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EVALUATION OF ACETYLCHOLINESTERASE INHIBITORY ACTIVITY AND MODULATORY EFFECT ON NICOTINIC ACETYLCHOLINE RECEPTORS OF ALKALOIDS ISOLATED FROM *Rhodolirium andicola*

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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Evaluation of acetylcholinesterase inhibitory activity and modulatory effect on nicotinic acetylcholine receptors of alkaloids isolated from *Rhodolirium andicola*

Esta tesis fue realizada bajo la supervisión del director de Tesis Dra. ANA MUTIS TEJOS, y bajo la co-tutela del Dr. EMILIO HORMAZÁBAL URIBE, pertenecientes al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera, y es presentada para su revisión por los miembros de la comisión examinadora.

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Summary and outline of this thesis

Traditionally, pharmacological treatment for neurodegenerative diseases, such as Alzheimer and mild to moderate vascular dementia or similar diseases related to a deficit of the neurotransmitter acetylcholine, are treated through acetylcholinesterase (AChE) inhibitors. In this regard several compounds from synthetic and natural origin have been approved as AChE inhibitors by Food and Drug Administration in the United States. Although AChE inhibitors have demonstrated high efficacy in Alzheimer treatment, compounds from synthetic origin, such as donepezil and rivastigmine have reported several side-effect in patients from hepatotoxicity to gastrointestinal symptoms, in contrast with the fewer side-effects reported by natural compounds galanthamine and physostigmine. In this regard, after the discovery of Amaryllidaceae-derived alkaloid galanthamine (Reminyl®) as a competitive and reversible inhibitor of AChE from several Amaryllidaceae species, these have been studied as new sources of alkaloids with potential AChE inhibitory activity. Additionally, with the purpose of enhancing the cholinergic system damaged by Alzheimer's disease, several studies suggest that allosteric modulators of nicotinic acetylcholine receptors (nAChR) could be used as therapy in addition to AChE inhibitors. Therefore, the aim of this thesis was to characterize the alkaloid composition of bulbs of *Rhodolirium andicola* (Poepp.) Traub, a native Chilean Amaryllidaceae specie, and assess their inhibitory activity on AChE by *in vitro* and *in silico* methodologies. Likewise, the effects of isolated compounds on the agonist responses of nAChR α7, α3β4, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ expressed heterologously in *Xenopus oocytes* were determinated by electrophysiological techniques. The alkaloids from bulb extracts along with isolated

compounds, galanthamine, haemanthamine and tazettine, were identified by gas chromatography-mass spectrometry (GC-MS). The AChE inhibitory activity assays of alkaloidal bulb extract and isolated compounds were carried out by Ellman method. Alkaloidal extracts from *R. andicola* exhibited an inhibitory activity with IC_{50} values between 11.25 ± 0.04 and 57.78 ± 1.92 μg/ml that included isolated alkaloid, galanthamine (2.3 ± 0.18 μg/ml). Additionally, 12 alkaloids were detected using GC-MS and identified by comparing their mass fragmentation patterns with literature and database NIST vs.2.0. *In silico* results suggested that alkaloids, such as lycoramine, norpluvine diacetate and 6α-deoxy-tazettine expand the list of potential AChE inhibitors besides galanthamine. On the other hand, using nAChR expressed heterologously in *Xenopus oocytes* in conjunction with two-electrode voltage clamping, we found that galanthamine inhibits the function of nAChRs assayed through a mix competitive and non-competitevely. The nAChR α7 was significantly more sensitive to inhibition (17 ± 0.6 μM), whereas the heteromeric receptor α3β4 was the less affected (90 ± 3.4 μM). Moreover, neither haemanthamine nor tazettine were more potent than galanthamine. Finally, the findings of the present research show that this endemic plant is a potential source of AChE inhibitors that could be used eventually for treatment of neurodegenerative diseases. In addition, we found that galanthamine is an inhibitor of nAChR, contrary to studies that have found that this alkaloid enhances agonist responses of α7 and $(\alpha4)_3(\beta2)_2$ receptors through an allosteric mechanism.

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CHAPTER I

General introduction

1.1 General Introduction

The Acetylcholinesterase (AChE) inhibitors are the most used approved drugs for treating of patient with Alzheimer's disease (AD) (McGleenon et al., 1999). Additionally, with the objective to enhance the cholinergic system damaged in AD (Bourin et al., 2003), several studies suggest that allosteric modulators of nicotinic acetylcholine receptors (nAChRs) could be used as therapy in addition to AChE inhibitors (Maelicke et al., 2001; Pereira et al., 2002**;** Kihara et al., 2004; Texidó et al., 2005). After the isolation of Amaryllidaceae-alkaloid galanthamine, an AChE inhibitor approved by USA Food and Drug Administration (FDA), the focus has been on the identification and isolation of new compounds from Amaryllidaceae family with prominent use in AD (López et al., 2002, Ortiz et al., 2012, de Andrade et al., 2016). Pharmacological and biological properties, such as antitumor, antiviral, antibacterial, antifungal, antimalarial, analgesic, cytotoxic and AChE inhibitory activities, are attributable to plants from Amaryllidaceae family in which the presence of isoquinolinic alkaloids in bulbs appear to play a key role (Bianchi and Cole 1969; Jimenez et al., 1976; Castilhos et al., 2002; Lubbe and Verpoorte 2011; de Andrade et al., 2011; Cheesman et al., 2012; Ortiz et al., 2012; He et al., 2015).

