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# Alkaloids of *Phaedranassa dubia* (Kunth) J.F. Macbr. and *Phaedranassa brevifolia* Meerow (Amaryllidaceae) from Ecuador and its cholinesterase-inhibitory activity

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## ABSTRACT

Alzheimer's disease is considered the most common cause of dementia and, in an increasingly aging population worldwide, the quest for treatment is a priority. Amaryllidaceae alkaloids are of main interest because of their cholinesterase inhibition potential, which is the main palliative treatment available for this disease. We evaluated the alkaloidal profile and the *in vitro* inhibitory activity on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) of bulb alkaloid extract of *Phaedranassa dubia* and *Phaedranassa brevifolia* collected in Ecuador. Using gas chromatography coupled to mass spectrometry (GC-MS), we identified typical Amaryllidaceae alkaloids in these species, highlighting the presence of lycorine-type alkaloids in *P. dubia* and haemanthamine/crinine-type in *P. brevifolia*. The species *P. dubia* and *P. brevifolia* showed inhibitory activities against AChE (IC<sub>50</sub> values of  $25.48 \pm 0.39$  and  $3.45 \pm 0.29 \mu\text{g.mL}^{-1}$ , respectively) and BuChE (IC<sub>50</sub> values of  $114.96 \pm 4.94$  and  $58.89 \pm 0.55 \mu\text{g.mL}^{-1}$ , respectively). Computational experiments allowed us to understand the interactions of the alkaloids identified in these samples toward the active sites of AChE and BuChE. *In silico*, some alkaloids detected in these Amaryllidaceae species presented higher estimated binding free energy toward BuChE than galanthamine. This is the first study about the alkaloid profile and biological potential of *P. brevifolia* species.

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## 1. Introduction

Alzheimer's disease is a type of progressive and irreversible dementia, showing memory loss as an early symptom (Konrath et al., 2013; Gabriel et al., 2017). Although its etiology is still not clear, multiple factors are known to cause this pathology, such as the deterioration of cholinergic neurotransmission in the brain caused by a

decrease in acetylcholine levels (Konrath et al., 2013; Gabriel et al., 2017). This disorder is considered the most common cause of dementia (WHO, 2019). Most of the pharmacological treatments used for this disease are focused on the inhibition of the hydrolytic enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) that play a fundamental role in the cholinergic neurotransmission in the brain by reducing the metabolism of acetylcholine (Li et al., 2017). Studies have shown that a significant number of compounds produced by plants inhibit acetylcholinesterase and some of them are used as treatments of Alzheimer's disease (Konrath et al., 2013).

The Amaryllidaceae plant family produces specific alkaloids with a broad range of physiological effects, among them is the central nervous system action, which often is correlated with the inhibitory activity of AChE (Cortes et al., 2015). Amaryllidaceae species have become a well-known source of alkaloids that inhibit AChE and to a

**Abbreviations:** AChE, Acetylcholinesterase; AE, alkaloid extract; ATCI, acetylthiocholine iodide; BTCl, butyrylthiocholine iodide; BuChE, butyrylcholinesterase; CD, circular dichroism; DTNB, (5,5'-dithio-bis-[2-nitrobenzoic acid]); EtOAc, ethyl acetate; Et<sub>2</sub>O, diethyl ether; GAL, galanthamine; GC-MS, gas chromatography coupled to mass spectrometry; IUCN, International Union for Conservation of Nature; MeOH, methanol; MS, mass spectrometry; NMR, nuclear magnetic resonance; UV, ultraviolet

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lesser extent, BuChE (Ortiz et al., 2016). The alkaloid galanthamine, specific to this plant family, is a competitive inhibitor of AChE, currently used for the palliative treatment of Alzheimer's disease (López et al., 2002). Work in medicinal chemistry today focused on the quest for new inhibitors of both cholinesterases with multifunctional activities that can potentially be used in the treatment of Alzheimer's disease (Huang et al., 2014).

There are 36 species of Amaryllidaceae family in Ecuador (from Amaryllidoideae subfamily), 15 of which are endemic to the country (Meerow, 1990; Oleas, 2011a; Minga et al., 2015). The genus *Phaedranassa* Herb. includes eleven species, eight are found in Ecuador, three in Colombia and one Costa Rica (Meerow, 1990; Oleas, 2011b; Minga et al., 2015; Jiménez et al., 2018). *Phaedranassa* are mountainous species, often growing along roadsides, cliffs, and other open areas along the inter-andean valleys in northern Andes (Meerow, 1990). These species are geophytes, with bulbs covered by a brown tunic and contractile roots. Their leaves are elliptical or lanceolate, with a sharp apex and a narrow base towards the petiole (Meerow, 1990). *Phaedranassa* species usually flower after the dry summer season (Meerow, 1990; Minga et al., 2015).

*Phaedranassa dubia* (Kunth) J.F. Macbr. (Fig. 1a) commonly known as 'vulture's onion' or 'wolf's potato' is native to Colombia and Ecuador (Meerow, 1990; Oleas, 2011b). In the latter it is found in Carchi, Imbabura, Pichincha and Napo provinces, from 2000 to 4000 m of altitude. This species has a bulb of 6 × 4.5 cm, two to three leaves rarely present in anthesis, petiole up to 10 cm long, six flowers up to 6 cm long, campanulate and tubular, pink tepals, green with yellow towards the margins and with a thin yellow stripe near the ovary, fruits are capsules of up to 15 × 27 mm, with black and shiny seeds (Meerow, 1990). *Phaedranassa brevifolia* Meerow (Fig. 1b) is morphologically the smallest of all the genus, with leaves up to 12 cm long, including petiole (Meerow, 1990). Also, this species is known by only eight populations in an area of 16 km<sup>2</sup> (Oleas et al., 2014). Because of the small number of populations and its restricted distribution area, *P. brevifolia* is categorized as Endangered under IUCN criteria (Oleas 2011a).

This study aimed to evaluate the alkaloidal profile and the cholinesterase potential of *P. dubia* and *P. brevifolia* collected in Ecuador. Gas Chromatography coupled to Mass Spectrometry (GC-MS) has been used to identify the alkaloids present in these samples. The potential of these species in Alzheimer's disease therapeutics was estimated by determining the AChE and BuChE inhibitory activity of both samples. Molecular docking studies were applied to understand the theoretical interactions between the alkaloids identified in *P. dubia* and *P. brevifolia* toward the active sites of AChE and BuChE.

