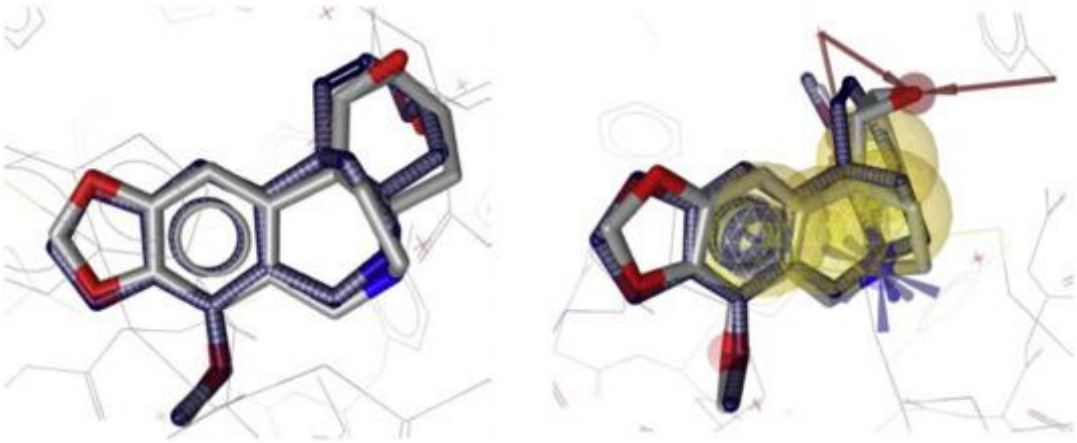


Fig. 5. The second-best ranked pose of undulatine (left) with the best-ranked pose of buphanidrine (right), indicating a nearly identical binding orientation.

4. Conclusions

Four compounds were successfully isolated from the acid base extracts of *A. belladonna* and their chemical structures were characterised using one and two dimensional ^{13}C and ^{1}H NMR. The NMR data were in agreement with the previous published data. Compounds [1] to [4] were identified as undulatine, buphanidrine, belladine and acetylcaranine, respectively. All four isolated compounds were tested for AChE activity using the microplate assay. Acetylcaranine was found to be the most active of the isolates (IC_{50} = 11.7 ± 0.7 μM) and the activity compared favourably with galanthamine, while belladine was the least active. Molecular docking successfully predicted the binding modes of ligands within receptor binding sites and confirmed the IC_{50} values obtained. Both qualitative and quantitative assays have indicated that several species of the South African Amaryllidaceae are noteworthy acetylcholinesterase inhibitors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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