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Clinanthus microstephus, an Amaryllidaceae species with cholinesterase inhibitor alkaloids: structure-activity analysis of haemanthamine skeleton derivatives

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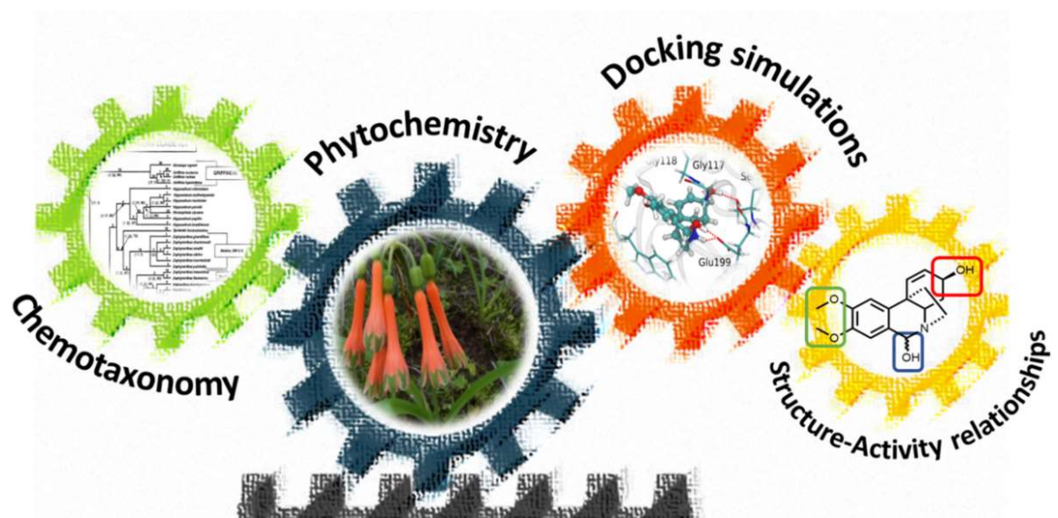
Abstract.

Plants of the Amaryllidaceae family are well-known (not only) for their ornamental value but also for the alkaloids they produce. In this report, the first phytochemical study of *Clinanthus* genus was carried out. The chemical composition of alkaloid fractions from *Clinanthus microstephus* was analyzed by GC-MS and NMR. Seven known compounds belonging to three structural types of Amaryllidaceae alkaloids were identified. An epimeric mixture of a haemanthamine-type compound (6-hydroxymaritidine) was tested as an inhibitor against acetyl and butyryl cholinesterase enzymes (AChE and BChE, respectively), two enzymes relevant in the treatment of Alzheimer's Disease, with good results. Structure-activity relationships through molecular docking studies with this alkaloid and other structurally related compounds were discussed.

Graphical abstract



Keywords

Amaryllidaceae alkaloids; *Clinanthus microstephus*; Cholinesterase inhibitors; Structure-activity relationship.

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Introduction

Amaryllidaceae species are known as ornamental plants that produce alkaloids with a wide range of interesting biological activities, such as antimalarial, antitumoral, antiviral, cholinesterase inhibition, among others.[1-3] Many Amaryllidaceae species have been extensively used in traditional medicine to treat a variety of health problems and as a promising source for new and bioactive molecules.[4] Within all the bioactive alkaloids reported in this family,[5, 6] the compound galanthamine worth to be mentioned. It was approved as a prescription drug by the FDA for the treatment of Alzheimer´s disease (AD) due to its high inhibitory efficacy, through a competitive, reversible and selective mechanism on acetylcholinesterase enzyme (AChE).[7, 8]

AD is the most predominant cause of dementia in the elderly population. Recent studies indicate a clear increase in its prevalence. Today, 47 million people live with dementia worldwide. This number is projected to increase to more than 131 million by 2050, at a similar rate as the increase in life expectancy.[9] Therefore, AD is a major public health concern and has engaged significant research efforts.

AD patients present a deficit of the cholinergic functions, such as memory impairments and loss of intellectual abilities, due to decreased levels of the critical cationic neurotransmitter acetylcholine (ACh) in the cortex.[10] This neurotransmitter is quickly hydrolyzed by AChE, finishing with the nerve impulse transmission at cholinergic synapsis. For this reason, a palliative strategy employed to treat symptoms of the disease is to inhibit the action of AChE, increasing the level of ACh in presynaptic space.[11, 12] Also, the inhibition of this enzyme is related to other types of neurodegenerative diseases such as senile dementia, ataxia, myasthenia gravis, and Parkinson´s disease.[13] The enzyme butyrylcholinesterase (BChE) is also involved in the metabolic degradation of ACh. In AD patients, the AChE/BChE ratio depends on the brain region and the stage of the disease progression. BChE can compensate AChE activity when its levels are decreased. Since BChE activity increases as AD progresses, this enzyme may also play an important role in cholinergic dysfunction, particularly in the later stages of AD.[14]

In recent years, alkaloids from the Amaryllidaceae family have received great attention due to their well-known anticholinergic activity, which generally have in common the presence of nitrogen atoms in a heterocyclic ring. The chemical structures of these alkaloids are unique due to their variability and their pharmacological properties. This fact

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has motivated the search of more potent and effective AChE inhibitors (AChEIs) from Amaryllidaceae species leading to the isolation of different active alkaloids. [15, 16]

From this perspective, one of the plant families of our interest is the Amaryllidaceae family. This botanical family is richly represented in the tropics and has significant centers of diversity in the Andean region of South America.[17] In Peru, the varied climatic and geographical conditions have allowed a great diversity of flora to explore, with a great number of species to be discovered. This region is a major center of diversity for the genus *Clinanthus* which contains between 15 and 20 non-monographed species.[18] Therefore, the need for preliminary studies is imperative in order to know their phytochemical composition.