## 2. Materials and methods

### 2.1. Plant material and alkaloid extraction

Bulbs from *P. dubia* (Kunth) J.F. Macbr. were collected in Cuicocha (Imbabura, Ecuador) during the flowering period, while *P. brevifolia* Meerow were collected on the Ibarra – San Lorenzo road (Imbabura, Ecuador), approximately 30 km from Ibarra, the samples were infertile. The samples were authenticated by Dra. Nora Oleas, the person responsible for reviewing herbarium specimens and fresh material (Peñafiel 187,7, 453 MO, Oleas 1010, 1011, 1012 QCNE). Collections and research were approved by the Ministerio del Ambiente del Ecuador under permits MAE-DNB-CM-2015-0054 and MAE-DNB-CM-2018-0086.

Fresh bulbs were cleaned and cut into pieces of approximately 2 cm, then dried at 60°C on RedLine stove (Binder, York, UK) for 48 h. Once the plant material was completely dry, it was ground in a rotating blade mill (Arthur H. Thomas CO). The powdered, dry material was macerated with methanol for 72 h at room temperature, applying ultrasonic baths at intervals of 1 to 2 h. The mash was filtered and

evaporated to dryness under reduced pressure. Crude extract of the bulbs was acidified with H<sub>2</sub>SO<sub>4</sub> (2%, v/v) to pH 2 and extracted with Et<sub>2</sub>O to remove neutral material. The aqueous solution was basified with NH<sub>4</sub>OH (25%) to pH 9 and extracted with EtOAc (alkaloid extracted, AE).

### 2.2. Identification and quantification of Alkaloids by GC-MS

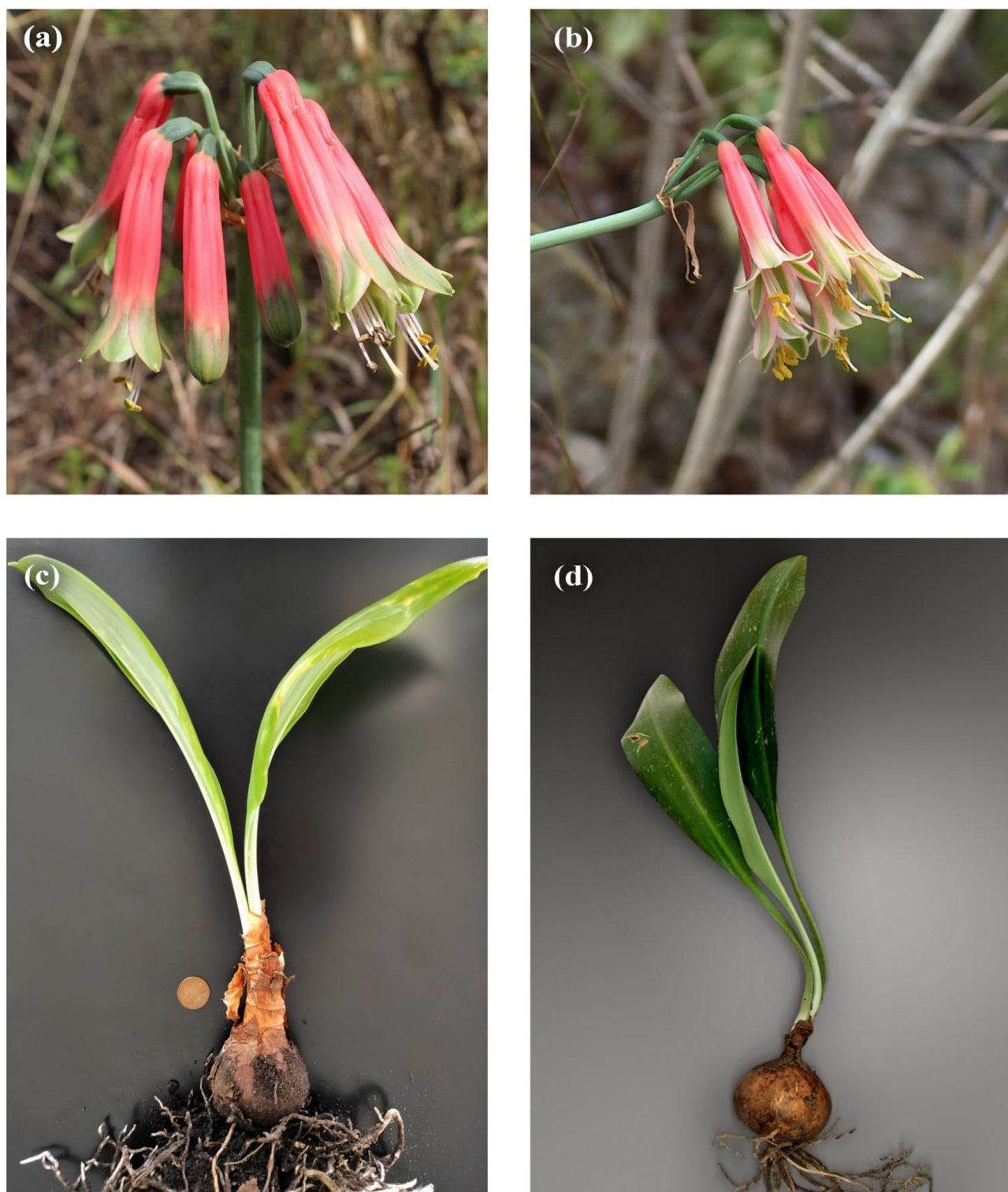
GC-MS data were obtained on an Agilent 6890 N GC 5975 coupled with MSD5975 inert XL, operating in EI mode at 70 eV (Agilent Technologies, Santa Clara, CA, USA). A Sapiens-X5 MS column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. The temperature gradient was as follows: 12 min at 100°C, 100–180°C at 15°C min<sup>-1</sup>, 180–300°C at 5°C min<sup>-1</sup> and 10 min hold at 300°C. The injector and detector temperatures were 250 and 280°C, respectively, and the flow-rate of carrier gas (He) was 1 mL min<sup>-1</sup>. Two mg of each alkaloid extract was dissolved in 1 mL of MeOH:CHCl<sub>3</sub> (1:1, v/v) and 1 μL was injected using the splitless mode. Codeine (0.05 mg mL<sup>-1</sup>) was used as an internal standard in all the samples. The chromatograms were analyzed using the software AMDIS 2.64. The alkaloids were identified comparing their GC-MS spectra and Kovats retention index (RI) values with those of authentic Amaryllidaceae alkaloids previously isolated and identified by spectroscopic and spectrometric methods (NMR, CD, IR, UV, MS) in the Natural Products Laboratory (University of Barcelona), the NIST 05 Database, or literature data.

