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Article

Chemical evaluation and anticholinesterase activity of *Hippeastrum puniceum* (Lam.) Kuntz bulbs (Amaryllidaceae)

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Hippeastrum puniceum is a species that belongs to the Amaryllidaceae family. A particular characteristic of this family is the consistent and very specific presence of isoquinoline alkaloids, which have demonstrated a wide range of biological activities such as antioxidant, antiviral, antifungal, antiparasitic, and acetylcholinesterase inhibitory activity, among others. In the present work, fifteen alkaloids were identified from the bulbs of *Hippeastrum puniceum* (Lam.) Kuntz using a GC-MS approach. The alkaloids 9-*O*-demethyllycoramine, 9-demethyl-2α-hydroxyhomolycorine, lycorine and tazettine were isolated through chromatographic techniques. The typical Amaryllidaceae alkaloids lycorine and tazettine, along with the crude and ethyl acetate extract from bulbs of the species were evaluated for their inhibitory potential on α-amylase, α-glucosidase, tyrosinase and acetylcholinesterase activity. Although no significant inhibition activity was observed against α-amylase, α-glucosidase and tyrosinase from the tested samples, the crude and ethyl acetate extracts showed remarkable acetylcholinesterase inhibitory activity. The biological activity results that correlated to the alkaloid chemical profile by GC-MS are discussed herein. Therefore, this study contributed to the knowledge of the chemical and biological properties of *Hippeastrum puniceum* (Lam.) and can subsidize future studies of this species.

Keywords: *Hippeastrum puniceum*. Amaryllidaceae. GC-MS. Acetylcholinesterase inhibitory activity.

INTRODUCTION

Amaryllidaceae (J. St.-Hil.) plants are a large bulbous species with a pantropical distribution and a great sphere of influence in areas as diverse as horticulture, agriculture, ethnobotany, traditional medicine and pharmacology (Bastida *et al*., 2006; Nair *et al*., 2013). It is well established that the exclusive group of isoquinoline Amaryllidaceae alkaloids are metabolites that consistently have a wide range of effects, and the Amaryllidaceae plant species are used in traditional folk medicine due to the presence of this kind of metabolites (Nair *et al*. 2013). These alkaloids have demonstrated a great variety of biological activities, such as antiviral, antimicrobial, antiparasitic, antitumoural, psychopharmacological, and cholinesterase inhibitory activity, among others (Bastida *et al*., 2006; da Silva *et al*., 2006; de Andrade *et al*., 2016; Giordani *et al*., 2010; Jin *et al*., 2013; Kirilenko and Evidente, 2008).

Acetylcholinesterase inhibitory (AChE) activity is one of the most promising targets for Amaryllidaceae alkaloid evaluations, since the well-known

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Amaryllidaceae compound galanthamine was approved for palliative therapy of mild-moderate Alzheimer's disease (AD) in 2001 (Heirich and Teoh, 2004). Due to the great biological potential of Amaryllidaceae alkaloids, some Amaryllidaceae compounds have been studied in a variety of enzymatic systems, as in the case of interactions with human cytochrome P450 3A4 (McNulty *et al*., 2009). In this attempt, tyrosinase is the rate-limiting enzyme of melanin synthesis and the main target of antihyperpigmentation compounds. Much interest has been focused on compounds that can inhibit tyrosinase activity, particularly natural products, for which there is an increasing demand in the fields of cosmetics and pharmaceuticals (Burlando *et al*., 2017). Furthermore, the enzymes α-amylase and α-glucosidase are responsible for cleaving large malto-oligosaccharides to maltose and maltose to glucose, respectively. The inhibition of these enzymes plays a major role in managing postprandial hyperglycaemia in diabetic patients (Shinde *et al*., 2008).

Amaryllidaceae plant alkaloid-enriched extracts have recently been chemically characterized by means of chromatographic/spectrometric methods, and the gas chromatography-mass spectrometry (GC-MS) technique has been particularly useful to the study of these isoquinoline derivatives (Berkov *et al*., 2008; Torras-Claveria *et al*., 2014). The high resolution of the capillary column technique in gas chromatography (GC) together with the ready availability of libraries of electron impact mass spectrometry (EI-MS) data in the literature facilitate the rapid identification and quantification of known alkaloids. Thus, the native Brazilian species *Hippeastrum puniceum* (Lam.) Kuntze were collected and extracted to obtain the alkaloid-enriched fractions. All enriched fractions including the crude extract were studied by GC-MS, and in the course of the phytochemical procedure, four alkaloids were purified from the bulbs of the species. A total of fifteen compounds were identified from *H. puniceum*. The plant extracts and the isolated alkaloids lycorine and tazettine were tested against α-amylase, α-glucosidase, tyrosinase and AChE. The crude extract and the ethyl acetate fraction from the bulbs of *H. puniceum* showed remarkable AChE activity, displaying values of 72.96% (±1.26) and 78.31% (±1.04) of enzyme inhibition at 100 $mg.mL^{-1}$, respectively. The ethyl acetate fraction exhibited the lowest IC_{50} - necessary concentration to reach a half of the maximum of enzyme inhibition (21.97 $mg.mL^{-1}$, ±1.05). In summary, *H. puniceum* was able to synthesize some galanthamine-type derivatives in addition to other typical Amaryllidaceae alkaloids. The relationship between the presence of galanthamine-type derivatives and the AChE activity is discussed in the present work. Considering it is important to know about biological and chemical properties of plant species, the purpose of this work was to describe the chemical study and evaluate the inhibition of α-amylase, α-glucosidase and tyrosinase enzymes of extracts and alkaloids of the bulbs from *Hippeastrum puniceum* (Lam.).