1.1.1 Colinergic nervous system

The cholinergic system is an excitatory pathway using acetylcholine (ACh) as a neurotransmitter in the parasympathicus, sympathicus and central nervous systems (Pohanka, 2012). Cholinergic neurons in the mammalian central nervous system play an important role in fundamental brain functions, such as learning, memory, sleep and movement (Woolf et al., 1986; Woolf, 1991). The neurotransmitter ACh is synthesized from choline and acetylcoenzyme A by enzyme choline *O*-acetyltransferase (ChAT) (Oda

and Nakanishi, 2000; Gabrielle et al., 2003). As is the case for all nerve terminal proteins, ChAT and ACh can be found throughout the neuron. However, the highest concentration is found mainly in the axon terminals (Schmidt and Rylett, 1993). The rate-limiting steps in ACh synthesis is the availability of choline and acetyl-CoA. During increased neuronal activity the availability of acetyl-CoA from the mitochondria is upregulated as is the uptake of choline into the nerve ending from the synaptic cleft (Bahr and Parsons, 1986). The Calcium ion (Ca^{2+}) appears to be involved in both of these regulatory mechanisms (Parsons et al., 1993). Consequently, much of the choline used for ACh synthesis comes from the recycling of choline from metabolized ACh (Gabrielle et al., 2003). The majority of the ACh in nerve endings is contained in vesicles. Vesicle-bound ACh is not accessible to degradation by AChE (Llona, 1995). Thus, the uptake of ACh into storage vesicle occurs through an energy-dependent pump that acidifies the vesicle (Parsons, 1993). Subsequently, the acidified vesicle uses a vesicular ACh transporter (VAChT) to exchange protons for ACh molecules (Parsons, 1993). The release of ACh occurs through Ca^{2+} stimulated docking, fusion, and fission of the vesicle with the nerve terminal membrane (Llona, 1995). ACh binds only briefly to the pre- or postsynaptic receptors followed by dissociation from the receptor, and a rapid Ach hydrolyzation by the enzyme AChE (Stryer, 1995).

1.1.2 Acetylcholinesterase

The enzyme acetylcholinesterase (AChE), localized to the cytoplasm and outer cell membrane of blood and neural synapses its known by its rapid hydolisis of neurotransmitter acetylcholine (ACh) in the cholinergic neurotransmission (Barnard, 1974, Stryer, 1995). According with its physiological role, AChE has an unusually high specific activity, especially for a serine hydrolase, operating at a rate approaching that of a diffusion-

controled reaction (Quinn, 1987). Furthermore, AChE occurs in an array of molecular forms, differing in both quaternary structure and the mode of anchoring within the synapse (Silman and Futherman, 1987), probably to satisfy the individual requirements of diferents type of synapses (Magazanik et al., 1984). As consequence of its key physiological role, AChE is the target of a several natural or synthetic toxins (Čolović, et al., 2013).

At the present, the structural knowledge concerning AChE is mostly based on several oligomeric AChE forms obtained from *Electrophorus electricus* and *Torpedo californica* electric organs (Bon et al., 1979). In this regard, considering the 88.52% global sequence identity between structures of AChE from *T. californica* 1C2B.pdb and human AChE (PDB: 4PQE) (Moraga-Nicolás et al., 2018) several *in vitro* works has been carried out using *T. californica* AChE as enzyme model.

The knowledge of the 3D structure of AChE is, therefore, essencial for understanding its remarkable catalytic efficacy, for rational drug desing (Silman and Sussman, 2005). Its active site is located at the bottom of a deep and narrow cavity 20 Å under enzyme surface, lined by 14 conserved amino acid recidues (Dvir et al., 2010). Several kinetics and computacional studies have indicated that the active site of AChE contains two subsites, the first of them called esteratic subsite and the second one anionic subsite (Nachmansohn and Wilson, 1951; Dvir et al., 2010) corresponding, to the catalytic machinery (Ser, His and Glu) and the choline-binding pocket, respectively. The choline-binding pocket or so-called anionic subsite highlight by indole ring from trytophan residues, with which the quaternary group of choline moiety of acetylcholine make a π-cation ineteraction. Several researches have demostred the π-cation interaction between quaternary inhibitors and the anionic subsite from AChE. In this regard, Wilson and Quan (1958) reported that the anionic subsite interacts with the charged quaternary group of endrophonium, which act as

competitive inhibitor of AChE. Additionaly, Mooser and Sigman (1974) described the same effect of AChE obtained from chemicals studies with *N*-methylacridinum.

In addition, with the esteratic and the anionic AChE subsites, several authors have described that AChE possess additional binding sites for ACh or others quaternary ligands called 'peripheral anionic site' (PAS), "Acyl pocket" and "Oxyanion hole". In this regard, Taylor and Lappi (1975) by use of the fluorescent probe, propidium, established the 'peripheral anionic site' (PAS), clearly distinct from the choline-binding pocket of the active site. Enzymatic studies showed that the peripheral anionic site could be associated with substrate inhibition characteristic of AChE (Reiner et al., 1991). Additionaly, Szegletes and colleagues (1999) suggest that the first step in the acetylthiocholine (ATCh) catalytic pathway could be through of a transiently bound with PAS, however high ATCh concentrations in the PAS could produce to substrate inhibition. In the other hand, at lower substrate concentrations, substrate binding to the PAS could actually accelerate the acylation step in the catalytic pathway (Johnson et al., 2003). Additionaly, to peripheral anionic site, crystallographic and NMR studies suggest the existence of additional binding site from AChE called oxyanion hole (Steitz and Shulman, 1982). These authors suggest that the oxyanion hole could be formed main by chain nitrogens from two glycine and one alanine residues, interacting with the ACh carbonyl oxygen. This step will be key for the interaction bettwen the ACh with the imidazole ring from histidine in the active site (Houghton et al., 2006). The additional bending site called acyl pocket, consists of two phenylalanine residues, which are believed to play a role in limiting the dimension of substrates, which are able to enter the active site (Kua et al., 2002).