Given the increased inhibitory activity shown by the alkaloids of this family on the AChE enzyme and considering that the genus *Clinanthus* does not possess any previous phytochemical studies to date, in this manuscript we report the first phytochemical study of the Peruvian bulbous plant *Clinanthus microstephus* (Ravenna) Meerow in the search of bioactive alkaloids and with the aim to contribute to the taxonomy of the Clinanthus genus.[19] The purpose of the study was to begin the revision of recently described species of the Peruvian flora.

In addition, to understand the differences in the AChE inhibitory activity displayed by different alkaloids from Amaryllidaceae family, molecular modeling studies were performed.

Results and Discussion

One fresh bulb of *C. microstephus* was extracted exhaustively with methanol (1 L). After concentration and subsequent acid-base extractions of the crude extract, an alkaloidal fraction (approximately 100 mg) was obtained. This residue was fractionated by a combination of chromatographic techniques, ultimately giving nineteen fractions of very low mass (see experimental section for details). According to the ^{1}H-NMR analysis, most of them were a complex mixture of alkaloids and in many cases, they could not be purified given the low quantities available. Only three of them could be analyzed by CG-MS (renamed A, B, and C). Despite the complexity of these fractions, some reported alkaloids were identified (they are summarized in Table 1). Their identification was performed by comparison of retention times and mass spectra, with the information from reference databases. A complete set of 1D and 2D-NMR experiments allowed the structural

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elucidation of 6-hydroxymaritidine (hereafter named as compound **1** isolated in fraction C almost as main component), recently isolated from *Hippeastrum reticulatum* (Amaryllidaceae).[20] This compound (figure 1) was isolated as a β:α epimeric mixture, 83:17.[21] All the spectral data (NMR assignments, HRESIMS and mass fragmentation pattern observed in the GC-MS) are in agreement with the information reported in the work of Tallini *et al.*[20]

Figure 1. β:α epimeric mixture of 6-hydroxymaritidine

The remaining fractions (A and B) were analyzed by GC-MS to identify the alkaloids in the obtained mixtures, due to their low quantity and complexity. GC-MS analysis has demonstrated to be a useful, robust and efficient technique for the rapid identification and/or quantification of many complex mixtures of alkaloids from Amaryllidaceae plant extracts.[22, 23]

Seven alkaloids of three structural types were identified by GC-MS analysis (Table 1). The area of the GC-MS peaks depends not only on the corresponding compounds, but also on the intensity of their MS fragmentation (response factor). Thus, data given in the table represent relative ratios that can be used for comparison between samples, though they do not correspond strictly to a quantification.

Table 1. Alkaloids identified by GC-MS analysis of fractions A, B and C

Fraction	Compound	t_R (min)	$[M]^+$ [*m/z* (% relative intensity)]	MS [*m/z* (% relative intensity)]	% TIC[a]	Complementary ID by:
A	Anhydrolycorine (I)	20.8	251 (35)	250 (100), 192 (15), 191 (10), 97 (10)	5.4	

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	11,12-Dehydroanhydrolycorine (II)	22.3	249 (50)	248 (100), 191 (15), 192 (30), 96 (20)	1.3	
	Lycorine derivative	23.6	301 (100)	286 (20), 254 (20), 226 (15), 172 (25)	4.5	
	Hippeastrine (III)	26.4	315 (3)	126 (5), 125 (85), 124 (10), 96 (100), 94 (10)	23.7	1D and 2D NMR spectra.
	1-O-Butenoyllycorine (IV)	27.0	355 (65)	268 (40), 227 (70), 226 (100), 69 (35)	1.3	
	1-O-(3´-hydroxybutanoil) lycorine (V)	28.5	373 (60)	268 (30), 250 (20), 227 (65), 226 (100)	40.6	1D and 2D NMR spectra.
B	Anhydrolycorine (I)	21.3	251 (35)	250 (100), 192 (20), 191(10), 97 (10)	2.3	
	11,12-dehydroanhydrolycorine (II)	23.0	249 (50)	248 (100), 191 (15), 192 (30), 96 (20)	1.1	

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	Lycorine (VI)	25.2	287 (40)	288 (15), 286 (15), 268 (20), 227 (65), 226 (100)	78.6	
C	6-hydroxymaritidine (**1**)	22.1	303 (15)	285 (15), 260 (25), 259 (100), 256 (25), 241 (70), 115 (30), 77 (20)	43.6	1D and 2D NMR spectra

[a] Values are expressed as a percentage of the total ion current (TIC) of every fraction.