MATERIAL AND METHODS

General Experimental Procedures

NMR spectra were recorded on a Varian 400 MHz (Palo Alto, CA, USA) instrument using deuterated chloroform ($CDCl_3$) as a solvent and tetramethylsilane (TMS) as an internal standard. The chemical shifts are reported in δ units (ppm) and coupling constants (J) in Hertz (Hz). The GC-MS spectra were obtained on a Shimadzu GC-17A QP 5000 operating in the EI mode at 70 eV (Kyoto, Japan) using a DB1 MS column (30 m x 0.25 mm x 0.25 µm, Shimadzu). The temperature programme was as follows: 100–180 ºC at 15 ºC min^{-1}, 1 min hold at 180 ºC and 180–300 ºC at 5 ºC min^{-1} and 40 min hold at 300 ºC. The injector temperature was kept at 280 ºC. The flow rate of carrier gas (helium) was 0.8 mL min^{-1}.

For thin layer chromatography (TLC), silica gel F_{254} was used as the stationary phase and a plate dimension of 20 cm x 20 cm x 0.20 mm was used for analytical TLC (Macherey-Nagel) and 20 cm x 20 cm x 0.25 mm for semi-preparative TLC (Macherey-Nagel). Exclusion chromatography was performed using a Sephadex® LH-20 and methanol as the mobile phase.

Plant material

Samples of the species *Hippeastrum puniceum* (Lam.) Kuntze were collected in November 2014 from a population located at São Caetano city, Pernambuco Province state, Brazil. The species was collected and identified by Dr. Anderson Alves-Araújo, and the voucher specimen of *H. puniceum* was deposited in the herbarium of the Federal University of Espírito Santo (Brazil) under the reference number VIES 37595.

Identification of alkaloids by GC-MS

The Amaryllidaceae alkaloids were identified by comparing the GC-MS spectra and Kovats retention indices (RI) to our library database. This library has been regularly updated with alkaloids isolated and unequivocally identified via physical and spectroscopic methods (Berkov *et al*., 2008; de Andrade *et al*., 2016). NMR data for the described known alkaloids reported in this work were compared with the literature data (Bastida *et al*., 1990, 2006; Evidente, 1986; Wang *et al*., 2010). Mass spectra were deconvoluted using AMDIS 2.64 software (NIST), and RIs were recorded using a standard *n*-hydrocarbon calibration mixture (C9-C36). The proportion of individual components in the alkaloid fractions are expressed as a percentage of total alkaloid content. GC-MS peak areas are dependent on the concentration of the injected alkaloid and the intensity of its mass spectral fragmentation. Although the data in Table I are not representative of a validated alkaloid quantification method, they can be used for relative comparison purposes.

Extraction and isolation of alkaloids

Fresh bulbs (1.8 kg) of *Hippeastrum puniceum* were air-dried for 48 h in over at 38-40^{0}C, crushed and thrice extracted for 48 hours with methanol at room temperature, and the combined macerate was filtered and evaporated under reduced pressure to yield the crude extract (2.5% w/w). The crude extract was then acidified with sulfuric acid (2%) to pH 2 and extracted with ethyl ether (4 x 150 mL) and ethyl acetate (4 x 250 mL) to remove neutral material. The aqueous solution was basified with ammonia (25%) up to pH 10 and extracted with *n*-hexane (8 x 150 mL) which provided the *n*-Hexanic fraction (570 mg). Another extraction using ethyl acetate (20 x 150 mL) yielded the ethyl acetate fraction (3.2 g). Finally, a final extraction using an ethyl acetate - methanol mixture in the proportion of 3:1 (v/v) was performed (3 x 150 mL) to give the ethyl acetate - methanol fraction (2.4 g).