A calibration curve of galanthamine (10, 20, 40, 60, 80, and 100 μg mL<sup>-1</sup>) was applied to quantify each single constituent detected in the chromatogram, using codeine (0.05 mg mL<sup>-1</sup>) as the internal standard. Peak areas were manually obtained, considering selected ions for each compound (usually the base peak of their MS, i.e., *m/z* at 286 for galanthamine and 299 for codeine). The ratio between the values obtained for galanthamine and codeine in each solution was plotted against the corresponding concentration of galanthamine to obtain the calibration curve and its equation ( $y = 0.0112x - 0.0469$ ;  $R^2 = 0.9995$ ). All data were standardized to the area of the internal standard (codeine), and the equation obtained for the calibration curve of galanthamine was used to calculate the amount of each alkaloid. Results are presented as mg GAL (galanthamine), which was finally related to the alkaloid extract (AE). As the peak area does not only depend on the corresponding alkaloid concentration but also on the intensity of the mass spectra fragmentation, the quantification is not absolute. However, the method is considered suitable to compare the specific alkaloid amount between samples (Torrás-Clavería et al., 2014).

### 2.3. Microplate Assay for AChE- and BuChE-Inhibitory Activities

AChE and BuChE-Inhibitory activities were assayed as described by Ellman et al. (1961) with some modifications by Ortiz et al. (2016). 50 μL of AChE in buffer phosphate (8 mM K<sub>2</sub>HPO<sub>4</sub>, 2.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) and 50 μL of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature before the addition of 100 μL of the substrate solution (acetylthiocholine or butyrylthiocholine, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.5 M DTNB, 0.6 mM ATCI in Millipore water, pH 7.5). The absorbance was read in a ELx800 Biotek microplate reader (Vermont, USA) at 405 nm after three minutes. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor. The inhibitory data were analyzed with the software Prism (Graph Pad Inc., San Diego, CA, USA). IC<sub>50</sub> values are means ± SD of three individual determinations each performed in triplicate.

The enzymes used for the test were AChE of the electric eel *Electrophorus electricus* (C3389) and Equine serum BuChE (C7512), while the substrates in each case were acetylthiocholine iodide (A5751) and butyrylthiocholine iodide (20820). Dithionitrobenzoic acid (DTNB, D-8130) was added to generate the color reaction.



**Fig. 1.** (a) *Phaedranassa dubia* flowers; (b) *P. brevifolia* flowers; (c) *P. dubia* bulb and leaves; (d) *P. brevifolia* bulb and leaves. Photos: Karen Acosta León and Nora Oleas.

Galanthamine was the reference compound. All the reagents of this assay were purchased from Sigma-Aldrich (Missouri, USA).

#### 2.4. Molecular docking

Molecular docking simulations for the principal alkaloids identified from *P.* species were performed to investigate the binding mode into the active site of two different enzymes, *Torpedo californica* AChE

(TcAChE) and human BuChE (hBuChE), PDB codes 1DX6 (Greenblatt et al., 1999) and 4BDS (Nachon et al., 2013). Three-dimensional (3D) structures of the alkaloids were recovered from the PubChem database and submitted to a geometrical optimization procedure at PBE0 (Adamo and Barone, 1999; Ernzerhof and Scuse-ria, 1999)/6–311 + g\* (Petersson et al., 1988) level of theory using the program Gaussian 09 (Frisch et al., 2013). All optimized alkaloids were confirmed as a minimum on the potential energy surface.

Docking simulations for the set of optimized ligands were performed using the AutoDock v.4.2 program (Molecular Graphics Laboratory, La Jolla, USA) (Moris et al., 2009) as described at Moreno et al. (2020).

### 3. Results and discussion

#### 3.1. GC-MS analysis of alkaloids extracts

A total of eleven known alkaloids were identified in both *Phaedranassa* Herb. species (Table 1 and Fig. 2). About 70% of them belong to two different alkaloid types: lycorine- and haemanthamine/crinine-type; and the others belong to three different alkaloid types: galanthamine-, homolycorine- and montanine-type. The occurrence and quantification of the alkaloids in both samples are described in Table 1.

*Phaedranassa dubia* shows higher variety and concentration of lycorine-type alkaloids than *P. brevifolia* (127.63 and 6.21 mg GAL.g<sup>-1</sup> AE, respectively). Seven alkaloids were identified in *P. dubia*: anhydrolycorine (1), caranine (2), 2,4-didehydro-2-dehydroxylycorine (3), 11,12-dehydroanhydrolycorine (4), lycorine (5); galanthamine (9) and 2-hydroxyhomolycorine (10); and four unidentified compounds (Fig. 3a). The major component of this species was lycorine (5), 86.24 mg GAL.g<sup>-1</sup> AE (Table 1).

In general, lycorine-type alkaloids are the most diverse group among Amaryllidaceae alkaloids (Berkov et al., 2020). According to the literature, the compound lycorine (5), a lycorine-type alkaloid, is active against poliovirus, vaccinia smallpox virus and SARS-associated coronavirus (Li et al., 2005; Deng et al., 2007; Hwang et al., 2008). This structure is fatal to the protozoan parasite *Trypanosoma brucei* and it is more potent than indomethacin as an anti-inflammatory agent (Citoglu et al., 1998; Mackey et al., 2006; McNulty et al., 2009). This alkaloid also is known as a potential chemotherapeutic agent which presents antiproliferative potential against different cancer cell lines (Likhitwitayawuid et al., 1993; Lamoral-Theys et al., 2009; Nair and Van Staden, 2018). Cholinesterases activities appears

to be associated with the presence of two hydroxyl groups in some alkaloids with this structural type (Bastida et al., 2011).

