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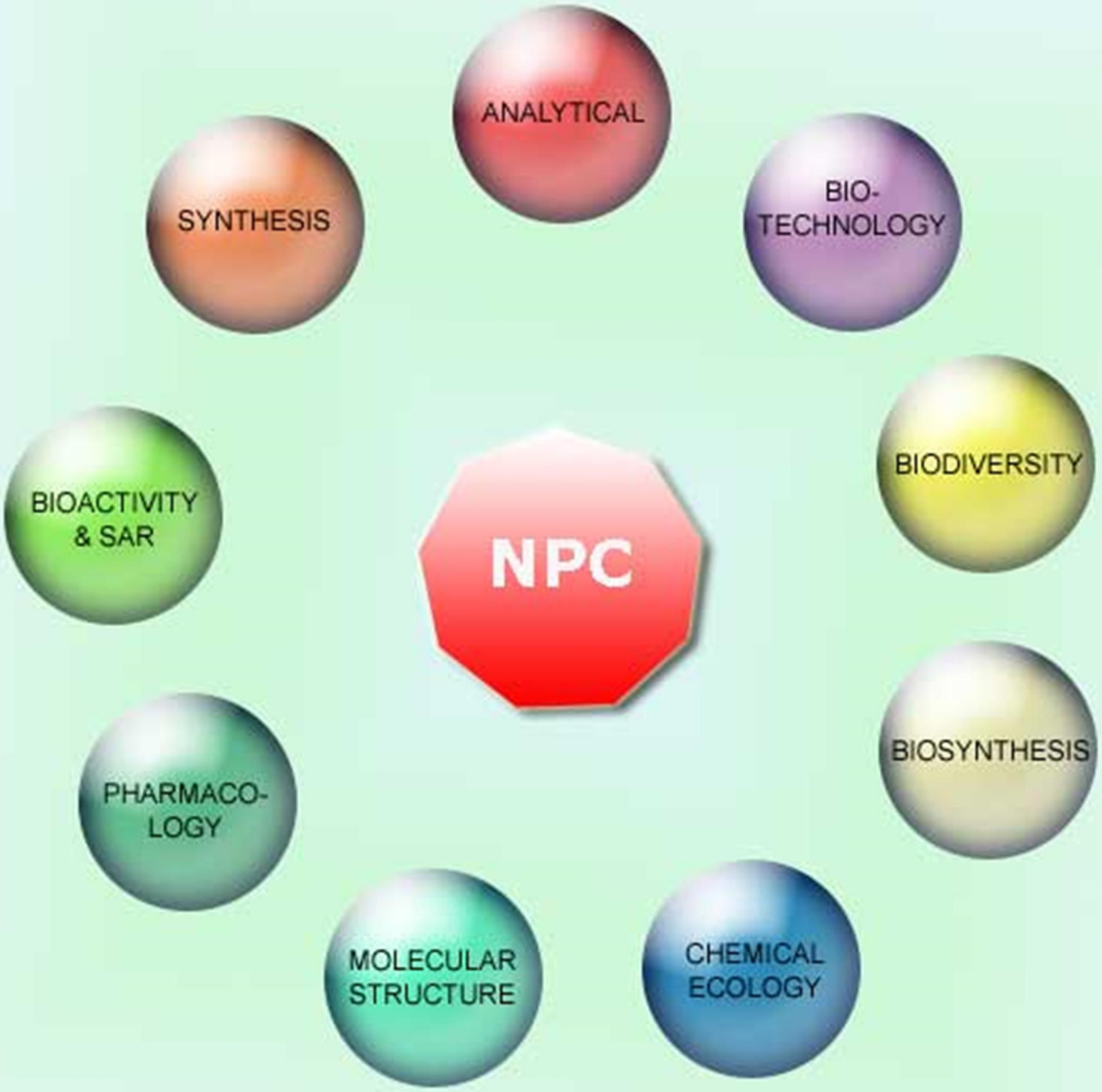

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Alkaloids from *Habranthus tubispathus* and *H. jamesonii*, two Amaryllidaceae with Acetyl- and Butyrylcholinesterase Inhibition Activity