In addition to knowledge of 3D structure form AChE several computacional studies suggest that AChE is characterized by a strong electrostatic dipole caused by the

asymmetric charge distribution (Dvir et al., 2010), it is unlikely that the choline produced by hydrolysis leaves the active site through the same path by which the substrate entered. For this reason the called "back door" mechanism has been proposed in order to account for these properties (Houghton et al., 2006). It has been proposed, based on molecular dynamics and site-directed mutagenesis, that the process occurs through the opening of the bottom of the AChE gorge to provide a channel through which the products can pass, so enabling a part flow of substrate through the enzyme active site (Gilson et al., 1994).

1.1.3 Nicotinic Acetylcholine Receptors, Galanthamine and Alzheimer disease

Nicotinic Acetylcholine Receptors (nAChRs) belong to the Cys loop ligand-gated ion channel family are key elements of cholinergic transmission. In mammalian brain, the most prevalent receptors found are α4β2 and α7 types (Séguéla et al., 1993, Cassels et al., 2005). Several researches have demostred a considerable variation in the density of nAChRs observed in patients with AD that involve difficulties in maintaining and sustaining attention, and profound cognitive impairments, such as loss of memory and learning ability (Coyle et al., 1983; Aubert et al., 1992). Numerous studies using nicotinic agonist have demostred beneficial results on patients with AD in functions such as verbal learning, memory on the selective reminding task and attention (Newhouse et al., 1993; Potter et al., 1999). These results have demonstrated the potential use of nAChRs as target for find new candidate AD drugs from natural or synthetic origin (Arneric et al., 2007).

In this regard, in addition to its effects on AChE, electrophysiological studies have suggested that the Amaryllidaceae alkaloid, galanthamine, allosterically enhances the function of a number of nAChR providing therapeutic benefits in the areas of cognition,

attention and antineurodegenerative activity (Samochocki et al., 2003). However, other studies using slightly different experimental procedures have found that galanthamine inhibits nAChR in a non-competitive manner (Smulders et al., 2005). This is in contrast to studies that have found that galanthamine enhances agonist responses of α7 and $(\alpha4)_3(\beta2)_2$ receptors through an allosteric mechanism (Maelickeet al. 2001; Texidó et al. 2005). In contrast to this results, Kuryatov et al. (2008) found no significant potentiating effects of galanthamine on $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ receptors, although they reported that galanthamine appeared to be a specific allosteric modulator of α5α4β2 nAChR. These discrepancies may well reflect differences in experimental conditions. For example, Texidó et al. (2005) tested the effects of galanthamine on the responses of α7 receptors to 500 μM ACh, a concentration that is well above the ACh EC_{50} for human α7 nAChR expressed heterologously in *Xenopus* oocytes (Chavez-Noriega et al., 1997) and, critically, observed significant potentiation at only one concentration of galanthamine. Further studies have to be carried out to resolve these discrepancies.

1.1.4 Acetylcholinesterase inhibition and Alzheimer disease

Inhibition of enzyme Acetylcholinesterase, is an important strategy for the treatment of diseases that involve cholinergic transmission deficit such as myasthenia gravis and Alzheimer's disease (AD) (Rahman and Choudhary, 2001; Mehndiratta et al., 2011). AD is the most common form of dementia in our society (World Alzheimer Report, 2015). Worldwide, it is currently estimated that 46 million people have AD or a related dementia, and considering that life expectancy will increase, it is estimated that people with AD will reach to 131.5 million by 2050 (World Alzheimer Report, 2015). Although AChE

inhibition is an established therapeutic strategy to ameliorate cognitive dysfunction and memory loss associated with AD (Rahman and Choudhary, 2001), only a few compounds, such as tacrine, donepezil, physostigmine and galanthamine (Zarotsky et al., 2003) have been approved by the Food and Drug Administration (FDA) in the United States. However, several side-effect such as hepatotoxicity and problems associated with gastrointestinal symptoms, have been reported for the synthetic drugs tacrine and donepezil, respectively (Schulz, 2003; Mehta et al., 2012). In contrast, physostigmine and galanthamine, both from natural origin, have fewer side effects in patients with mild to moderate AD (Mehta et al., 2012). Consequently, many research groups have focused their studies on finding new renewable sources of compounds with acetylcholinesterase inhibitory activity (Mukherjee et al., 2007). In this regard, after the isolation of natural compound galanthamine, a long-acting, selective, reversible and competitive AChE inhibitor, approved in 2001 by FDA (Razadyne®), for clinical treatment of mild and moderate AD, several Amaryllidaceae species have been evaluated as new sources of galanthamine or other alkaloids with potential AChE inhibitory activity (López et al., 2002; Rhee et al., 2004; Ortiz et al., 2012). Although the chemical synthesis of galanthamine is available (Marco and Carreiras, 2006; Bulger et al., 2008), current pharmaceutical production of this compound is mainly limited to the extraction of natural populations of the Amaryllidaceae *Leucojum aestivum* and *Narcissus* spp. (Heinrich and Teoh, 2004).