The alkaloid anhydrolycorine (1) also was detected in *P. dubia* extract (19.19 mg GAL.g<sup>-1</sup> AE). This structure is derived from lycorine-type alkaloids that contains several biological properties like the ability to inhibit the biosynthesis of ascorbic acid, the inhibition of growth and cell division in superior plants, algae, and yeasts (Bastida et al., 2006). The most important characteristic of anhydrolycorine (1) probably is its antitumor activity (Lamoral-Theys et al., 2009; Guo et al., 2016).

The second most abundant alkaloid identified in *P. dubia* was galanthamine (9) (23.31 mg GAL.g<sup>-1</sup> AE). This compound is a very important Amaryllidaceae alkaloid which was approved by FDA in 2001 and it is commercially used in the palliative therapy for mild-moderate Alzheimer's disease as a relatively selective inhibitor of acetylcholinesterase (Maelicke et al., 2001; Konrath et al., 2013). Galanthamine is still isolated from the bulbs of different Amaryllidaceae genera, such as *Narcissus* and *Leucojum*, by pharmaceutical companies that have generated an exponential interest in the isolation and characterization of alkaloids from the bulbs of several species of this plant family (Berkov et al., 2009).

The only homolycorine-type compound identified in this *P. dubia* sample was 2-hydroxyhomolycorine (10). Structures belonging to this alkaloid-type have demonstrated high biological potential as antiproliferative agents and could be a promising source of antitumor structures for cancer therapy (Chen et al., 2016). Also, this structure-type presents potential as antimalarial agent (Cedron et al., 2013).

It was not possible to identified four compounds detected in *P. dubia* by GC-MS (Table 1, Fig. 3a). The compound 13, *m/z* 266 [*M*<sup>+</sup>=267] (RI 2891.9) occurs in high quantity in this species (46.05 mg GAL.g<sup>-1</sup> AE) and probably belongs to lycorine-type alkaloids according to its fragmentation pattern, similar to other unidentified structures observed in this sample.

In a previous study, a similar alkaloid profile was reported for another *P. dubia* sample collected in Ecuador at the Pichincha

**Table 1**  
Alkaloids identified in *P. dubia* and *P. brevifolia* collected in Ecuador by GC-MS. Values in mg GAL.g<sup>-1</sup> AE.

| Alkaloid                               | [ <i>M</i> <sup>+</sup> ] | BP  | Rt     | RI     | <i>P. dubia</i> | <i>P. brevifolia</i> |
|----------------------------------------|---------------------------|-----|--------|--------|-----------------|----------------------|
| Lycorine-type                          |                           |     |        |        | 127.63          | 6.21                 |
| anhydrolycorine (1)                    | 251                       | 250 | 25.133 | 2544.1 | 19.19           | -                    |
| caranine (2)                           | 271                       | 226 | 25.413 | 2562.2 | 5.67            | -                    |
| 2,4-didehydro-2-dehydroxylycorine (3)  | 269                       | 250 | 25.608 | 2574.9 | 7.08            | -                    |
| 11,12-dehydroanhydrolycorine (4)       | 249                       | 248 | 26.655 | 2645.7 | 9.45            | -                    |
| lycorine (5)                           | 287                       | 226 | 29.060 | 2812.4 | 86.24           | 6.21                 |
| Crinine/haemanthamine-type             |                           |     |        |        |                 | 21.54                |
| vittatine (6a)/ crinine (6b)           | 271                       | 271 | 23.480 | 2469.3 | -               | 5.44                 |
| haemanthamine (7a)/ crinamine (7b)     | 301                       | 272 | 25.849 | 2628.1 | -               | 5.03                 |
| 11-hydroxivittatine (8a)/ hamayne (8b) | 287                       | 258 | 26.961 | 2702.7 | -               | 11.07                |
| Galanthamine-type                      |                           |     |        |        | 23.31           | 3.36                 |
| galanthamine (9)                       | 287                       | 286 | 23.418 | 2432.9 | 23.31           | 3.36                 |
| Homolycorine-type                      |                           |     |        |        | 6.35            | -                    |
| 2-hydroxyhomolycorine (10)             | 317                       | 125 | 30.644 | 2922.2 | 6.35            | -                    |
| Montanine-type                         |                           |     |        |        |                 | 8.11                 |
| pancratinine C (11)                    | 287                       | 174 | 25.168 | 2582.4 | -               | 8.11                 |
| Not identified                         |                           |     |        |        | 66.04           | 67.61                |
| NI, (lycorine-type)* (12)              | 285                       | 272 | 24.603 | 2509.7 | 8.06            | -                    |
| NI, (lycorine-type)* (13)              | 267                       | 266 | 30.207 | 2891.9 | 46.05           | -                    |
| NI, (lycorine-type)* (14)              | 281                       | 280 | 31.586 | 2987.6 | 5.76            | -                    |
| NI, (lycorine-type)* (15)              | 265                       | 264 | 31.699 | 2995.4 | 6.17            | -                    |
| NI, (crinine/haemanthamine type)* (16) | 303                       | 303 | 23.808 | 2491.3 | -               | 52.36                |
| NI, (crinine/haemanthamine type)* (17) | 273                       | 201 | 23.904 | 2497.8 | -               | 3.59                 |
| NI, (homolycorine-type)* (18)          | 301                       | 109 | 28.285 | 2791.4 | -               | 9.00                 |
| NI, (homolycorine-type)* (19)          | 315                       | 125 | 29.406 | 2866.6 | -               | 2.66                 |
| Total                                  |                           |     |        |        | 223.33          | 106.83               |

\* proposed structure-type according to the fragmentation pattern; BP: base peak; Rt: retention time; RI: Kovats Retention Index; NI: not identified.