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Alzheimer´s disease (AD) is a neurodegenerative disorder associated with memory impairment and cognitive deficit. Most of the drugs currently available for the treatment of AD are acetylcholinesterase (AChE) inhibitors. Plants of the Amaryllidaceae family are known to synthesize alkaloids, which have shown AChE inhibitory activity. *Habranthus tubispathus* and *H. jamesonii* are two Amaryllidaceae that can be found growing wild to the southwest of Buenos Aires in Argentina. Acetyl- and butyrylcholinesterase inhibition was observed for the extracts obtained from bulbs of *H. tubispathus* and bulbs and aerial parts of *H. jamesonii*. The strongest cholinesterase inhibition was observed for the alkaloid extract obtained from the aerial parts for *H. jamesonii* (AChE IC_{50}= 0.7 μg/mL; BChE IC_{50}= 6.7 μg/mL). The AChE inhibition observed for *H. jamesonii* could be explained by the presence of galanthamine and sanguinine, two potent AChE inhibitors. The levels of lycorine and hippeastidine, moderate AChE inhibitors, observed in the bulbs of *H. tubispathus* could be responsible for the significant AChE inhibition observed. The alkaloids present in these Amaryllidaceae were identified by means of GC-MS analysis. In the case of *H. tubispathus*, hippeastidine and 3-*O*-demethylhippeastidine, were isolated and completely characterized by ^{1}H and ^{13}C NMR spectroscopy.

Keywords: *Habranthus tubispathus*, *Habranthus jamesonii*, Amaryllidaceae, Acetylcholinesterase inhibition, Butyrylcholinesterase inhibition, Alkaloids.

Alzheimer´s disease (AD) is a progressive neurodegenerative disorder associated with memory impairment and cognitive deficit. It is characterized by low levels of the neurotransmitter acetylcholine (ACh) in the brain of AD patients. The inhibition of acetylcholinesterase (AChE), the enzyme that catalyzes ACh hydrolysis, is the main therapeutic strategy used to treat AD. AChE inhibitors can alleviate AD symptoms by improving cholinergic functions in AD patients. In the healthy brain, another enzyme, namely butyrylcholinesterase (BChE), is involved in the metabolic degradation of ACh. BChE activity increases as AD progresses. Thus, both enzymes, AChE and BChE, are considered legitimate therapeutic targets for treating the cholinergic deficit characteristic of AD [1].

Amaryllidaceae alkaloids represent a large and still expanding group of isoquinoline alkaloids that have attracted considerable attention due to their interesting pharmacological properties such as antiprotozoal, antibacterial, anticonvulsant, antitumor, antiviral and anticholinesterase activities [2,3]. The Amaryllidaceae family comprises about 1100 species and is widely distributed through tropical and warm regions of the world [4]. Among the Amaryllidaceae genera found in Argentina, the genus *Habranthus*, commonly known as "rain lilies", is represented by 27 species, of which 24 are endemic [5]. Typically, they appear from late spring through autumn, in response to rain. *H. tubispathus* (L´Hér.) Traub and *H. jamesonii* (Baker) Ravenna can be found growing wild to the southwest of Buenos Aires province [6]. *H. tubispathus* is found in grasslands and disturbed areas. In Argentina, its distribution covers the provinces of Corrientes, Entre Ríos, Santa Fe and Buenos Aires; it can also be found in Chile, Paraguay and Uruguay [7]. *H. jamesonii* is found in sandy soils in the west and center of Argentina (provinces of Catamarca, La Rioja, San Juan, San Luis, Mendoza, La Pampa, Río Negro, Chubut and in the south of Buenos Aires) [7,8].

In the present study, as part of our ongoing phytochemical and biological activity studies of Argentinean flora, *H. tubispathus* and *H. jamesonii* were investigated for their AChE and BChE inhibitory activity using Ellman´s method [9]. The alkaloids present in both extracts were also investigated and, in the case of *H. tubispathus,* two of the main alkaloids were isolated using chromatographic methods and identified by ^{1}H and ^{13}C NMR and GC-MS analysis.

Bulbs of *H. tubispathus* and bulbs and aerial parts (flowers and leaves) of *H. jamesonii* were extracted separately. The results observed for AChE and BChE inhibition are summarized in Table 1. The alkaloid extract from the aerial parts of *H. jamesonii* showed the highest AChE and BChE inhibitory activity. The alkaloid extract obtained from the bulbs of *H. jamesonii* also exhibited potent AChE and BChE inhibition.

Table 1: Acetyl- and butyryl-cholinesterase inhibition of *Habranthus* species and isolated compounds, expressed as IC_{50} values.