Biological activity, molecular docking and structure–activity relationship

One of the main objectives of our research group is the exploration of the chemical diversity of natural compounds in search of bioactive metabolites. Due to the marked biological activity as cholinesterase inhibitors that this family of compounds presents and since the epimeric mixture of 6-hydroxymaritidine was obtained in appropriate quantity and purity, it was re-evaluated for their AChE and BChE inhibitory activities (using galanthamine as reference compound). In addition, a docking study was performed including a group of alkaloids structurally related and previously reported. The main purpose was to deepen the analysis of interactions with the enzyme through a detailed structure-activity analysis.

Acetyl- and Butyrylcholinesterase inhibitory activities

Compound **1** as an epimeric mixture, was tested for *in vitro* inhibition of cholinesterase on the commercially available *eel* AChE and horse serum BChE (results are summarized in Table 2). The activity was determined according to the method of Ellman *et al*. with slight modifications.[24] The inhibition percentages obtained after 3 replications at 25 μM, were shown to be interesting (70.1% for AChE and 87.9% for BChE) and IC_{50} was calculated in both assays. For comparison purposes, galanthamine was used as a reference inhibitor.

It is noteworthy that this IC_{50} value for compound **1** is quite different from those recently reported for this alkaloid.[20] This different outcome may be attributed to the differences in the experimental conditions. As can be observed in numerous reports, [25-30]

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although the conditions are similar, the results for the same compound (for example galanthamine) present variations (in Supporting Information, Table S1 summarizes some differences found). In this study, MeOH (2.5 %) was used as a cosolvent to improve and guarantee alkaloid solubility in the buffer where the assay was carried out. Also, the incubation of the enzyme along with the inhibitors for longer periods of time before adding the reactive according to the optimized Ellman protocol in our research group (See experimental section and Supporting Information for a more detail explanation). Furthermore, it is interesting to note that it is essential to carry out the measurement of positive control in each assay or study in particular.

Table 2. AChE and BChE inhibitory activity of the tested alkaloids expressed as IC_{50} values.

Compound	IC_{50} AChE (μM)	IC_{50} BChE (μM)
1	10.53 ± 1.19	9.51 ± 1.24
Galanthamine	0.76 ± 0.01	23.85 ± 1.12

All results are expressed as IC_{50} values (μM). Each value is the mean of three replications.

IC_{50} values for AChE inhibition for compound **1** and related alkaloids (previously reported in bibliography) are summarized in Table 3 for comparison purposes and to obtain structure-activity relationships. As it can be observed, beyond galanthamine, epimeric mixture **1** showed the best IC_{50} value (10.35 ± 1.19 μM) among the other related structures for AChE inhibition.

Molecular modeling studies were performed to investigate key interactions of these compounds with the enzymes and the role that the structural differences between them may play in the enzyme inhibition. This verifies the reliability of the experimental result found.

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In addition, the contribution and the differential composition of the isomers according to the physiological pH was also evaluated.

Structure-activity analysis through Molecular Docking

To date, there is information from hundreds of isolated alkaloids in the Amaryllidaceae plant family. Some of them with very promising activities as AChEIs. Performing structure-activity relationship studies from molecular modeling results is sometimes difficult. This is due to the high variability of compounds and the quantity and structural diversity of isolated nuclei in the same species.[31] In the area of medicinal chemistry, the "similarity paradox"[32] establishes that small structural changes in molecules can drastically impact their biological activity. So, it is important to carry out an analysis of the molecular interactions between inhibitor and protein that determine the activity found.[33, 34]

Numerous reports consider that alkaloids are the most promising candidates within the products of natural origin for use in AD.[35] This is because of their complex structures that contain at least one nitrogen atom.[36] Structure-activity studies have demonstrated that the ability of the nitrogenous functionalities to be positively-charged at physiological pH is one of the most important characteristics related to the potency of these compounds. This ensures the interaction with key sites of the AChE enzyme cavity.[37]

AChE inhibitors are interesting since they could restore the cholinergic deficit by blocking the catalytic site of the enzyme. Several studies showed that one of the remarkable features of the structure of the AChE enzyme[38] is the presence of a "catalytic cavity" of approximately 20 Å depth and 4.5 Å narrow channel. This one possesses an active site where the neurotransmitter is hydrolyzed. The residues responsible for the catalytic activity (Ser, His and Glu), are known as “catalytic triad". In the active site there are also three additional subsites; anionic, oxyanionic and acyl binding. At the surface of the cavity, there is a region called peripheral anionic site (PAS, allosteric site) that is related to the inhibition of other neurodegenerative processes.[39] Between the active site and PAS there is a region called "bottleneck" formed by Phe and Tyr that regulates the entrance of different substrates (see Figure S3 in Supporting Information for visualization).

As mentioned above, AChE plays an important biological function and knowing the possible interactions of the substrates in the three-dimensional structure of the enzyme is important to achieve a more efficient inhibition. To evaluate this effect, compound **1** (four

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structural types: two epimers, as their neutral and protonated forms) together with some structurally related structural compounds and galanthamine were subjected to molecular docking studies.