1.1.5 Chilean Amaryllidaceae family

The Amaryllidaceae family have been cultivated for centuries as ornamental plants for their colourful flowers and fragrant oils, but also for their efficacious medicinal properties (Ding et al., 2017). This family includes about 1600 native species from Asia, Europe,

North Africa, Oceania and South America (Heywood, 1985; Meerow and Snijman 1998) distribute on three subfamilies: Amaryllidoideae, Agapanthoideae and Allioideae (Chase et al., 2009). In Chile there are more than 30 native species from the subfamily Amaryllidoideae covering a wide variety of eleven genera (Ravena, 2003). Despite the variety of Amaryllidoideae species in Chile, there are a few studied species in relation to chemical constituents (Pacheco et al., 1978, 1981, 1982; Sepúlveda et al., 1982; Pacheco and Silva, 1992; Lizama-Bizama et al., 2018; Tallini et al., 2018). Particularly, *Rhodolirium andicola* (Poepp.) Traub, part of endemic Amaryllidoideae species growing in Chile could be consider as new source of secondary metabolites with potential AChE from that there are not chemicals studies yet. Considering the limited knowledge on secondary metabolites regarding to a native Amaryllidoideae species, this work will contributed to knowledge of metabolites presents on native Amaryllidoideae species.

1.1.6 Chemical constituents of the Amaryllidoideae subfamily

The chemical constituents isolated and identified from *Amaryllidaceae* species include alkaloids, anthocyanins, flavonoids and peptide (López et al., 2002; Rhee et al., 2004; Chu and Ng, 2004; Ortiz et al., 2012; Byamukama et al., 2006; Mikátková et al., 2014; Ortiz et al., 2016). However, the most bioactive constituents in the *Amaryllidaceae* family are the isoquinoline alkaloids (Bastida et al., 2006; Bastida et al., 2011; de Andrade et al., 2011). These compounds are identified primarily in steam and bulbs containing higher concentrations and variety of isoquinoline alkaloids (Bastida et al., 2011; de Andrade et al., 2011; Tallini et al., 2018). Some of the major alkaloids reported from various *Amaryllidaceae* species are Galanthamine, Tazettine, Haemanthamine and Lycorine, (Bastida et al., 2011; de Andrade et al., 2016: Ortiz et al., 2016).

The differences alkaloids content depending on geographical distribution (altitude). For example, geographical distribution of *Habranthus jamesonii* plants showed differences on galanthamine content and the authors suggest that is influenced by altitude, and season (Ortiz et al., 2012). Additionaly, Berkov et al. (2011) reported an intraspecies diversity in alkaloid profiles in *Galanthus elwesii* and *G. nivalis* populations collected in different locations in Bulgaria.

The biosynthesis of the isoquinoline Amaryllidaceae alkaloids (Figure 1) in begins with the enzyme preparation of precursors from the amino acids L-Phenylalanine (L-Phe) and L-tyrosine (L-Tyr). Although L-Phe and L-Tyr are closely related in chemical structure, they are not interchangeable in plants (Bastida et al., 2006).

In the biosynthesis pathway of Amaryllidaceae alkaloids, the fragment C_6-C_1 is derived from L-Phe, whereas, L-Tyr is the precursor of fragment, C_6-C_2-N. The conversion of L-Phe to the C_6-C_1 unit requires the loss of two carbon atoms from the side chain as well as the introduction of at least two oxygenated substituents into the aromatic ring, which occurs through cinnamanic acid or its derivatives, involving the participation of the enzyme phenylalanine ammonia lyase (PAL) (Bastida et al., 2006). The fragmentation of the cinnamic acid involves oxidation of the β-carbon to the ketone or acid level, where the final product is protocatechuic aldehyde. Additionaly, L-Tyr is degraded no further than tyramine before incorporation into the Amaryllidaceae alkaloids (Figure 1) (Machocho, 2000; Bastida et al., 2011). The second stage involves merging the biosynthesis of tyramine and the protocatechuic aldehyde, resulting in norbelladine by forming a Schiff's base. This reaction occupies a pivotal position since it represents the entry of primary metabolites into a secondary metabolic pathway (Figure 1) (Bastida et al., 2011). Norbelladine can undergo oxidative coupling of phenols in Amaryllidaceae plants, Norbelladine has been suitably

protected by methylation, which is considered as the third step. Finally, the last stage includes a series of sequential oxidative coupling resulting in the diversification into the other eight skeletons (Figure 2). Ortho-para' phenol oxidative coupling of O-methylnorbelladine results in the formation of a lycorine-type skeleton, from which homolycorine-type compounds proceed as well. The galanthamine-type is the only skeleton which originates from para-ortho' phenol oxidative coupling. The formation of Crinine, haemanthamine, tazettine, narciclasine and montanine structures derived from the para-para' phenol oxidative coupling (Figure 2) (Bastida et al., 2006). Subsequent transformations may involve oxidation, reduction, ring opening and rotation, where one alkaloid may be converted into another (Machocho, 2000).