Samples	IC_{50}	
	AChE [b]	BChE [b]
EE-***Ht***-b	0.9 mg/mL	> 1 mg/mL
AE-***Ht***-b	12.5 μg/mL	62.8 μg/mL
EE-***Hj***-b	34.4 μg/mL	> 2.5 mg/mL
EE-***Hj***-ap	29.6 μg/mL	> 2.5 mg/mL
AE-***Hj***-b	1.3 μg/mL	20.8 μg/mL
AE-***Hj***-ap	0.7 μg/mL	6.7 μg/mL
hippeastidine	104.6 μM	200.8 μM
3-*O*-demethylhippeastidine	>200 μM	>200 μM
eserine[c]	11.3 nM	14.3 nM

[a] EE: ethanolic extract, AE: alkaloid extract, *Ht*: *H. tubispathus*, *Hj*: *H. jamesonii*, b: bulbs, ap: aerial parts. [b] Results are the mean of three replications. [c] Reference compound.

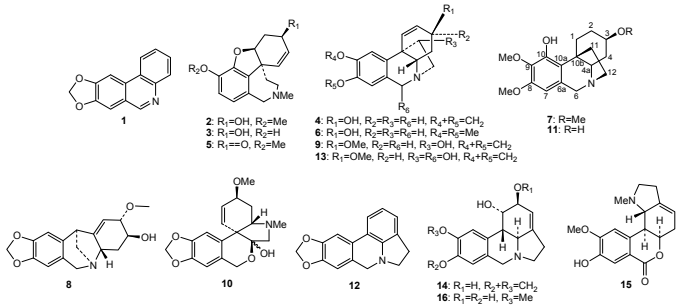

Figure 1: Alkaloids from *H. tubispathus* and *H. jamesonii*.

In order to identify the alkaloids present in these extracts a GC-MS analysis was performed for each sample. The majority of the alkaloids were identified by comparing their mass spectra with those available in either the spectrometer database or the literature [10, 11]. Sanguinine (**3**), vitattine (**4**), narwedine (**5**), 8-*O*-demethylhomolycorine (**15**) and lycorine (**14**) were observed to be the major alkaloids in the aerial parts of *H. jamesonii* (Table 2), whereas the bulbs of *H. jamesonii* proved to be rich in lycorine (**14**), 8-*O*-demethylhomolycorine (**15**), galanthamine (**2**) and sanguinine (**3**) (Table 2). It is notable that the alkaloid profile differs substantially between the bulbs and the aerial parts of *H. jamesonii*. For example, alkaloids **4** and **5**, which were present in traces in the bulbs, were detected in high proportions in the aerial parts. Similarly, while the bulbs of *H. jamesonii* were rich in galanthamine (**2**) and lycorine (**14**), these alkaloids were minor constituents of the aerial parts of this plant. Interestingly, our results differ dramatically from those reported by Ortiz *et al*. for *H. jamesonii* bulbs collected in two provinces in the Andean region of Argentina [12]. Our results confirm that alkaloid content depends on the geographical distribution of *H. jamesonii* populations. Intraspecies diversity in alkaloid profiles has also been observed for other Amaryllidaceae genera in populations collected from different locations [13]. The most important differences between our results and those reported by Ortiz *et al.*, are probably the higher levels of galanthamine and sanguinine that we have detected in our study of *H. jamesonii* bulbs, and the fact that in our case, the chemical composition of the aerial parts was also investigated.

When the alkaloid extract of *H. tubispathus* was submitted for GC-MS analysis nine alkaloids were detected (Table 2). Seven were clearly identified by comparison of their mass spectra with those published either in the literature or stored in the spectrometer database, namely maritidine (**6**), montanine (**8**), haemanthamine (**9**), tazettine (**10**), haemanthidine (**13**), lycorine (**14**) and pseudolycorine (**16**) [10, 11]. The presence of alkaloids **8**, **9** and **14** was in accordance with previous information about alkaloids from *H. tubispathus* [Prof. B. Maldoni, pers. comm. 2002]. The GC-MS results showed the presence of two compounds, **7** and **11**, exhibiting molecular ions at *m/z*= 319 and 305, respectively, with unidentified MS fragmentation patterns. Several chromatographic separations allowed the isolation and purification of **7** (5.1 mg) and **11** (3.6 mg), along with lycorine. Compounds **7** and **11** proved to be crinine-type alkaloids with the unusual feature of a hydroxyl group at C-10.