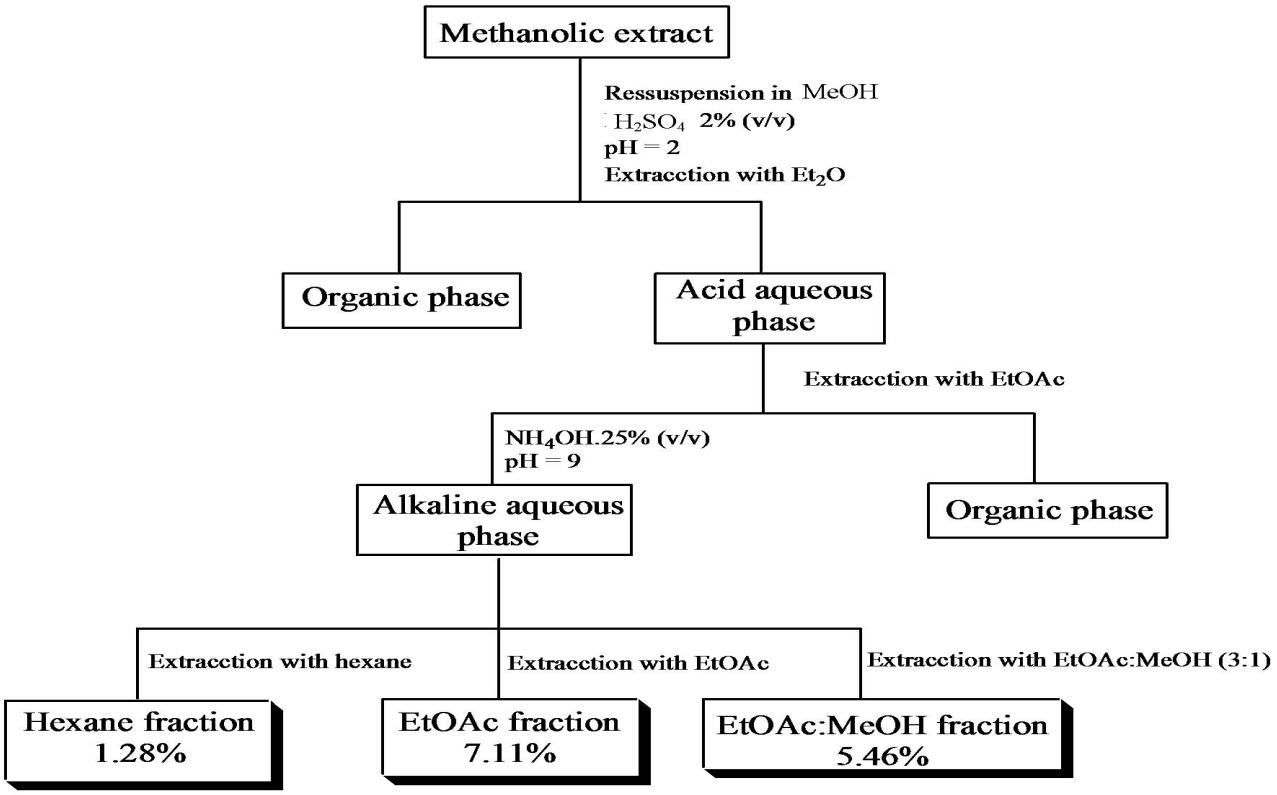

FIGURE 1 – Acid-base extraction process in the methanolic extract of the bulbsfrom *Hippeastrum puniceum*.

After methanol resuspension, the ethyl acetate fraction was filtered and provided 3.072 g of material. This extract was then submitted to Vacuum Liquid Chromatography (VLC, 50.0 x 3.0 cm) on silica gel 60 (70 – 90 mm), starting with *n*-hexane (100%) and increasing solvent polarity with ethyl acetate (0 → 50%). Thereafter, chloroform and ethyl acetate were gradually added until a chloroform - ethyl acetate ration of 1:1 was reached. Finally, the system was gradually supplemented with methanol (0 → 30%), yielding 180 fractions (50 mL each). The fractions were combined according their UV light λ 254 nm and Dragendorff's reagent stain profile by analytical TLC and yielded 19 sub-fractions. Sub-fraction 14 (from fraction 53 to 56, 820.2 mg) showed the presence of alkaloids and was submitted to three rounds of serial Exclusion Chromatography (EC) using Sephadex® LH-20 (methanol as eluent) as follows: i. bed column (31.2 x 1.6 cm) providing 458 aliquots (1 mL each), which were combined according their UV light λ 254 nm and Dragendorff's reagent stain profile by analytical TLC to yield sub-fraction A; ii. sub-fraction A (678.1 mg) was subjected to a 31.2 x 1.6 EC bed column affording 90 fractions (10 mL each) which after combining according their similar profiles by analytical TLC (Dragendorff's reagent, UV light λ 254 nm) yielded a sub-fraction named sub-fraction B (73.2 mg); iii. ultimately, a 31.2 x 1.5 cm EC bed column was used for sub-fraction B, yielding 20 fractions (5 mL each), which after combining according their similar profile by analytical TLC (Dragendorff's reagent, UV light λ 254 nm) yielded sub-fraction C (19.0 mg). The lycorine (3.0 mg) and tazettine (6.0 mg) were isolated from the sub-fraction C via semi-preparative TLC using the system dichloromethane - ethyl acetate - methanol - *n*-hexane (3:1:1:4, in NH3 atmosphere) and dichloromethane - ethyl acetate - methanol - acetone - *n*-hexane (2:3:2:1:2, in NH3 atmosphere), respectively.