To carry out the simulations, the geometry of the receptor was taken from the X-ray structure of the complex between AChE from *Torpedo Californica* (*Tc*AChE) and Galanthamine (PDB: 1W6R). In addition, for comparative purposes, docking calculations over BChE from *human* (*h*BChE) were performed (PDB: 1P0I). The geometries of the complexes with the inhibitors were obtained using *AutoDock4.2.6* program (Gasteiger charges were applied). After docking calculations, a minimization of each complex was made with *Sander* program and the binding energy re-scored within the MM-GBSA method (*Amber16* package).

The calculated pKa value for compound **1** is 7.24 so at physiological pH, both the neutral and protonated species could be found. Furthermore, in solution the compound **1** is presented as an epimeric mixture on C-6. Therefore, there are four species that may be participating in the interaction with the enzyme. As the protonation state can affect the interaction, it must be considered. For this reason, all these species were included in the docking calculations.

The lowest binding energies obtained with MM-GBSA method were used for the analysis. The results suggest that the protonated form of the β-epimer (β +), with more negative binding energy, is the species that presents a more stable binding mode with the protein. Interestingly, this is the major epimer of the isolated mixture (as shown by the NMR essays). To estimate the conformers ratio in water (the media in which the IC_{50} were determined) quantum calculations were carried out with the protonated species. The estimated ratio obtained by DFT calculations is ~4:1 in accordance with the experimental value found in chloroform. In order to know more about the relative stability of the epimeric species, the energy difference between the two epimers with two DFT functionals was calculated (B3LYP and M06-2X). It was observed that in both cases the β-epimer is the most stable. In one case an energy difference of 0.73 kcal/mol was obtained with a 0.77:0.23 molar ratio; in the second case, the energy difference is 0.94 kcal/mol with a 0.83:0.17 molar ratio. This is another important point to consider since the composition of the isolated epimeric mixture is slightly different from those recently reported for this alkaloid (slightly larger for the β-epimer).[20] As it was obtained from the modeling studies, the most active isomer appears in greater proportion.

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For these reasons, the analysis of docking results and interactions with the protein was carried out with the β + species, Figure 2 A. The results suggest three fundamental interactions; basically, van der Waals type interactions between skeleton rings A, B, and Trp84 belonging to the anionic subsite. Also, this type of interaction was observed between rings B-C, and residues Gly117 and Gly118. Besides, a hydrogen bond between the hydroxyl of C-3 with Ser200 of the catalytic triad. In this case, hydroxyl group participates as hydrogen bond donor to the oxygen of the side chain of the Ser200. The most important interaction is established with Glu199 of the anionic subsite (binding decomposition energy of -21.51 kcal/mol). This interaction can be split into two main contributions: firstly, the carboxylate of Glu199 establishes a salt bridge interaction with $(R_3N\text{-}H)^+$ group and secondly interacts with the hydroxyl group of C-6.

The analysis of the obtained complexes with BChE reveals that the α-epimer, in its protonated form, presents the most stable binding mode (binding energy: -59.27 kcal/mol), see Figure S5 in Supporting Information for details. The docking showed that this compound binds to the bottom of the active site of the enzyme. From the energy decomposition, it is possible to see that the most important interaction is with Glu197. The relevance of this interaction is a consequence of the sum of two important interactions. One of them is a hydrogen bond between carboxylate of this residue and the hydroxyl group of C-6; and the second one, is a salt bridge with $(R_3N\text{-}H)^+$ group. In addition, van der Waals interaction is observed between Trp82 belonging to the anionic subsite and two hydrogen bond with Gly116 (oxyanionic subsite) and His438 (catalytic triad).[33]

On the other hand, galanthamine binds at the base of the active site gorge in both enzymes. Particularly, the calculated geometry for AChE was close to the experimental with a low RMSD, thus validating the protocol used. In Figure S4 (supplementary material) AChE-galanthamine complex geometry is shown as an example. It is possible to distinguish hydrogen bond type interactions with Glu199 and the C-3 hydroxyl group (1.58 Å). The rest of interactions are van der Waals type. Particularly, rings A and D of the inhibitor interact with Trp84, the rings A-B-C with Gly117 and Gly118, and C and D with Tyr121 and Phe330. The main differences in the poses adopted by both inhibitors (galanthamine and compound **1**, most stable epimer and protonated state) in the enzyme cavity, together with the binding energy decomposition graphical are shown in figure 2 A-B.