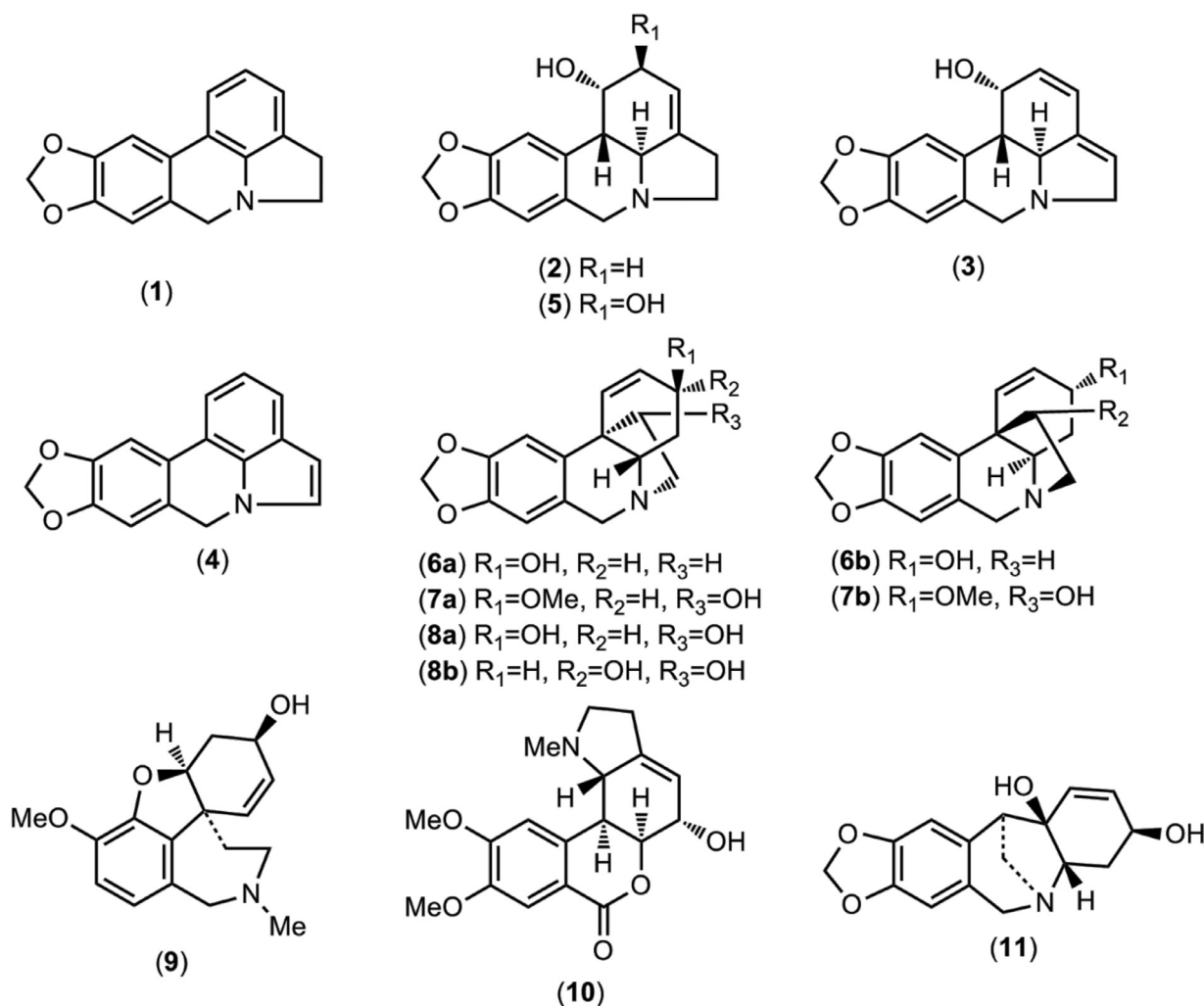


Fig. 2. Alkaloids identified in *P. dubia* and *P. brevifolia* collected in Ecuador by GC-MS.

(Pululahua) locality (Moreno et al., 2020). The authors described the presence of anhydrolycorine, 11,12-dehydroanhydrolycorine, lycorine, galanthamine, *N*-demethylgalanthamine and one unidentified compound,  $m/z$  268 [ $M^+=329$ ] in this species (Moreno et al., 2020). In another work, a different alkaloidal composition was reported for *P. dubia* collected in Colombia, which presented seven alkaloids: phaedaranamine, haemanthamine, pseudolycorine, ungeremine, zefbetaine, sanguinine, galanthamine and epinorgalanthamine

(Osorio et al., 2010). Differences in the occurrence and quantification of alkaloids also have been reported among *Lapiedra martinezii* species collected in different Spain localities, as well as among *Rhodophiala andicola* species collected in different Chilean localities (Ríos et al., 2013; Tallini et al., 2018).

According to Table 1, a total of six alkaloids have been identified in *P. brevifolia*: lycorine (5), vittatine (6a)/crinine (6b), haemanthamine (7a)/crinamine (7b), 11-hydroxyvittatine (8a)/hamayne (8b), galanthamine (9) and pancratinine C (11). It was not possible to identify four compounds detected in this species (Fig. 3b). The compound 16,  $m/z$  303 [ $M^+=303$ ], which has been not identified, was the major structure detected in this species (52.36 mg GALg<sup>-1</sup> AE) and probably belongs to haemathamine/crinine-type alkaloids according to its fragmentation pattern. The second highest amount compound was 11-hydroxyvittatine (8a)/ hamayne (8b), 11.07 mg GALg<sup>-1</sup> AE. Often, nuclear magnetic resonance (NMR) and circular dichroism (CD) analyses are required to confirm the structures of alkaloids belonging to the haemanthamine/crinine-type. In this work, the alkaloid profiles of the samples have been evaluated only by GC-MS, so that haemanthamine/crinine-type alkaloids have been described as isomers (see Table 1 and Fig. 2). This alkaloid-type was the most representative in this sample (21.54 mg GALg<sup>-1</sup> AE). This structure-group has interesting potential as antiproliferatives against different tumoral cells (Bastida et al., 2006; Cedrón et al., 2015; Doscocil et al., 2015). The alkaloids vittatine (6a) and crinine (6b) have shown cytotoxic activities in cancer cells and haemanthamine (7a) has been studied

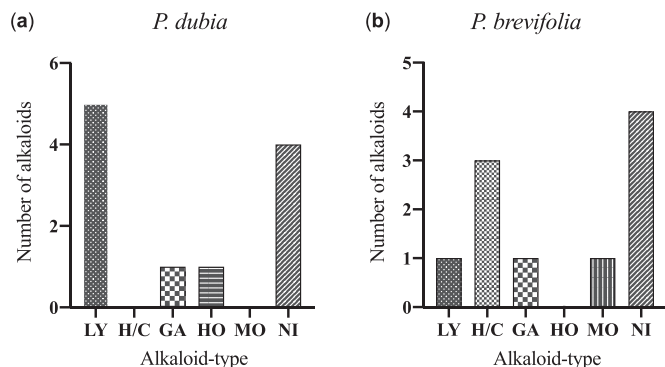


Fig. 3. Number of alkaloids detected in *P. dubia* (a) and *P. brevifolia* (b) separated by alkaloid-type. LY: lycorine-type; H/C: Haemanthamine/crinine-type; GA: galanthamine-type; HO: homolycorine-type; MO: montanine type; NI: not identified.

as a novel anticancer agent due its ability to overcome cancer cell resistance to apoptosis (Nair and Staden, 2018; Pellegrino et al., 2018). The alkaloid profile of *Stenomesson aurantiacum* (Amaryllidaceae) species collected in Ecuador showed the presence of twelve alkaloids in the bulbs of this species by GC-MS, among them was haemanthamine, which was the most abundant (Acosta et al., 2014).