Sub-fraction 15 (from fraction 57 to 60, 269.3 mg) also showed the presence of alkaloids under UV light at λ 254 nm and Dragendorff's reagent stain by analytical TLC and was then submitted to column chromatography (2.5 x 50.0 cm) on silica gel (70 – 90 mm), starting with *n*-hexane (100%) and increasing solvent polarity with dichloromethane- methanol (1:1) (0 → 100%). Thereafter, methanol was gradually added to reach 70%, and 253 fractions were collected in total (10 mL each). Two major sub-fractions were combined according to their similar profile by analytical TLC (Dragendorff's reagent, UV light λ 254 nm) as follows: i. sub-fraction 1 (from fraction 1 to 98) was submitted to semi-preparative TLC (dichloromethane - ethyl acetate - methanol - acetone - *n*-hexane – 1.1:5:1.1:1:1.1, in NH_3 atmosphere) and 9-*O*-demethyl-2α-hydroxyhomolycorine was obtained (1.1 mg); ii. sub-fraction 2 (from fraction 99 to 118) was submitted to semi-preparative TLC (dichloromethane - ethyl acetate - methanol - acetone - *n*-hexane – 5:3:2:2:6, in NH_3 atmosphere) providing 9-*O*-demethyllycoramine (7.0 mg).

The *n*-hexane and ethyl acetate - methanol fractions (Table I) were not submitted to chromatography fractionation because the GC-MS results showed little or no alkaloid content, respectively.

Biological activity

Samples: the isolated compounds galanthamine (positive standard for AChE inhibition assay, donation from Libbs Farmacêutica Ltda, Brazil), lycorine and tazettine along with the crude and ethyl acetate extract were prepared at concentrations of 1000 $mg.mL^{-1}$ (1000 ppm) in methanol (HPLC grade - stock solution). All tested samples at a concentration of 100 $mg.mL^{-1}$ were screened against tyrosinase, α-glucosidase and α-amylase enzymes, and only galanthamine along with the crude and ethyl acetate extract were tested against AChE enzyme. The samples displaying inhibition values above 70% in the tested targets were submitted to IC_{50} calculations. For IC_{50} measurements, concentrations from 100 mg.mL to 1.5 $mg.mL^{-1}$ were prepared (aqueous solution with methanol 5% and DMSO 0.1%).

Tyrosinase inhibition assay

The samples were tested for tyrosinase inhibition ability using the methodology described by Kathib *et al*. (2005) with some modifications (Freitas *et al*., 2016). Briefly, 60 µL of 50 mM phosphate buffer (pH 6.5), 10 µL of sample solution and 30 µL of 250 $U.mL^{-1}$ of tyrosinase (EC 1.14.18.1, 2.500 $U.mg^{-1}$ solid, Sigma Aldrich) solution (in 50 mM phosphate buffer, pH 6.5) were added to each well of a 96-well plate. After pre-incubation at 25 ºC for 5 min, 100 µL of 2.0 mM L-tyrosine were added to each well. The 96-well plate was incubated for another 20 min at 25 ºC, and the absorbance of the reaction mixture was determined at 475 nm using a Multimode Plate Reader (EnSpire, Perkin-Elmer, Singapore). Kojic acid (Sigma-Aldrich) was used as a positive control (0.8 – 50 $µg.mL^{-1}$).

α-Glucosidase inhibition analysis

The α-glucosidase (E.C. 3.2.1.20) inhibition was performed based on the method reported by Shinde *et al*. (2008). A volume of 20 µL of sample or standard was added to 10 µL of 1 $U.mL^{-1}$ α-glucosidase from *Saccharomyces cerevisae* (19.3 $U.mg^{-1}$ solid, Sigma-Aldrich) solution (in 50 mM sodium phosphate buffer with pH 6.8). The mixture was pre-incubated at 25 ºC for 5 min, followed by the addition of 40 µL of 1 mM 4-nitrophenyl-α-D-glucopyranoside solution (in 50 mM sodium phosphate buffer with pH 6.8), before the mixture was again subjected to incubation at 37 ºC for another 30 min. The enzymatic reaction was stopped by adding 100 µL of 1 M Na_2CO_3. The absorbance was measured at 400 nm using a Multimode Plate Reader (EnSpire, Perkin-Elmer, Singapore), and 1-deoxynojirimycin (Sigma-Aldrich) was used as a reference (1.6 - 200 $\mu g.mL^{-1}$).