The mass spectrum of **7** showed a molecular ion peak at *m/z* 319 (100%), indicating a molecular formula $C_{18}H_{25}NO_4$, and a fragmentation pattern similar to that observed for hippeastidine, an alkaloid isolated from *Hippeastrum añañuca* [14]. Pacheco *et al*. reported the structural elucidation of hippeastidine based on X-ray diffraction of its picrate and provided limited information about its mass spectrum and ^{1}H NMR spectrum (100 MHz) [14]. In order to confirm the identity of compound **7**, ^{1}H and ^{13}C NMR data were recorded (Table 3). Signals were assigned with the aid of correlations observed in ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments. We observed that the spectroscopic data obtained for alkaloid **7** was all in agreement with the structure proposed by Pacheco *et al.* in 1978 for hippeastidine [14]. This structure was confirmed by Watson *et al*. in 1982 through X-ray diffraction of a crystalline sample of this alkaloid [15]. Alkaloid **7** was, therefore, unambiguously identified as hippeastidine and a complete assignation of ^{1}H and ^{13}C NMR signals was achieved.

Interestingly, although a large number of alkaloids has been isolated or detected in plants belonging to the Amaryllidaceae family over the past decades, as far as we know, hippeastidine has only been isolated from *Hippeastrum añañuca*, until now, when we found it as one of the main alkaloids in *H. tubispathus* [3a, 14].

The other unidentified alkaloid isolated from *H. tubispathus*, compound **11**, showed a molecular ion peak at *m/z* 305 (100%) in

Table 2: Alkaloids identified in *H. tubispathus* and *H. jamesonii*

Alkaloid	RT (min)	M^+ and *m/z* (rel. int.)	Species			Ref. [b]
			H. tubispathus (%)	***H. jamesonii*** (%)		
			bulbs	bulbs	aerial parts	
Trisphaeridine (**1**)	18.73	223(100), 222(40), 167(15), 165(14), 164(20), 138(32)	-	< 1	< 1	[10]
Galanthamine (**2**)	20.61	287(80), 286(100), 270(11), 244(19), 230(15), 216(37)	-	18.5	3.5	[10]
Sanguinine (**3**)	21.26	273(100), 272(85), 256(22), 202(41), 160(50)	-	10.9	21.9	[10]
Vittatine (**4**)	21.74	271(100), 252(29), 199(68), 187(65), 173(26)	-	< 1	20.6	[10]
Narwedine (**5**)	21.81	285(84), 284(100), 242(23), 228(12), 216(30), 199(27)	-	< 1	14.7	[10]
Maritidine (**6**)	22.29	287(91), 268(8), 244(34), 215(100), 203(61), 189(24), 128(24), 115(30), 71(2), 56(20)	2.1	-	-	[11]
Hippeastidine (**7**)	23.73	319(100), 304(18), 288(39), 258(20), 233(84), 218(30), 206(27), 163(16), 115(14)	42.6	< 1	< 1	[14]
Montanine (**8**)	23.79	301(100), 270 (82), 257(7), 252(26), 223(26)	< 1	-	-	[10]
Haemanthamine (**9**)	23.99	301(13), 272 (100), 240(24), 225(11), 211(21)	8.6	-	-	[10]
Tazettine (**10**)	24.27	331(22), 316(8), 298(22), 247(100), 230(12)	< 1	-	-	[10]
3-*O*-demethylhippeastidine (**11**)	24.30	305 (100), 304 (26), 290(17), 288(18), 277(28), 276(28), 246(17), 234(50), 233(72), 232(33), 218(28), 217(20), 206(30),115(16), 91(18)	15.0	-	-	
Anhydrolycorine (**12**)	24.34	251(66), 250(100), 192(14), 191(15), 165(5), 164(3), 139(3), 124(4)	-	-	4.5	[11]
Haemanthidine (**13**)	25.15	317(63), 284(57), 233(62), 211(64), 201(93), 199(90), 181(52), 173(66), 115(100), 56(46)	< 1	-	-	[11]
Lycorine (**14**)	25.57	287(23), 286(13), 268(20), 250(13), 227(63), 226 (100)	28.6	33.8	9.1	[10]
8-*O*-demethylhomolycorine (**15**)	26.74	301(-), 192(1), 164(5), 110(9), 109(100), 108(31), 94(6), 82(7)	-	29.8	22.8	[11]
Pseudolycorine (**16**)	26.80	289(26), 288(15), 270(18), 252(7), 230 (10), 229(67), 228 (100)	< 1	-	-	[10]

[a] For GC conditions see the Experimental section, [b] Reference for MS data.

Table 3: NMR data for compounds **7** and **11**.