Lycorine (**5**), galanthamine (**9**) and pancratinine C (**11**) (6.21, 3.36 and 8.11 mg GAL.g<sup>-1</sup> AE, respectively) also have been identified in the alkaloid extract of *P. brevifolia* by GC-MS (Table 1). The compound pancratinine C (**11**) belongs to the montanine-type alkaloids, which have interesting biological proprieties, such as anxiolytic, antidepressive, anticonvulsive, antitumoral and anti-inflammatory effects (Da Silva et al., 2006; Cedrón et al., 2015; Farinon et al., 2017; Govindaraju et al., 2018).

### 3.2. AChE and BChE inhibitory activity

Alkaloid extracts of *P. dubia* and *P. brevifolia* showed inhibitory activities against AChE with IC<sub>50</sub> values of 25.48 ± 0.39 and 3.45 ± 0.29 µg.mL<sup>-1</sup>, respectively (Fig. 4). Comparing the results obtained for both *Phaedranassa* alkaloid extracts with galanthamine, the greater inhibitory activity of the reference compound is remarkable, thus corroborating the chemical affinity of this alkaloid in pure form towards cholinesterases (Cortes et al., 2015).

Regarding the inhibitory activity on BuChE, the alkaloid extracts of *P. dubia* and *P. brevifolia* showed IC<sub>50</sub> values of 114.96 ± 4.94 and 58.89 ± 0.55 µg.mL<sup>-1</sup>, respectively (Fig. 4). According to Ortiz et al. (2016), the alkaloids of Amaryllidaceae family usually inhibit BuChE to a lesser extent than AChE. It could allow us to explain the difference in IC<sub>50</sub> values produced by *Phaedranassa* Herb. species for both cholinesterases, being greater the inhibition of AChE. Although it has been proven that BuChE has the capacity to temporarily replace inhibited AChE and thus hydrolyze the accumulated acetylcholine, research focused on BuChE inhibition is still scarce (Walsh et al., 2011; Gabriel et al., 2017).

The AChE and BuChE results obtained for *P. dubia* sample contrast with Moreno et al. (2020), which obtained IC<sub>50</sub> values of 2.20 ± 0.08 and 14.26 ± 2.71 µg.mL<sup>-1</sup>, respectively, for the same species. Using computational experiments, the authors explained that the presence of two alkaloids with a high-energy ligand-protein interaction, galanthamine and *N*-demethylgalanthamine, in this sample indicates that *in vitro* results could have been enhanced by a synergistic mechanism (Moreno et al., 2020). Difference in cholinesterase inhibitory activity also have been reported among *Rhodophiala andicola* species collected in different Chile localities (Tallini et al., 2018).

It has been observed that *P. brevifolia* presented stronger inhibitory activity against both cholinesterases than *P. dubia* (Fig. 4). The

difference between the alkaloid profile of these *Phaedranassa* species (Table 1), represented mainly by the high amounts of lycorine-type and haemanthamine/crinine-type alkaloids in *P. dubia* and *P. brevifolia*, respectively, as well as by the difference between the profile of unidentified compounds detected in these species, could be correlated with their different cholinesterase inhibitory ability.

### 3.3. Molecular docking

Eleven Amaryllidaceae alkaloids were identified in *P. dubia* and *P. brevifolia* extracts by GC-MS and both species presented inhibitory activity against AChE and BuChE. Computational experiments were carried out to better explore these results. The theoretical affinity of the alkaloids identified in both samples toward the active sites of AChE and BuChE together with values reported in the literature are listed in Table 2.

Based on the Amaryllidaceae alkaloids identified in *P. brevifolia* extract, the best estimated free energy of binding to AChE enzyme was the alkaloid crinine (**6b**), with theoretical values very close to haemanthamine (**7a**) and crinamine (**7b**) molecules (see Table 2). A synergistic effect among different alkaloids could be expected, increasing the enzymatic inhibition of the AChE enzyme (Cortes et al., 2018). To understand the different interactions responsible for stabilizing the alkaloid crinine (**6b**) in the gorge of the active site of AChE, a ligand-protein interaction diagram for the galanthamine (**9**) and crinine (**6b**) alkaloids is presented in Fig. 5a and 5b, respectively. *In silico*, the crinine (**6b**) structure is stabilized at the active site of the AChE enzyme by one hydrogen bridge with the His440 residue at 2.0 Å. Furthermore, inhibition of AChE is enhanced by the hydrophobic interactions of crinine (**6b**) with Phe330, Tyr130 and Ile444 residues, similar to those seen for galanthamine (**9**) (Fig. 5).

Molecular simulation of ten alkaloids identified in *P. dubia* and *P. brevifolia* on 4BDS structure theoretically showed higher enzymatic inhibition against BuChE than galanthamine (**9**). These were caranine (**2**), 2,4-didehydro-2-dehydroxylycorine (**3**), lycorine (**5**), vittatine (**6a**), crinine (**6b**), haemanthamine (**7a**), crinamine (**7b**), 11-hydroxyvittatine (**8a**), hamayne (**8b**) and 2-hydroxyhomolycorine (**10**) (Table 2). Vittatine (**6a**) and crinine (**6b**) showed higher theoretical inhibitory activity against BuChE than galanthamine (**16**) by 1.09 and 0.91 kcal.mol<sup>-1</sup>, respectively. The docking results obtained for vittatine (**6a**) suggest that the presence of a hydroxyl group at C-3 position and/or a 5,10b-ethano bridge, both in an  $\alpha$ -orientation, increase the BuChE inhibition on the 4BDS structure in comparison with crinine (**6b**) by 0.18 kcal.mol<sup>-1</sup>. It is possible to confirm the presence of