α-Amylase enzyme assay

The α-amylase (E.C. 3.2.1.1) inhibition was conducted following the method described by Bernfeld (1955) with minor modifications (Rodrigues *et al*., 2017). In brief, 20 µL of sample or standard, 50 µL of 40 $U.mL^{-1}$ α-amylase from porcine pancreas (500 $KU.mg^{-1}$ solid, Sigma-Aldrich) (in 0.02 M sodium phosphate buffer containing 6.7 µM NaCl, pH 6.8) and 930 µL of phosphate buffer (pH 6.8, containing 6.7 µM NaCl) were mixed and pre-incubated at 25 ºC by 30 min. Then, a 250 µL aliquot of the pre-incubated mixture was added to a tube containing 500 µL of 1% starch solution and 250 µL of phosphate buffer, followed by incubation at 40 ºC by 20 min. Then, 500 µL of 33 mM 3,5-dinitrosalicylic acid (containing 350 mM of sodium hydroxide; 770 mM of potassium sodium tartrate and 57 mM of sodium bisulfite) was added before the mixture was placed in a boiling water bath for 5 min. The mixture was cooled in an ice bath for 15 min. The reaction mixture was then diluted with 4.5 mL of distilled water, and the absorbance was detected at 540 nm in a UV-VIS spectrophotometer instrument (Evolution™ 60S UV-Visible Spectrophotometer, Thermo Fisher Scientific, USA). Acarbose (Sigma-Aldrich) was used as a positive control (0.8 - 50 µg.mL).

Acetylcholinesterase inhibitory activity

Acetylcholinesterase (E.C. 3.1.1.7, AChE) inhibition activity was measured as described by (López *et al*., 2002) with modifications. Briefly, 50 µL of phosphate buffer (containing 8 mM K_2HPO_4, 2.3 mM NaH_2PO_4 and 0.15 M NaCl, pH 7.6), 50 µL of 0.25 $U.mL^{-1}$ AChE from *Electrophorus electricus* type IV (137 $U.mg^{-1}$ solid, Sigma Aldrich) in buffer phosphate, and 50 µL of the sample or standard were added to each well of a 96-well plate. Then, the plates were incubated for 30 min at room temperature. Subsequently, 100 µL of substrate solution (containing 0.24 mM of acetylthiocholine iodide, 0.2 mM of 5,5'-dithiobis[2-nitrobenzoic acid], and 0.04 M of Na_2HPO_4) was added to each well and incubated for 10 min. The absorbance was determined at 405 nm using a Multimode Plate Reader (EnSpire, Perkin-Elmer, Singapore). Galanthamine was used as a positive control (0.03 – 2 $\mu g.mL^{-1}$).

Statistical analyses

The inhibition activity was expressed as a percentage inhibition of enzyme activity. All analyses were performed in triplicate (two independent experiments), and data were expressed as the mean ± standard deviation. However, to calculate IC_{50}, only extracts presenting at least 70% inhibition at 100 $\mu g.mL^{-1}$ were considered. The inhibition curves to determine the enzyme activity were plotted and IC_{50} values were obtained using GraphPad 6.0 software.

RESULTS AND DISCUSSION

Chemical evaluation

GC-MS analysis was applied to identify alkaloids from the crude extract and the alkaloid-enriched fraction of the species *H. puniceum*. The GC-MS approach revealed that the *n*-Hexanic extract had low alkaloid content, showing only lycoramine (**2**) as an identified alkaloid (Table I). Notwithstanding, sixteen alkaloids were detected from the ethyl acetate extract and the comparison with the in-home spectral database allowed the identification of fifteen compounds. Lycorine (**12**) was the major component and the alkaloids pseudolycorine (**13**), 9-*O*-demethyllycoramine (**3**) and pancratinine C (**7**) were found in significant proportion at 15.3, 13.4 and 10.4%, respectively (Table I). Lycoramine was again detected in the ethyl acetate extract (7.4%) and the crinane compounds 8-*O*-demethylmaritidine (**4**) and 11-hydroxyvittatine (**11**) derivatives along with pancracine (**10**) were found in proportions of 2 to 6%. The absolute configuration of the compounds **4** and **11**

cannot be achieved by GC-MS. Considering the very minor components, galanthamine (**1**) was detected as a trace compound, as well as tazettine/pretazettine (**8**/**9**), kirkine (**5**), assoanine (**6**) and the homolycorine derivatives 9-*O*-demethyl-2α-hydroxyhomolycorine (**15**) and 2α-hydroxyhomolycorine (**14**). The most striking EI-MS fragmentation pattern of homolycorine-type derivatives is a base peak from a retro-Diels-Alder rearrangement at ring C. The base peak corresponds to the pyrrolidine ring together with any substituent at C-2, and the other less abundant fragment encompasses the aromatic lactone or hemilactone moiety. There is a notably low abundance of the molecular ion peak in all alkaloids with a double bound $\Delta^{3,4}$ (Bastida *et al*., 2006). A very slight signal was detected as a homolycorine-type derivative even though its identification was not possible using only GC-MS (*undefined homolycorine-type alkaloid*, Table I). Further collection of plant material and guided purification might provide isolation and complete characterization conditions using other spectroscopy techniques for this homolycorine-type derivative. Worth mentioning is the quantification of pretazettine as tazettine due to its well-known epimerization after acid-base extraction or under GC-MS conditions (de Andrade *et al*., 2012).