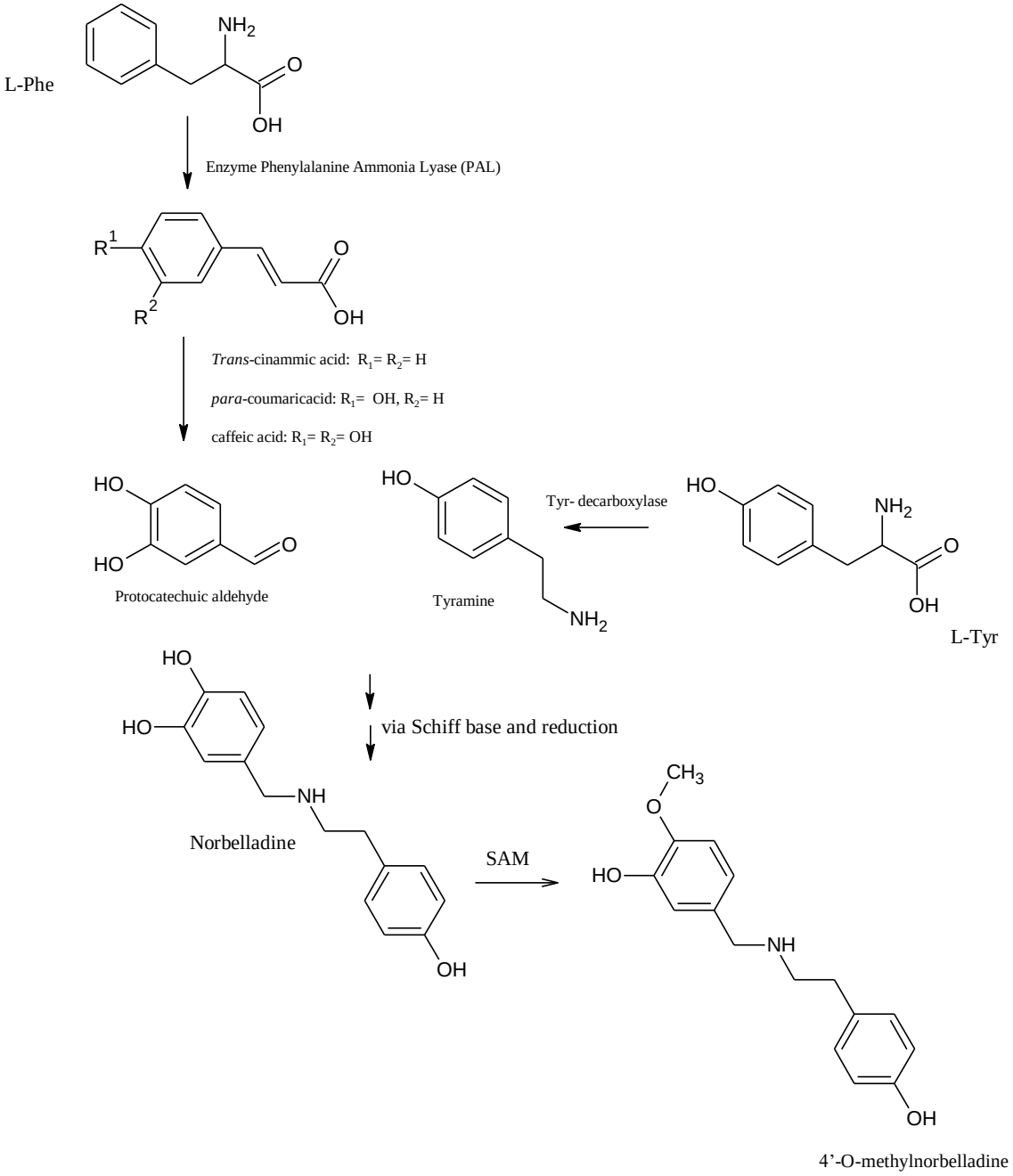

Figure 1. Biosynthetic patways of commun precursor of isoquinoline alkaloids identified in *Amaryllidaceae* species, 4'-*O*-metylnorbelladine. Unbroken arrows from L-Phe and L-Tyr indicate single enzymatic conversions. PAL enzyme phenylalanine ammonia lyase, Tyr-decarboxylase, enzyme Tyrosine-decarboxylase (Adapted from Bastida et al., 2006).