	7		**11**	
Position	δ_C[a]	δ_H[b] mult (*J* in Hz)	δ_C[a]	δ_H[b] mult (*J* in Hz)
1	25.9 t	1.76 t (14.5,4.2) 3.28 d (14.5)	26.4 t	1.79 m 3.27 d (14.4)
2	26.9 t	1.42 m 2.02 d (13.2)	30.3 t	1.52 m 1.91 os
3	76.7 d	3.22 m	67.6 d	3.57 m
4β	31.6 t	1.27 dd (23.7, 11.6)	35.6 t	1.33 dd
4α		2.23 dd(23.7, 11.2)		2.08 br s
4a	68.1 d	3.38 d (11.6)	67.8 d	3.19 t
6β	59.9 t	4.00 d (15.8)	60.7 t	3.86 d (15.4)
6α		4.59 d (15.8)		4.49 d (15.4)
6a	123.7 s		124.9 s	
7	101.2 d	6.23 s	100.8 d	6.22 s
8	135.5 s		135.0 s	
9	151.7 s		151.2 s	
10	148.1 s		147.8 s	
10a	124.2 s		126.6 s	
10b	44.1 s		43.4 s	
11endo	34.2 t	1.98 os	34.9 t	1.95 os
11*exo*		2.44 m		2.38 m
12*endo*	51.8 t	3.13 m	51.4 t	2.96 br s
12*exo*		3.67 t (12.2, 11.8)		3.53 os
3-OCH_3	54.9 q	3.35 s		
8-OCH_3	59.9 q	3.72 s	59.7 q	3.77 s
9-OCH_3	55.1 q	3.77 s	54.8 q	3.79 s

[a] Recorded at 75 MHz in methanol-d_4; multiplicity by DEPT [b] Recorded at 300 MHz in methanol-d_4.

its mass spectrum, suggesting a molecular formula $C_{17}H_{23}NO_4$ (Table 2). The ^{1}H and ^{13}C NMR spectra of **11** were very similar to those recorded for alkaloid **7**, except for the absence of the ^{1}H and ^{13}C signals corresponding to the methoxy group attached to C-3 and differences observed in the chemical shifts of ^{1}H and ^{13}C resonances corresponding to positions 2 and 4 (Table 3). Based on this information, compound **11** was identified as 3-*O*-demethylhippeastidine. This alkaloid was isolated also from *H. añañuca*, but in this case the identification was based on limited spectroscopic data: only four proton resonances (100 MHz, $CDCl_3$) and two fragmentations from the molecular ion in the mass spectrum were reported [16].

The presence of the alkaloids galanthamine (**2**) and sanguinine (**3**) could be related to the high AChE inhibitory activity of *H. jamesonii*. Alkaloid **2** has been shown to be a potent AChE inhibitor (IC_{50} 1.07 μM) while alkaloid **3** (9-*O*-demethylgalanthamine) is ten times more potent than **2** as an AChE inhibitor [3b]. The higher levels of **2** found in the aerial parts of *H. jamesonii* could be responsible for the better cholinesterase inhibition observed in this case, compared with the activity observed for the bulb extract. Among the alkaloids present in *H. tubispathus* bulbs, hippeastidine (**7**) was observed to be the most active, while 3-*O*-demethylhippeastidine (**11**) proved to be inactive against both AChE and BuChE, at the tested concentrations (Table 1). Both species produce lycorine (**14**) in good amounts (Table 2), which has shown significant AChE inhibition in previous reports with IC_{50} values ranging from 152 μM to 450 μM [3b, 17, 18].

In conclusion, we have observed that both species exhibit potent AChE and BChE inhibition. The best activity was observed for the aerial parts of *H. jamesonii.* We have also studied the alkaloids profile for both species, and this work is the first report of a phytochemical study of *H. tubispathus*. Our results show that the *H. jamesonii* population found in the southwest of Buenos Aires province is a rich source of galanthamine, one of the approved drugs for AD treatment. The cholinesterase inhibition observed could be explained by the main alkaloids present in these Amaryllidaceae. In addition, we have isolated the main alkaloids of *H. tubispathus,* completely characterized them using spectroscopic methods, and evaluated them as cholinesterase inhibitors.

Experimental

General: NMR measurements, including COSY, HSQC, HMBC and NOESY experiments, were carried out on a Bruker ARX300 spectrometer. Chemical shifts are given in ppm (δ) with TMS as an internal standard. Silica gel 60 (Merck) was used for CC. Analytical TLC was performed on Silicagel 60 F_{254} sheets (0.2 mm thickness, Merck). *p*-Anisaldehyde-acetic acid spray reagent, Dragendorff's reagent and UV light (254 and 366 nm) were used for detection. UV spectra were recorded on a JASCO V-630BIO spectrophotometer. Acetylcholinesterase from electric eel (type VI-S), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI) and eserine were purchased from Sigma. Butyrylcholinesterase (horse serum) was purchased from MP Biomedicals.