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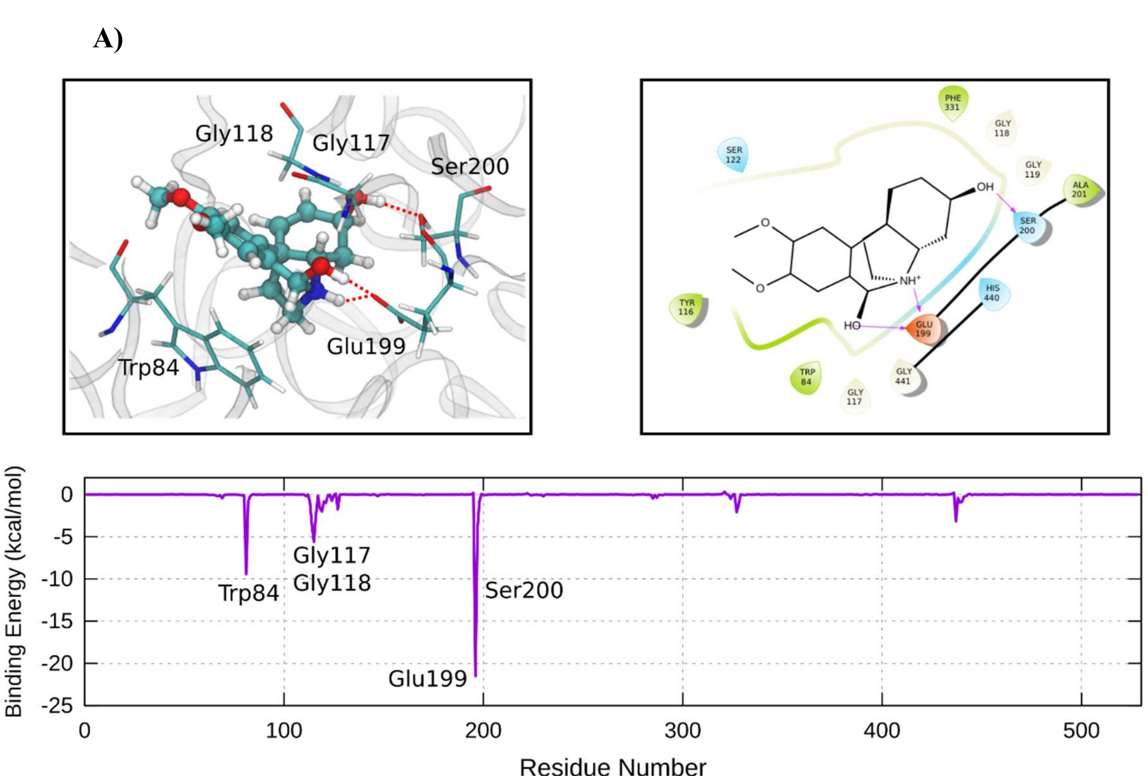
If compound **1** and galanthamine are compared, a certain structural similarity can be observed. Variable rigidity-flexibility characteristics in rings A-B-C-D are observed, this fact based on the different biogenetic approximations of both compounds. These distinctive arrangements result from *para-para´* (haemanthamine skeleton type) or *para-ortho´* (galanthamine skeleton type) phenol oxidative coupling.[40] Regarding alkaloidal skeleton type, in this compound there is a differential rigidity of rings. This means that the A ring has an arrangement almost perpendicular to the plane formed by the other rings. This feature generates differential interactions from those observed for compound **1** in a totally different geometry. The most notable difference relies on the more favorable interaction with Glu199 for compound **1** respect to galanthamine (from -21.51 kcal to -6.62 kcal, respectively). Probably since there is no direct relationship between the binding energy and the inhibitory activity found for both compounds, as claimed by other authors, the high affinity displayed by galanthamine for AChE appears to come from several moderate to weak favorable interactions. [41, 42]

A)



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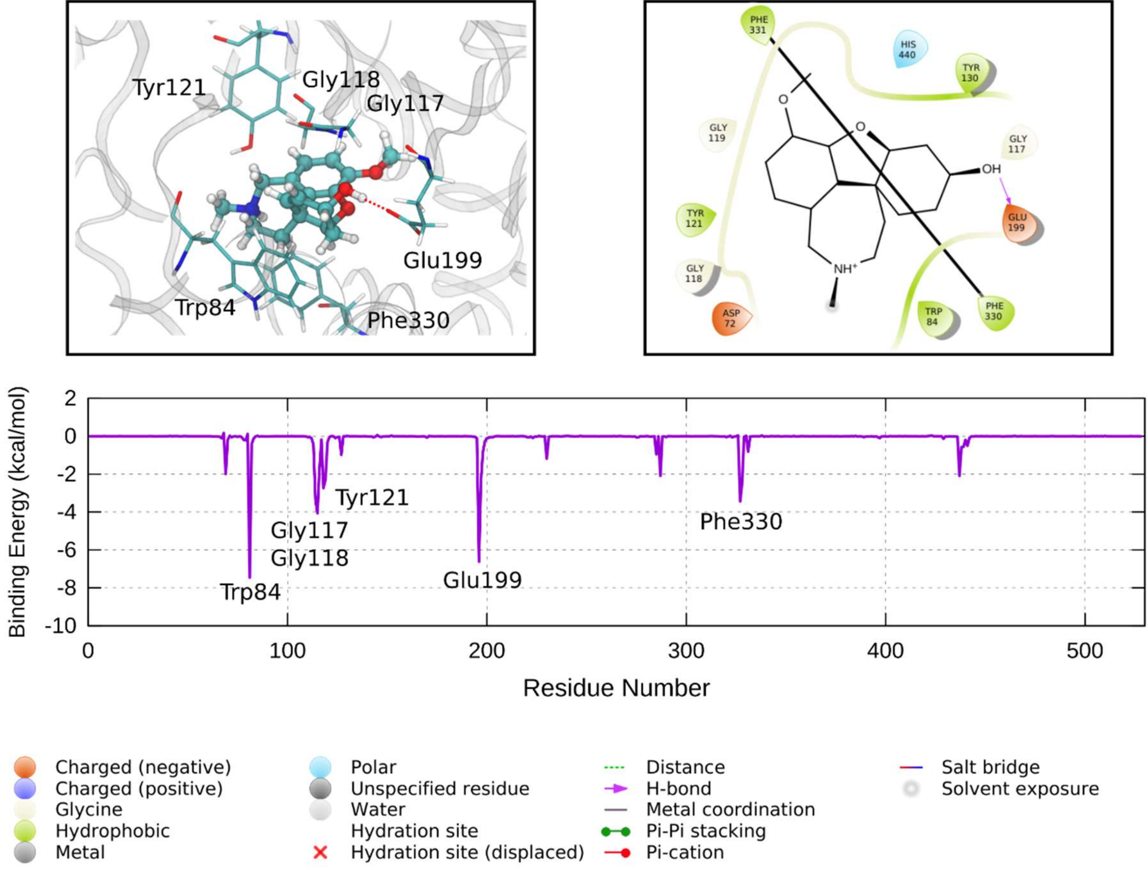