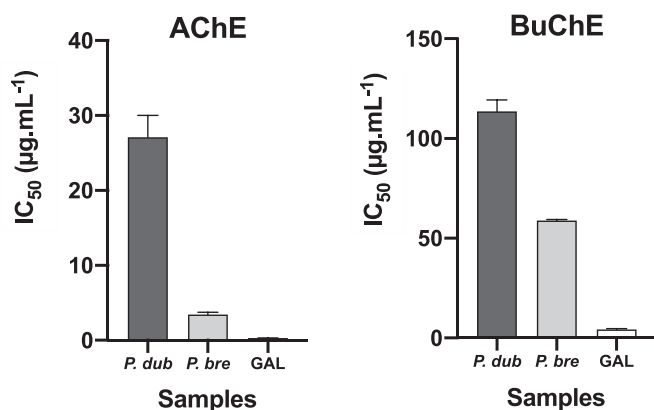


Fig. 4. Inhibitory activity of *P. dubia* (*P. dub*) and *P. brevifolia* (*P. bre*) alkaloid extracts against AChE and BuChE expressed as IC<sub>50</sub> (µg.mL<sup>-1</sup>). The data presented are the mean values SD (n=3). GAL = galanthamine (reference compound).

Table 2

Estimated binding free energy in molecular docking of alkaloids identified in extracts of *P. dubia* and *P. brevifolia* toward cholinesterases (AChE and BuChE). Values expressed in kcal.mol<sup>-1</sup>.

| Alkaloid                              | AChE <sup>a</sup> | BuChE <sup>b</sup> | Reference             |
|---------------------------------------|-------------------|--------------------|-----------------------|
| anhydrolycorine (1)                   | -8.38             | -8.14              | Cortes et al. (2018)  |
| caranine (2)                          | -8.66             | -9.13              | Calculated values     |
| 2,4-didehydro-2-dehydroxylycorine (3) | -8.76             | -9.34              | Calculated values     |
| 11,12-dehydroanhydrolycorine (4)      | -8.41             | -7.44              | Tallini et al. (2018) |
| lycorine (5)                          | -8.82             | -8.94              | Tallini et al. (2018) |
| vittatine (6a)                        | -8.51             | -9.32              | Calculated values     |
| crinine (6b)                          | -8.98             | -9.14              | Calculated values     |
| haemanthamine (7a)                    | -8.80             | -8.34              | Tallini et al. (2018) |
| crinamine (7b)                        | -8.71             | -8.61              | Calculated values     |
| 11-hydroxyvittatine (8a)              | -8.43             | -9.03              | Tallini et al. (2018) |
| hamayne (8b)                          | -8.28             | -8.54              | Tallini et al. (2018) |
| galanthamine (9)                      | -10.10            | -8.23              | Calculated values     |
| 2-hydroxyhomolycorine (10)            | -9.73             | -8.78              | Calculated values     |
| pancratinine C (11)                   | -8.53             | -8.12              | Moreno et al. (2020)  |

<sup>a</sup> PDB code: 1DX6

<sup>b</sup> PDB code: 4BDS.

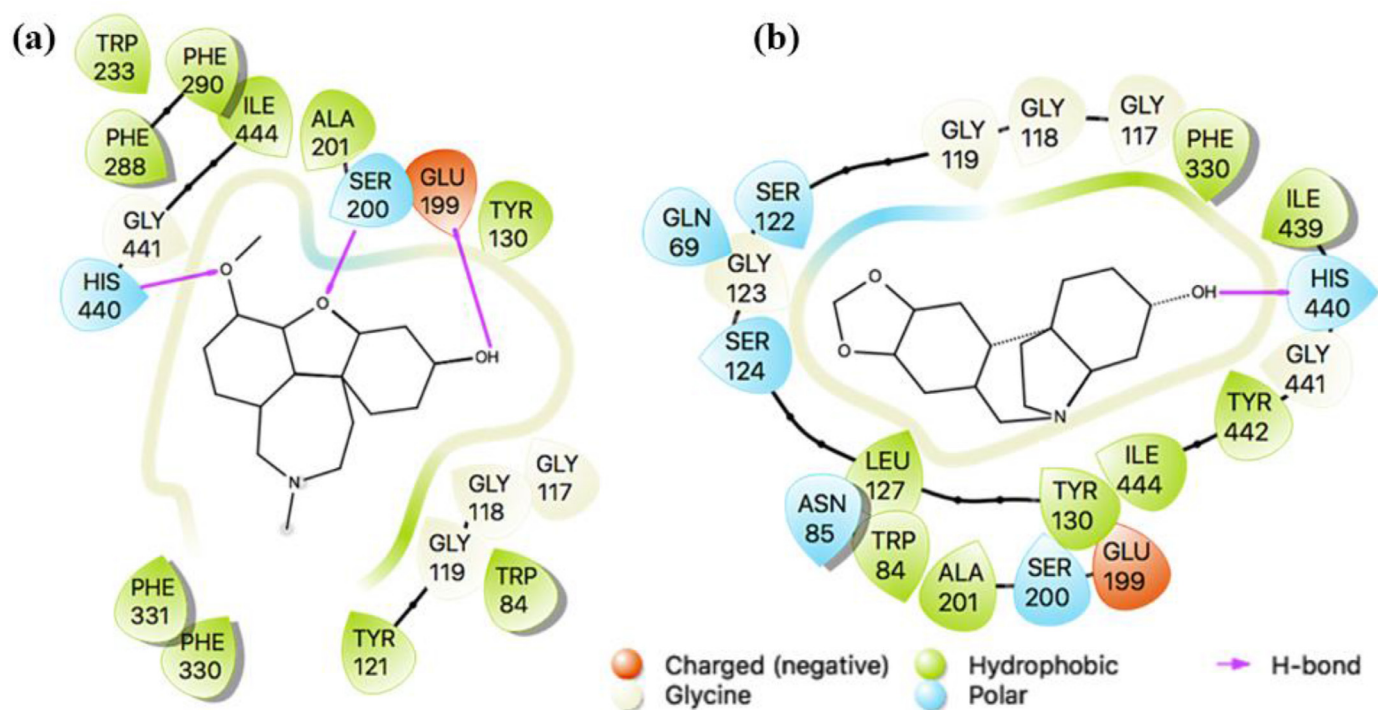


Fig. 5. Graphical representations of the binding of (a) galathamine (9) and (b) crinine (6b) in the gorge of the active site of AChE.