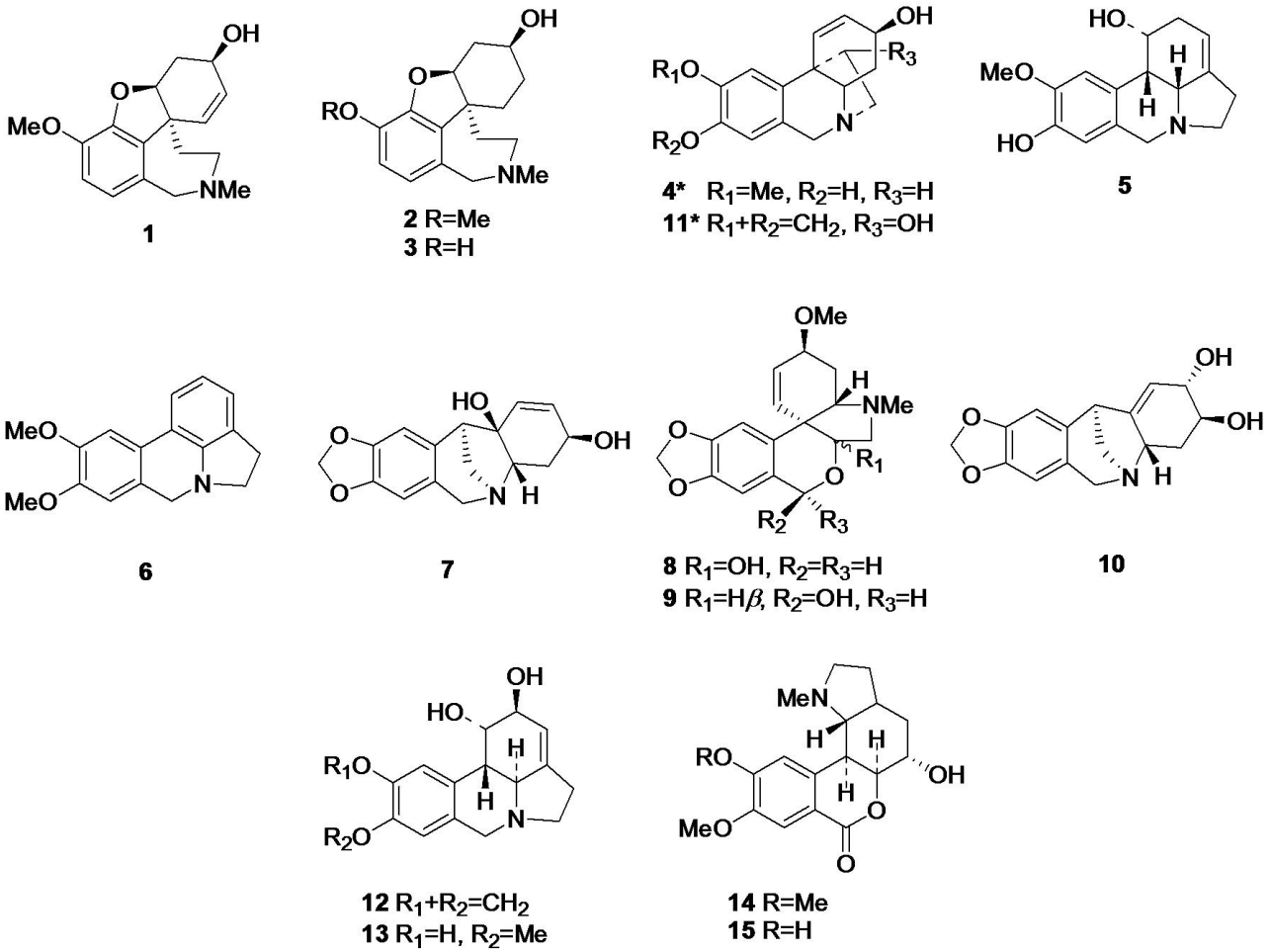

FIGURE 2 – Alkalois identified from *Hippeastrum puniceum*.
* the absolute configuration of compounds 4 and 11 cannot be achieved by GC-MS

TABLE I – GC–MS data for *Hippeastrum puniceum*. Values are expressed as a relative percentage of total ion current (TIC)