Figure 2. Calculated position of compound **1** (A) and galanthamine (B) in the binding pocket of the *Tc*AChE and most relevant residue interactions. Per-residue contributions to the binding energy calculated with MM-GBSA method. The most stable epimer of **1** in its protonated state formed the most stable complex, that is represented in this figure.

Analysis of docking results for compound **1** and structurally related alkaloids, allows establishing some structure-activity relationships, as discussed below (see Figures S6 to S11 in Supporting Information where calculated complexes in the binding pocket of *Tc*AChE and most relevant inhibitor-protein interactions for compounds **2**-**7** are presented).

Compound **2** [43, 44] differentiates itself from **1**, by an *O*-methyl group on C-3 instead of the hydroxyl. This structural change is responsible for the loss of the stabilizing hydrogen bond interaction with Ser200. It establishes a van der Waals type interaction, but the stabilizing effect is smaller. In addition, the interaction with Gly117 (van der Waals type) is weaker. The depletion in the interactions, in comparison to **1**, causes that this compound loses the AChE inhibition activity.

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With respect to **2**, compound **3** [43, 44] has the reduced the C-6 position, losing the hydrogen bond interaction with Glu199 of the anionic site, but still retaining the salt bridge interaction between the group $(R_3N\text{-}H)^+$ and the carboxylate of this residue. In the binding energy decomposition graphic, could be seen that this interaction decreased from -21,5 (compound **1**) to -17.4 kcal/mol.

Although the inhibitor **4**[45] does not possess the hydrogen bond interactions with Ser200 and Glu199, demethylation of hydroxyl group in the aromatic ring, allowed the formation of a new hydrogen bond interaction with Ser122 belonging to the peripheral anionic site. In this case, hydrogen of the aromatic hydroxyl group is the donor to oxygen of hydroxyl group of Ser122 side chain. Although in terms of inhibitory activity this change is not appreciable, possibly, this new interaction could be responsible for making this compound active compared with the compounds **2** and **3**.

In compound **5**,[43, 44] as it was in compound **3**, hydroxyl group of C-6 is absent. According to this, primary interaction with Glu199 is substantially reduced (from -21.5 to -16.9 kcal/mol) as well as van der Waals type interaction with Gly117. The attenuation of the interactions could be the reason whereby this compound does not possess inhibitory activity.

Compared to structure **5**, compound **6** [46, 47] has a demethylation of one of $O\text{-}CH_3$ aromatic group (C-8) making possible a hydrogen bond interaction with the carbonyl group of Trp84 residue. For this reason, interactions with this residue were reinforced going from -9.2 to -11.7 kcal/mol. This new interaction, product of a simple modification of compound **5**, could be the cause why compound **6** becomes an inhibitor of moderate activity.

On the other hand, if the simple chemical modification of the addition of the aromatic OH is taken as a basis to enable hydrogen bridge interaction with Ser 122 (as observed when introducing this modification of compound **3** to **4**), no substantial inhibitory activity improvement is obtained for compound **7** [45] compared with **5**.

In general terms, considering the interactions with AChE described for the analyzed compounds, the haemanthamine skeleton could not reach the efficient levels of inhibition presented by galanthamine. However, the IC_{50} value showed by compound **1** is just one order less active than that of galanthamine, being the best among the other structurally related alkaloids analyzed. Regarding the importance of the substituents of the haemanthamine skeleton, it is possible to infer that at least two hydroxyl groups are

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necessary to establish a good interaction with the enzyme, being C-3 and C-6 the most favorable positions.

Table 3. Summary of *in vitro* AChE inhibition activity of compound **1** and reported related compounds.[a] Binding energy calculated by rescoring of the geometries obtained with molecular docking.

Compound	Structure	IC_{50} (µM)	Reference	Binding energy (kcal/mol)
1	OH; C; O; A; H; D; B; N; O; OH	10.53 ±1.19 [a]		-60.9
2	O; O; H; N; O; OH	> 500	[43, 44]	-43.3
3	O; O; H; N; O	> 500	[43, 44]	-48.0
4	OH; O; O; H; N; O	104.6	[45]	-49.8
5	OH; O; H; N; O	> 500	[43, 44]	-55.0
6	OH; O; H; N; HO	57.4	[46, 47]	-56.1
7	OH; OH; O; H; N; O	> 200	[45]	-51.1

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Galanthamine	[chemical structure: O, OH, A, B, C, D, N]	0.76 ±0.01[a]		-50.5

[a] Results are expressed as IC_{50} values (μM). Each value is the mean of three replications.