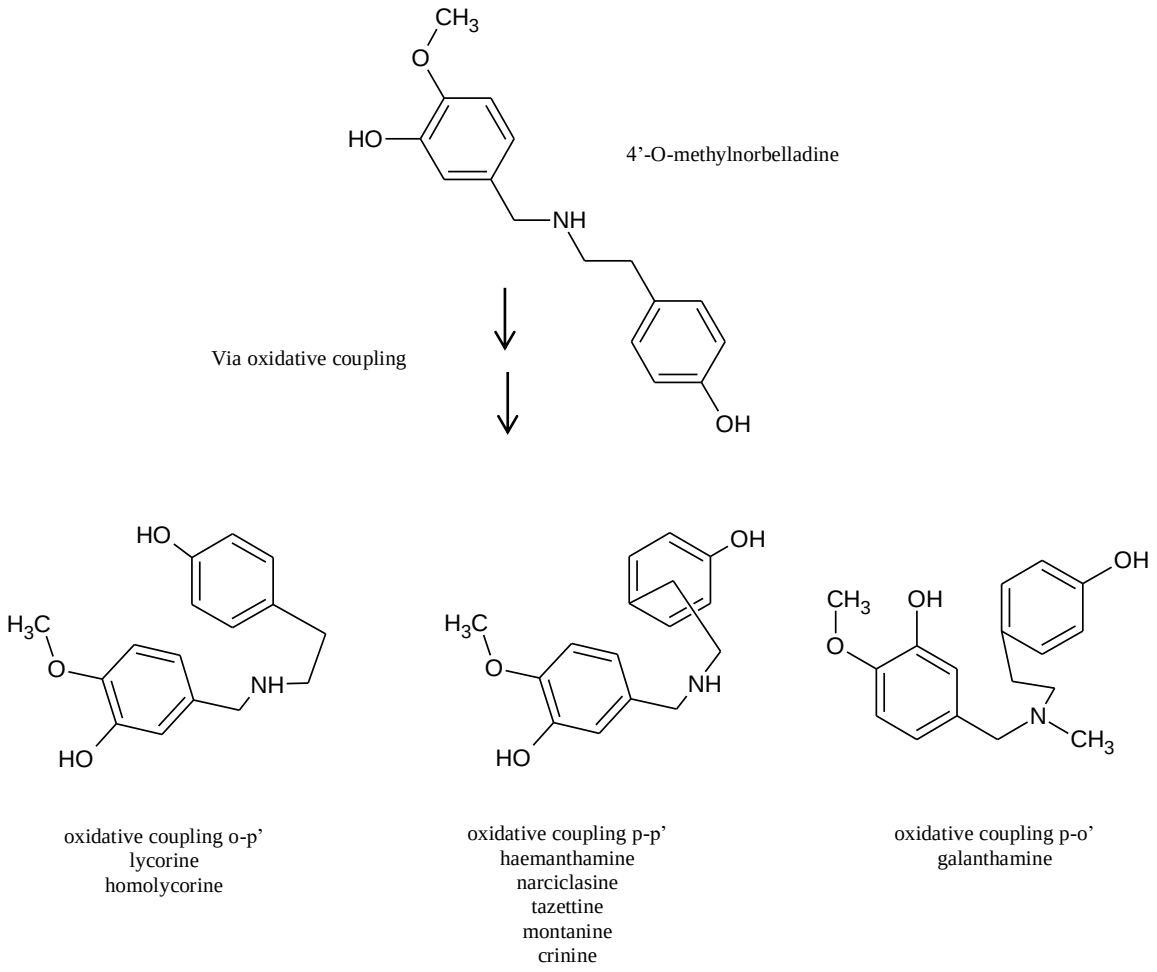

Figure 2. Phenol oxidative coupling in Amaryllidaceae alkaloids (Adapted from Bastida et al., 2006)

1.1.7 Acetylcholine receptors

Nicotinic acetylcholine receptors (nAChR) are distributed throughout the central nervous system (CNS), peripheral nervous system (PNS) and additionally in non-exitable cells (Gahring and Rogers, 2005; Bschleipfer et al., 2008; Song and Spindel, 2008). Like many other ligand-activated neurotransmitter receptors, consist of two major subtypes: the metabotropic muscarinic receptors and the ionotropic nicotinic receptors (Albuquerque et

al., 2009). Both major subtypes are activated by the endogenous neurotransmitter acetylcholine (ACh) (Alkondon and Albuquerque, 1991; Gotti and Clementi, 2004). Nicotinic acetylcholine receptors belongs to the Cys loop ligand-gated ion channel family that also includes the muscle nAChR, γ−aminobutyric acid receptors type A and C, glycine receptors and serotonin type 3 receptors (Taly et al., 2009).

The nAChR are transmembrane glycoproteins of 290 kDa, formed by bending of five sub-units, positioned symmetrically inserted into the lipid membrane around a pore that constitutes the ion channel. The neuronal subunits are divided into the alpha (α2–α7, α9, and α10) and beta (β2–β4) classifications based on the presence of adjacent cysteine groups in the extracellular domain of only the α subunits (Albuquerque, et al., 2009; Dani and Balfour, 2011). Each one of this sub-units are formed by four hydrophobic segments called M1, M2, M3 and M4 composed by alpha helices through the membrane (Blanton and Cohen, 1994; Corbin et al., 1998). Several sub-units combinations in diferents stoichiometry build a functional nAChR (Dani, 2015).

The relatively long extracellular N-terminal domain formed by 200 amino acid contributes to ligand binding, followed by the three hydrophobic transmembrane regions formed by 90 amino acids (M1–M3), a large intracellular loop, a fourth transmembrane region (M4), and ultimately a short extracellular C-terminus (10 to 30 amino acids) (Sargent, 1993; Lindstrom et al., 1995).

Several analysis of electron microscopy on nAChR isolated from the electric fish *Torpedo marmonata* showed a pentameric pseudosymmetric transmembrane assembly, formed by the bendig of five sub-units in torn to central pore conformed by transmembrane region M2 and extracellular N-terminal segment M1 (Zhang and Karlin 1997; Unwin, 2005).

1.1.7.1 Agonist bending site on nAChR

The receptor activation is carried out when a ligand (agonist) is capable to binding a specific sequence from the receptor structure, and generate with this a conformational change, opening the central pore, allowing the flow of ions (Dani, 2015). Several authors have suggest that the site of recognition of the ligand is located between of α subunit of the receptor and a complementary site of another subunit, and in particular implies a series of amino acids within the polypeptide chain (Dennis at al., 1988; Middleton and Cohen, 1991). However, for this interaction between quaternary group from ACh and the binding site from receptors is necessary a high density of electrons from the recognition site. In this regard, Celie and colleagues (2004) showed that both ACh (isolated from *Lymnaea stagnalis*, *Bulinus truncatus* and *Aplysia californica*) and Carbachol to bind by π-cation interactions to the same recognition site conformed by a conserved aromatic amino acid residue.

1.1.7.2 Physiological role of nAChR

The *nAChR* are involved in several cognitive functions from CNS such as learning memory, attention, control of motor activity, sensory perception, pain and corporal temperature regulation (Giacobini, 1990; Gotti et al., 1997). Generally, these effects are due to the existence of nAChR (which usually contain the subunit α7) in the presynaptic terminal, which act by modulating of neurotransmitters secretion (Wonnacott, 1997).

The most relevant action of nAChR is the neuroprotection, mediated mainly by α7 subtype, inducing the synthesis of nervous factors growth or to reduce the cytotoxicity mediated by Glutamate (Flores et al., 1992; Shimohama et al., 1996).

Additionally has also been demonstrated the involvement of nAChR in neurodegenerative diseases such as Alzheimer's or Parkinson's disease, and even in cerebral dysfunctions such as autism or schizophrenia, anxiety or analgesia (Court et al., 2000; Cassels et al., 2005).

1.1.7.3 Agonist and Antagonist of nAChR

The first known agonists acting on nAChR were molecules isolated from natural origin such as (-)-Nicotine, Anabaseína-a, (-)-cytisine. Despite the potency of these natural agonists, their therapeutic use is limited by their high toxicity, linked to their lack of selectivity, and dependency problems, although this would depend strongly on the route of administration (Daly, 2005; Cassels et al., 2005).