Plant material: *H. tubispathus* and *H. jamesonii* were collected during the flowering period (November 2009 and 2012, respectively) in, respectively, Bahía Blanca and *Salitral de la Vidriera*, an area with a low shrubby halophytic steppe near the estuary, 20 km from Bahia Blanca city, Argentina. Voucher specimens were identified by Dra Maria Gabriela Murray and deposited in the Herbarium of the Universidad Nacional del Sur (BBB) in Bahía Blanca, Argentina, under the numbers *Murray, M.G.* 545 (*H. tubispathus*) and *Murray M.G.* 541 (*H. jamesonii*).

Alkaloids extraction and isolation: Fresh bulbs of *H. tubispathus* (1 kg) were cut into small pieces and macerated with ethanol (96% v/v) at room temperature for 2 weeks and then extracted with boiling ethanol for 3 h. Both extracts were combined and evaporated to dryness under reduced pressure. Dry extract (73 g) was dissolved in 2% HCl (600 mL) and filtered 1 h later. The remaining solution was basified (pH 9) with $NaHCO_3$ 3 M and extracted with CH_2Cl_2 (3 x 600 mL). The dichloromethane extract was evaporated under reduced pressure and tested for alkaloids on TLC (Silicagel 60, CH_2Cl_2:MeOH:H_2O 85:14:1). This CH_2Cl_2 extract (0.28 g) was Dragendorff's positive, so was submitted for GC-MS analysis.

Bulbs of *H. jamesonii* (544 g) were extracted with ethanol at room temperature for 2 weeks. After evaporation of the solvent under reduced pressure, the ethanolic extract (36 g) was treated with 2% HCl (450 mL) for 1 h and then filtered. A 3 M $NaHCO_3$ solution was added until pH 9 and the aqueous solution was extracted with CH_2Cl_2 (3 x 500 mL). The alkaloid extract (0.15 g) was analyzed by GC-MS. The aerial parts of *H. jamesonii* (162 g) were treated in a similar way to obtain the ethanolic extract (2.3 g) and the alkaloid extract (24 mg).

A portion of *H. tubispathus* alkaloid extract (226.4 mg) was subjected to CC over silicagel 60 (70-230 mesh, 11.3 g) eluting with CH_2Cl_2 gradually enriched with MeOH. Fractions of 5 mL were collected (190 in total) and combined according to their TLC profiles monitored by UV light (254 nm and 366 nm) and Dragendorff´s reagent. Fractions 122-123 (CH_2Cl_2:MeOH 94:6) redissolved in MeOH led to spontaneous crystallization of lycorine (**14**) (7.4 mg). Hippeastidine (**7**) (5.1 mg) was isolated from fractions 143-148 (CH_2Cl_2:MeOH 87.5:12.5). Fraction 190 (MeOH 100%) was submitted to preparative TLC (20 cm x 20 cm x 0.25 mm, silica gel F_{254}, *n*-BuOH:AcOH:H_2O 12:3:5) and 3-*O*-demethylhippeastidine (**11)** (3.6 mg) was obtained.

GC-MS analysis: Gas chromatography-mass spectrometry analyses were performed on a Hewlett Packard 6890/MSD 5972 instrument operating in EI mode at 70 eV, equipped with a capillary column (HP-5 30 m x 0.25 mm, 0.25 μm film thickness). Mass range was

from *m/z* 35 - 500 amu. The carrier gas was helium with a flow rate of 1 mL/min. The GC oven temperature program was held at 100°C for 2 min, programmed at 15ºC/min to 180 °C, hold for 1 min, and then programmed at 5ºC/min to 280°C, and held for 10 min at 280°C. The injection block temperature was 280°C. Samples were dissolved in MeOH. Results were analyzed using NBS75K.LMSDATA. Alkaloids were identified by comparing their MS and retention times with those reported in the literature.

Cholinesterase inhibition assay: Electric eel (*Torpedo californica*) AChE and horse serum BChE were used as sources of both cholinesterases. AChE and BChE inhibitory activities were measured as previously reported [19]. All reactions were performed in triplicate. IC_{50} values were determined with GraphPad Prism 5. Eserine (99%) was used as the reference AChE/BChE inhibitor.

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