Conclusions

To our knowledge, this is the first phytochemical study of a species from the *Clinanthus* genus, representing a starting point in the exploration of new chemotaxonomic markers at a generic level in this family. Indeed, in this study it is shown that the genus *Clinanthus* as well as others of the Amaryllidaceae family possess at least alkaloids of lycorine, homolycorine, and haemanthamine types. This reaffirms the fact that these metabolites are chemotaxonomic markers of this family. In this respect, the exploration of native Peruvian flora is an interesting topic to be addressed because of the variability of alkaloidal structures.

For the classification of plant species, traditional systematic focus mainly on morphological and anatomical characteristics; usually chemical content and chemotaxonomy play a minor role in the classification. However, secondary metabolites can provide valuable information for the characterization of taxa at different levels. Several authors have carried out interdisciplinary approaches combining the chemical and botanical aspects, and in fact, the chemosystematics has been used in order to solve various taxonomic problems or to support the botanical findings. Due to the previously exposed reasons, the contribution to the systematic classification of plant species through their phytochemical study represents a subject of relevance for natural sciences.

AChE still represents an attractive target for rational drug design and discovery of mechanism-based inhibitors for the treatment of Alzheimer´s disease. Our results summarize reports of other groups, tending to improve the structure-activity relationships for the finding of increasingly active derivatives and suggest that these alkaloids could be potential candidates for further development of new drugs against AD. Indeed, evidence suggest that a minimal structural variation (different isomers for example), could impact in biological activity in this compound´s family. The results obtained from docking studies are in agreement with the experimental biological activity observed for the epimeric mixture **1**

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with the enzymes *Tc*AChE and *h*BuChE. Structure-activity analysis help to explain the differences in the inhibitory activity of alkaloids with galanthamine skeleton type (galanthamine) and alkaloids with haemanthamine skeleton, compounds **1** to **7**.

For all these reasons, *C. microsthepus* is an interesting species to be exhaustively addressed from other perspectives of importance due to the presence of active metabolites.

Materials and methods

General

Optical rotation was measured on JASCO P-1010 polarimeter. IR spectra were obtained in a Nicolet 5-SXC spectrophotometer (each compound was dissolved in a minimum amount of solvent and a drop of solution was added to the AgCl IR plates). NMR experiments were performed on Bruker AVANCE II 400 MHz instrument. Multiplicity determinations (HSQC-DEPT) and 2D spectra (COSY, HSQC and HMBC) were obtained using standard Bruker software. Chemical shifts are expressed in ppm (δ) units using tetramethylsilane as the standard. HR-ESI-QTOF-MS were measured on a Micro QTOF II Bruker Daltonics (MA, USA) mass spectrometer. Chromatographic separations were performed by column chromatography on silica gel 60 (0.063–0.200 mm), and preparative TLC on silica gel 60 F_{254} (0.2 mm thick) plates. Presence of alkaloids was revealed by Dragendorff's reagent. Acetylcholinesterase from *electric eel* (type VI-S), 5,50-dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI) and galanthamine (99%) were purchased from Sigma. BChE (horse serum) was purchased from MP Biomedicals.

Plant material

C. microstephus was collected in Departamento La Libertad, Otuzco, Salpo region and El Tablón-Rayampampa, Perú (8º00'39,9"S y 78º40'04,1" W, 1927 m) in January 2015 (code: *S. Leiva & M. Leiva 5703* (HAO, HUT); North of Rayampampa, 8º00'47,0"S y 78º40'06,2" W, 1981 m, February 2015 *S. Leiva & M. Leiva 5716* (CORD, HAO).[19] A voucher specimen of *C. microstephus* was identified by Professor Segundo Leiva and was deposited at the herbarium of Museo de Historia Natural y Cultural, Universidad Privada Atenor Orrego, Trujillo, Perú.

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Extraction and Isolation

One fresh bulb (109.5 g) was grounded and extracted with MeOH (1 L). The solvent was then evaporated under reduced pressure using a rotary evaporator. The total dried residue was diluted with aqueous HCl solution (200 mL, 10%). Diatomaceous earth was added, and the homogenate was placed at 2°C for 12h. Afterward, the aqueous phase was vacuum filtrated. The resulting fraction was partitioned in CH_2Cl_2 (3 x 100 mL). The pH of the aqueous acidic fraction was adjusted to 9 with NH_4OH and extracted with CH_2Cl_2 (5 x 100 mL). Organic extract was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness at reduced pressure. The residue was fractionated initially by silica gel 60 G chromatography. Elution with CH_2Cl_2: MeOH (10:0 to 8:2) afforded 7 fractions.