Currently there are a great interest in determining the structure and functional diversity of nAChR and developing selective agonists to subtypes that have potential as therapeutic agents for some neuropathologies and diseases. However, little attention has been paid to development of selective nAChR antagonists to subtypes. These antagonists could be effective in establishing the role of each receptor-specific subtype in its physiological function and for the treatment of some pathologies to which they are associated (Cassels et al., 2005).

1.1.7.4 Assays of biological activity against nAChR

Currently are performed mainly two assays: the displacement of a radioligation, which determines the affinity of tested compound for the *nAChR* and the electrophysiological, which allows to establish the functionality of a tested compound against *nAChR*, therefore

known if is agonist, antagonist, inverse agonist, positive allosteric modulator, competitive antagonist, or non-competitive antagonist.

There are several electrophysiological methods to determine the effect produced by ligands on excitable cells (voltage techniques) one of them called voltage clamp (Halliwell and Adams, 1982) a the other one known as patch-clamp (Neher and Sakmann, 1976).

The *Xenopus leavis* oocytes are widely used to study the behavior of ion channels of known molecular identity and have been the expression method of choice for studies of the structure-function relationships of ion channels (Stühmer, 1998). This technique was used for first time for Gurdon and colleagues (1971) for study of messenger RNA and its translation in living cells.

Since 1986 this technique has also been applied to express nAChRs and study its functional characteristics against different ligands reported as well as the new ligands synthetic that have been developed since them (Methfessel et al., 1986).

This electrophysiological method allows the control of the membrane potential to measure the streams flowing through the channels, or ionic pumps. In the process are implanted two borosilicate capillary glass inside the oocyte, one of them record the potential of membrane, which fixes the voltage, and the other to measure the current. The first electrode is connected to an amplifier where the signal is compared to the voltage value supplied by a generator. The amplified difference of this signal is applied as a current by means of the electrode through the membrane. When the channels are open, a flow of ions are transported inside of the oocyte, the change is measured as a deflection of the baseline of the current.

The compounds that by themselves can open the nAChRs channel are called agonists. Those who do not reach a maximum response to high concentrations are called partial agonists, which block the currents produced by agonists are called antagonists and those that increase the current produced by an agonist is they call positive allosteric modulators (Figure 3).

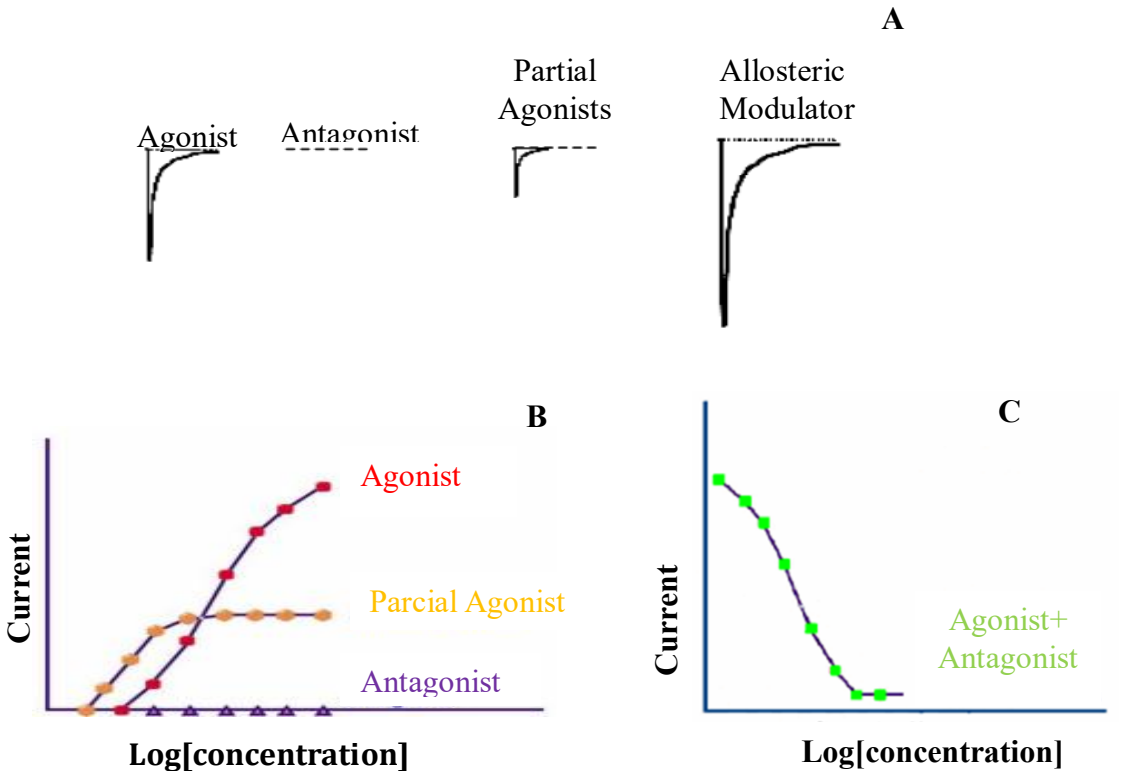

Figure 3: Behavior and analysis of ligands nAChRs. A. Currents produced by a single agonist, more antagonistic agonist and more agonist allosteric positive. B. Graphic analysis of the currents produced by an agonist, a partial agonist and an antagonist. C. Graphic analysis of inhibition produced by increased concentrations of an antagonist coapplied with an agonist (figure adapted from Pérez, 2008).