strong interactions, such as hydrogen bonds, between vittatine (6a) and crinine (6b) with the Trp82 residues, at 2.11 and 2.00 Å, respectively, in the gorge of the active site of BuChE. Likewise, both alkaloids present  $\pi$ - $\pi$  interactions with Trp82, but no electrostatic interactions with Phe330 (Fig. 6). Finally, the complexes BuChE-vittatine (6a) and BuChE-crinine (6b) seem to be stable due to the presence of two additional strong interactions with Gly78 and Trp430 (Fig. 6). Moreover, the hydroxylation of C-11 position in 11-hydroxyvittatine (8a) could theoretically decrease the BuChE inhibition on the 4BDS structure by 0.29 kcal.mol<sup>-1</sup>, compared with vittatine (6a)

(see Table 2). In another study, 11-hydroxyvittatine showed higher energy interaction with BuChE by molecular docking than galanthamine, but lower BuChE inhibition activity *in vitro* (Tallini et al., 2018). Comparing the estimated binding free energy of 11-hydroxyvittatine (8a) on the 4BDS structure with haemanthamine (7a) values, the methylation of C-3 position seems to decrease the theoretical inhibitory of BuChE by 0.69 kcal.mol<sup>-1</sup> (Tab. 2).

Although the best *in vitro* AChE and BuChE inhibitory activity results have been obtained with the alkaloid extract from *P. brevifolia* sample, *P. dubia* also presented some activity against both

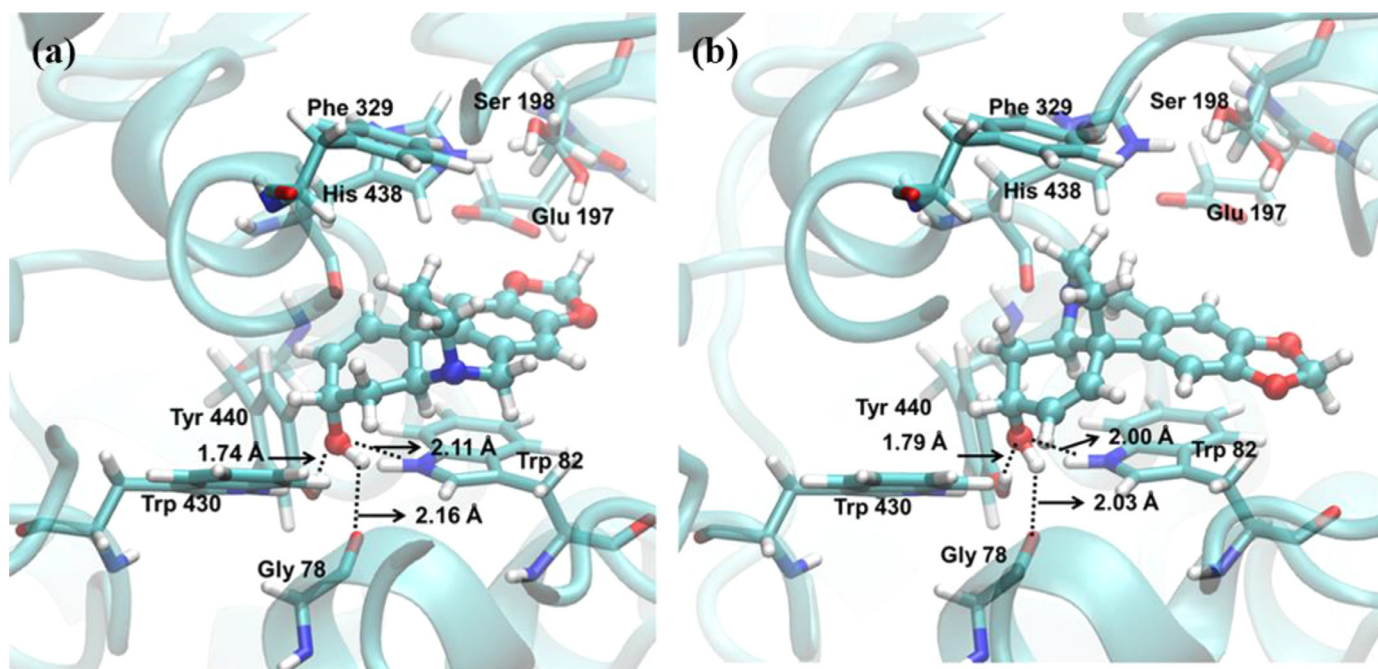


Fig. 6. Graphical representations of the binding of (a) vittatine (6a) and (b) crinine (6b) in the gorge of the active site of hBuChE.

cholinesterases (Fig. 4). The molecular docking experiments indicated that the alkaloids 2-hydroxyhomolycorine (**10**) and 2,4-didehydro-2-dehydrolycorine (**3**) identified in *P. dubia* extract present an interesting estimated binding free energy toward the active sites of AChE and BuChE (Table 2), respectively, however both compounds were detected in low concentrations in this species (Table 1).

#### 4. Conclusion

In summary, we identified eleven alkaloids in the bulbs extracts of *P. dubia* and *P. brevifolia* collected in Ecuador. *Phaedranassa dubia* had high concentrations of lycorine-type alkaloids, while higher levels of haemanthamine/crinine-type alkaloids were observed in *P. brevifolia*, and both species could be considered as interesting sources of new Amaryllidaceae alkaloids. These plant extracts also presented remarkable inhibitory activity against AChE and BuChE enzymes. *In silico* experiments showed the theoretical interactions of the alkaloid crinine detected in *P. brevifolia* toward the active site of AChE. In addition, considering the alkaloidal profile of these species, it would be advisable to study the potential of these *Phaedranassa* species in other pharmacological areas, such as anti-inflammatory, antiviral, or antitumor assays.

#### Declaration of Competing Interest

The authors have declared that there is no conflict of interest.

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#### Authors' contributions

KA designed the study, collected plant sample, ran the laboratory work, and drafted the paper. AI and JR contributed with extraction of alkaloids, biological studies and analysis of the data under supervision of KA. NO contributed with plant identification, sample locality information and critical reading of the manuscript. EO carried out the molecular docking experiments. JB and LRT carried out the chromatographic analysis. All the authors have contributed to the writing of the article, have read the final manuscript, and approved the submission.

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