All these fractions were purified by preparative TLC (CH_2Cl_2: MeOH, sequentially 10:0 to 8:2). From the nineteen resulting fractions, only three of them could be analyzed by CG-MS (renamed A, B, and C). Despite the low amount of sample and the level of complexity, 1 and 2D NMR spectra could be obtained from some of the purified compounds (**1**, **III** and **V**). Compound **1** was obtained as an epimeric mixture [4.2 mg, 83(β):17(α), approximately obtained ratio by ^{1}H-NMR signal integration], from fraction C, and completely characterized by 1 and 2D NMR.[20] Analytical and spectral data obtained for this alkaloid agree with those reported in the literature for structurally related compounds.[48]

Cholinesterase inhibition assay

Electric eel AChE and horse serum BChE were used as a source of both the cholinesterases. AChE and BChE inhibiting activities were measured in vitro by the spectrophotometric method developed by Ellman with slight modification.[24] The lyophilized enzyme, 500U AChE /300U BChE was prepared in buffer A (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4) to obtain 5/3 U/mL stock solution. Further enzyme dilution was carried out with buffer B (8mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) to produce 0.126/0.06 U/mL enzyme solution. Samples were dissolved in buffer B with 2.5% of MeOH as cosolvent. Enzyme solution (300 μL) and sample solution (300 μL) were mixed in a test tube and incubated for 60/120 min at room temperature. The reaction was started by adding 600 μL of the substrate solution (0.5 mM DTNB, 0.6 mM ATCI/BTCI, 0.1 M Na_2HPO_4, pH 7.5). The absorbance was read at 405 nm for 180 s at

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27ºC. Enzyme activity was calculated by comparing reaction rates for the sample to the blank. All the reactions were performed in triplicate. IC_{50} values were determined with GraphPad Prism 5. Galanthamine was used as reference AChE/BChE inhibitor.

GC-MS analysis

Mass spectra were obtained on a Varian Saturn 2200, a CP3800 GC coupled to an ion trap MS Saturn 2000 operating in EI mode, using a HP-5 MS column (30m x 0.25mm x 0.25um, Agilent). The injector temperature was 280 ºC. The temperature program was as follows: 100-180 °C at 15 °C min^{-1}, 1 min hold at 180 °C, 180-300 °C at 5 °C min^{-1}, and 5 min hold at 300 °C. The flow rate of carrier gas (Helium) was 0.8 ml min^{-1} and a split ratio of 1:20 was followed. Data obtained were analyzed using AMDIS 2.64 software (NIST). The alkaloids were identified by comparing their GC-MS spectra with the information of the database of Amaryllidaceae alkaloids from the library developed by Dr. Bastida´s research group. The proportion of each individual component in the alkaloid fractions analyzed by GC-MS is expressed as a percentage of the total alkaloids in the fraction (TIC-total ion current) (Table 1).

Quantum calculations

Quantum calculations were carried out using *Gaussian 09* package.[49] Calculations were performed with full geometry optimization including in all cases the effect of the solvent (water as polar solvent) through the smd model,[50] B3LYP[51] and M06-2X[52] DFT functionals and 6-311+G (d,p) as basis set.[53, 54] We checked that the conformations obtained were minima by running frequency calculations. No imaginary vibrational frequencies were found. All energy values include zero-point correction.

Molecular docking studies

The complexes between ligands and receptors employed were obtained by molecular docking. The AChE from *Torpedo Californica* crystal structure (PDB: 1W6R) [41] and *human* BChE crystal (PDB: 1P0I)[55] were used to extract the geometries of the receptors, removing the ligand and water molecules.

The protonation states of residues in the protein were assigned with *H++* server.[56, 57] Before starting the docking simulations, the pKa of compounds were evaluated

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employing *MarvinSketch v17.15.0* software package, assuming a pH of 7.4 as physiological value. [58]

The program *Autodock 4.2.6* was used for the docking simulations, employed Gasteiger charges for the protein and ligands.[59] The search space was defined using *AutoGrid* around the entire catalytic cavity with a size of 78 × 90 × 90 points for *Tc*AChE and 68 x 68 x 60 for *h*BChE and a grid spacing of 0.375 Å. The search was made with genetic algorithm with the maximum number of energy evaluations was set to 5 x 10^6 and the maximum number of generations to 5 x 10^4 on a single population of 200 individuals. For each compound, the geometries generated and clustered with a RMSD threshold value of 2.0 Å.

For each complex, a refinement of the binding energy was performed with *Amber 16*.[60] The construction of the ligand units to be used was achieved with the *antechamber* module, using GAFF force field and AM1-BCC fitted charges. Input files for the simulations were built with the *xleap* package included in *ambertools*. For the refinement, two minimizations, of 5000 steps of the complexes were performed employing *Sander* software. The first one keeping the protein heavy atoms retrained at their initial positions and the second one with the whole system free. After the minimization steps, the binding free energies were calculated with molecular mechanics-Generalized Born surface area (MM-GBSA) approximations, using *MMPBSA.py*.[61, 62] The visualization of the docking result was performed with and *Vmd v1.9* [63] *and Maestro*.[64]

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Author Contribution Statement

S. L. recollected and identified the vegetal material. J. C. O. processed vegetal material, T. A. and M. G. performed the isolation, purification and elucidation experiments, analyzed the data and wrote the paper. N. P., J. B. and V. N. contributed reagents, materials, GC-MS analysis tools and analyzed the data. V. C. and A. M. performed the

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cholinesterase assays. J. B. and M. P. performed the molecular modelling experiments. M. G. conceived and designed the experiments.

Conflict of Interest

The authors declare no conflict of interest.

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