Alkaloid	RI (Retention index)	Enriched fractions (%)			M^+	MS
		Crude	*n*-Hex	EtOAc		
Galanthamine **(1)**	2395	-	-	tr	287(83)	286(100), 270(13), 244(24), 230(12), 216(33), 174(27), 115(12)
Lycoramine **(2)**	2422	1.0	7,7	7.4	289 (61)	288(100), 232(8), 202(14), 188(13), 128(12), 115(19), 77(8)
9-*O*-Demethyllycoramine **(3)**	2463	3.8	-	13.4	275 (55)	274(100), 174(10), 173(14), 145(10), 131(11), 115(14), 77(10), 44(35)
8-*O*-Demethylmaritidine derivative **(4)**	2510	-	-	2.8	273(100)	256(22), 230(22), 201(83), 189(42), 174(22), 128(23), 115(24)
Kirkine **(5)**	2534	-	-	tr	273(<1)	253(52), 252(100), 237(14), 222(3), 209(16), 180(6), 110(4)
Assoanine **(6)**	2580	-	-	tr	267(50)	266(100), 250(22), 222(12), 180(12), 154(5), 126(5), 96(5)
Pancratinine C **(7)**	2584	tr	-	10.4	287 (48)	203(41), 188(54), 176(100), 175(69), 174(98), 148(70), 89(24), 77(25)
Tazettine **(8)**/Pretazettine **(9)**	2653	-	-	tr	331 (31)	316(15), 298(23), 247(100), 230(12), 201(15), 181(11), 152(7)
Pancracine **(10)**	2718	-	-	4.3	287(100)	286(23), 270(18), 243(20), 223(30), 199(45), 185(50), 128(11), 115(13)
11-Hydroxyvittatine derivative **(11)**	2728	-	-	5.7	287(5)	258(100), 211(15), 186(20), 181(23), 153(13), 128(24), 115(23)
Lycorine **(12)**	2746	-	-	21.5	287 (31)	286(19), 268(24), 250(15), 227(79), 226(100), 211(7), 147(15)
Pseudolycorine **(13)**	2823	-	-	15.3	289(23)	270(21), 252(12), 228(100), 214(10), 147(17), 111(18), 82(10)
2-α-Hydroxyhomolycorine **(14)**	2870	-	-	tr	345(<1)	125(100), 124(8), 96(35), 81(3), 77(2), 67(2), 53(3)
9-*O*-Demethyl-2α-hydroxyhomolycorine **(15)**	2980	-	-	tr	319(<1)	125(100), 96(65), 81(2), 77(2)
Undefined – Homolycorine-type derivative	3099	2.7	-	-	nd	151(3), 126(8), 125(100), 97(3), 96(35), 82(3), 81(3)
Neutral metabolites	-	91.6	92.3	14.2		-

Crude: crude methanolic extract, *n*-Hex: *n*-hexane extract, EtOAc: ethyl acetate extract; tr: traces (less than 1% of TIC); nd: not detected; neutral metabolites: terpenes, fatty acids, sterols, among others

The metanolic extract of bulbs from *Hippeastrum puniceum* was chromatographed over sílica gel column and providing four substances: lycoramine (**2**), 9-*O*-demethyllycoramine (**3**), tazettine (**8**) and 9-*O*-demethyl-2 α-hydroxyhomolycorine (**15**) (Figure 1). Compounds **2**, **8** and **15** were identified by comparison of ^{1}H-NMR chemical shifts (chemical shift (δ) is in ppm and *J* values in Hertz (Hz)) with data reported in BASTIDA *et al*., 2006 and compound **3** was identified by comparison of ^{1}H-NMR chemical shifts with data reported in KIHARA *et al.* (1991).

Lycoramine (**2**) ^{1}H-NMR ($CDCl_3$. 400 MHz): δ 4.46 s (H-1); 4.16 s (H-2); 5.54 s (H-3); 2.87 d (10.8, H-4a); 3.53 brd (13.9, H-6α); 4.11 d (14.1, H-6β); 6.64 s (H-7); 6.88 s (H-10); 2.70 m (H-10b); 2.52 - 2.67 m (H-11α and H-11β); 2.43 m (H-12α); 3.33 m (H-12β); 5.91 s (OCH_2O).

9-*O*-demethyllycoramine (**3**) ^{1}H-NMR ($CDCl_3$. 400 MHz): 1.50 – 2.00 m (H-2α, H-4, H-4' and H-11); 2.30 s (NMe); 2.45 d (15.2, H-2β); 2.98 d (15.4, H-12α); 3.14 t (13.2, H-12β); 3.62 d (15.2, H-6); 4.00 d (15.2, H-6'); 4.12 m (H-3); 4.36 m (H-1); 6.52 d (8.0, H-8); 6.62 d (8.0, H-7).

Tazettine (**8**) ^{1}H-NMR ($CDCl_3$. 400 MHz): 5.61 ddd (10.4; 1.9 and 1.5, H-1); 6.14 ddd (10.4; 2.0 and 1.5, H-2); 4.13 m (H-3); 2.87 m (H-4a); 2.23 m (H-4α); 1.62 ddd (13.6; 10.1 and 2.4, H-4β); 4.63 d (14.8, H-6α); 4.95 dd (14.7 and 0.6, H-6β); 6.49 s (H-7); 6.85 s (H-10); 2.68 d (10.6, H-12α); 3.31 d (10.6, H-12β); 2.40 s (NMe); 5.89 s (OCH_2O).

9-*O*-demethyl-2-α-hydroxyhomolycorine (**15**) ^{1}H-NMR ($CDCl_3$. 400 MHz): 4.54 d (4.8, H1); 4.10 m (H2); 5.74 s (H3); 2.62 d (9.0, H-4a); 7.48 s (H-7); 7.21 s (H-10); 2.87 dd (9.0 and 1.2, H-10b); 2,45-2,62 m (H-11); 3.12 m (H-12α); 2.35 m (H-12β); 3.94 s (OMe); 2.02 s (NMe).

Biological results

The alkaloid-enriched ethyl acetate fraction and the crude extract along with the purified alkaloids lycorine and tazettine were tested against the enzymes tyrosinase, α-glucosidase, α-amylase and AChE (Table II). Both the extracts and the isolated compounds showed no significant inhibitory activity against the targets tyrosinase, α-glucosidase and α-amylase at a concentration of 100 $mg.mL^{-1}$. The results were not used for IC_{50} calculations. Nevertheless, the ethyl acetate extract and the crude extract showed an important inhibitory AChE activity at 100 $mg.mL^{-1}$. The IC_{50} for ethyl acetate and crude extracts were 21.97 $mg.mL^{-1}$ (±1.05) and 60.24 $mg.mL^{-1}$ (±3.14), respectively (Table III). The IC_{50} of *H. puniceum* ethyl acetate extract is comparable to the alkaloid-enriched fraction of the Turkish *Galanthus elwesii* (Buzkourt *et al*., 2017) and the Argentinean *Hieronymiella clidanthoides* species (Ortiz *et al*., 2018). The first species belongs to the very important Amaryllidaceae genus in terms of producing remarkable quantities of galanthamine (Bozkurt *et al*., 2017) and the genus *Hieronymiella*, which comprises only five species to date, has shown significant AChE inhibition ability, related at least in part by the content of galanthamine and sanguinine alkaloids (Ortiz *et al*., 2018). In the case of the highest-activity EtOAc extract from *H. puniceum*, the compound 9-*O*-demethyllycoramine (13.4% of TIC) displays a phenolic group at C-9 instead of the methoxyl group, as in galanthamine. It has been confirmed that a higher degree of polarization of the hydroxyl function is favourable for better interactions with O^{γ} of Ser200 and $N^{\varepsilon 2}$ of His440 in the AChE gorge, due to hydrogen bonding acceptor and donor, respectively (Bartolucci *et al*., 2001).

TABLE II – Percentage of inhibition of the samples tested at 100 µg.mL

Samples tested	Inhibition (%)			
	AChE	**Tyr**	***α*-Glu**	***α*-Amyl**
Crude	72.96±1.26	17.35±1.84	24.46±2.19	10.30±1.05
EtOAc	78.31±1.04	17.18±2.03	26.71±1.89	7.01±0.56
Lycorine **(12)**	n.d.	18.31±1.53	27.76±2.08	13.79±1.26
Tazettine **(8)**	n.d.	14.03±1.07	34.75±3.12	11.51±0.94

Crude: crude methanolic extract; EtOAc: ethyl acetate extract; AChE: acetylcholinesterase, Tyr: tyrosinase, α-Glu: α-Glucosidase and α-amyl: α-amylase. Data were expressed as the mean ± standard deviation. n.d: not determined. Kojic acid: IC_{50} 3.34±0.36 µg.mL (reference for tyrosinase inhibitory activity); 1-deoxynojirimycin: IC_{50} 31.32±2.43 µg.mL (reference for α-glucosidase inhibitory activity); acarbose: IC_{50} 4.11±2.43 µg.mL (reference for α-amylase inhibitory activity)

TABLE III – Percentage of inhibition of the samples tested at 100 µg.mL

Samples tested	IC_{50} (µg.mL)[a]
Crude	60.24±3.14
EtOAc	21.97±1.05
Galanthamine[b]	0.139±0.018

Crude: crude methanolic extract; EtOAc: ethyl acetate extract; [a] IC_{50} values correspond to the average of two independent experiments in triplicate. Data were expressed as the mean ± standard deviation; [b] Compound used as positive control for AChE inhibition screening

The Brazilian species *Hippeastrum puniceum* has been shown to be able to biosynthesize alkaloids from galanthamine-, lycorine-, homolycorine-, haemanthamine-, tazettine- and montanine-type skeletons. In summary, sixteen alkaloids were detected, emphasizing the high content of lycorine, pseudolycorine and the galanthamine derivative 9-*O*-demethyllycoramine. Although the alkaloid-rich extracts and isolated compounds lycorine and tazettine did not show a significant capability to inhibit the enzymes tyrosinase, α-glucosidase and α-amylase, the ethyl acetate and crude extract showed remarkable AChE inhibitory activity at a similar magnitude to the other well-known genus in producing galanthamine-type compounds. The AChE activity might be explained by the presence of galanthamine derivatives, even though montanine-type skeleton has already demonstrated activity as AChE inhibitors. The demand for renewable sources of new cholinesterases inhibitors has prompted the study of the species *H. puniceum*. New trends towards the use of natural extracts as medicines instead of pure drugs have recently increased and might be promising in terms of the study of the *Hippeastrum* genus as a source of bioactive compounds.

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