1.2 Hypothesis

Considering that Amaryllidaceae plants are a source of galanthamine type alkaloids and galanthamine is a powerful acetylcholinesterase inhibitor, and have modulator effects on nicotinic acetylcholine receptors the hypothesis of this work is: Bulbs from *Rhodolirium andicola (Poepp.) Traub,* (Amaryllidaceae) contain active alkaloids such as galanthamine or others that inhibit acetylcholinesterase activity and have modulator effects on nicotinic acetylcholine receptors.

1.3 General Objetive

To evaluate the potential acetylcholinesterase inhibitory activity and modulator effect on nicotinic acetylcholine receptors of *Rhodolirium andicola* (Poepp.) Traub alkaloids.

1.4 Specific Objetives

(i) To evaluate the AChE inhibitory activity of pure alkaloids isolated from *R. andicola*, by enzymatic assays.

(ii) To determine the interactions between bioactive alkaloids and AChE previously identified, by molecular docking.

(iii) To evaluate the pharmacological response of pure alkaloids on nicotinic acetylcholine receptors.

CHAPTER II

Alkaloid discovery as natural acetylcholinesterase inhibitors, from nature to molecular docking

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Alkaloid discovery as natural acetylcholinesterase inhibitors, from nature to molecular docking

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Abstract

Acetylcholinesterase (AChE) plays a key role as an essential enzyme in memory and cognition process through the hydrolysis of neurotransmitter acetylcholine. Although the physiological role of AChE in neural transmission is well known, its role as pharmaceutical target for the treatment of Alzheimer's disease (AD) is still matter of extensive research. It has been elucidated that cholinergic deficiency is associated with AD. Therefore, one of the major therapeutic strategies is to inhibit the biological activity of AChE, increasing the acetylcholine level in the brain. However, one of the major limitations of up-regulating AChE activity through acetycholinesterase inhibition is that repeated doses of acetylcholinesterase inhibitor (AChEi) lead to the development of tolerance. Despite its limited success, AChEi remains as the only approved treatment by Food and Drug Administration (FDA) for AD. This scenario has led to strong efforts to discover new AChEi from a vast number of plant species, which can provide new bioactive compounds for control strategies in AD. Thus, natural alkaloids have been shown to be potent AChEi. However, the process of AChEi-based drug development is challenging, time consuming, expensive, and requires consideration of many aspects (eg. chemical structure, identification and biological assay). Therefore, the computer-based methods are becoming increasingly important as they complement laboratory experiments in studying the structure

and function of biomolecules. Specifically, molecular docking has been highlighted as a frequently used tool in structure-based drug discovery. Although early efforts were hindered by limited possibilities in computational resources, the recent advance in high performance computing with virtual screening methods has become drug discovery more efficient. Therefore, the objective of this review is to summarize the usefulness of molecular docking related to alkaloid research as well as new approaches for alkaloid discovery using AChE as targets.

Keywords: Molecular docking, alkaloids, acetylcholinesterase inhibitors.

2.1 Introduction

Alzheimer's disease (AD) is the most common single cause of dementia (Ferris and Farlow, 2013). This illness is characterized by progressive neurodegenerative disorders, collapse of cognitive functions and formations of amyloid plaques along with neurofibrillary tangles (Auld et al., 2002; Blennow et al., 2006). The AChEi have been approved for the symptomatic treatment of AD for over a decade, which is highlighted by preventing the hydrolysis of acetylcholine (Zaheer-ul-Haq et al., 2010). However, it seems that all long-term studies have not shown clinical efficacy resulting in a loss of drug efficacy or the relentless progression of the disease (Zaheer-Ul-Haq et al., 2003). Thus, interest in the discovery of novel AChEi remains since the lack of perfection in the current ones.

Many of AChEi and structures known today are derived from natural products, such as galantamine and huperzine A (Lilienfeld, 2002; Ma and Gang, 2008). Natural products have played a significant role in the development of new drugs for the treatment of diseases

(Ingrassia et al., 2008). Thus, alkaloids can exhibit a wide range of bioactivities, such as antitumor, antiviral, antibacterial, antifungal, antimalarial, analgesic and AChEi (Evidente and Kornienko, 2009; Jin, 2011). It is thought that novel natural sources of drugs can be discovered by traditional extraction methods and bioassays associated.

The use of AChE as target and potential AChEi as ligands can involve an *in silico* approach called molecular docking, which is frequently used in structure-based drug discovery. Unfortunately, no perfect representation has been obtained for ligand-receptor systems since physicochemical conditions and dynamics have apparently been overlooked in diferent receptors (Guo et al., 2004). Likewise, the results of previous studies on AChE-ligand interactions seemed to be heavily dependent on the type of ligand (Berg et al. 2011), the protein conformation (Alonso et al.,2006) and the presence of water (Roberts and Mancera, 2008). Despite these difficulties, molecular docking of AChE ligands has been applied in numerous cases to explore the role of identified and subsequently synthesized compounds in terms of AChE-ligand interactions and quantitative structure-activity relationships (QSARs). However, to answer the question of how useful molecular docking could be for alkaloid discovery, some facts need to be addressed. Can alkaloids be successfully docked in to AChE crystal structures? Is it likely to achieve consistency between experimental and in silico AChE-alkaloid interactions? This review seeks to answer these and other questions summarizing the usefulness of molecular docking with successful results related to alkaloid research. Likewise, the best conditions along with new approaches for alkaloid discovery using AChE as targets are proposed.

2.2 Alkaloids and their impact for the treatment of human diseases.

Plants used in traditional medicine have the potential to provide pharmacologically active natural products such as alkaloids, which can be used to treat several diseases. This could be achieved by taking advantage of information available from traditional medicine and/or ethnobotanical knowledge (Elgorashi and Van Staden, 2004). Alkaloids are important molecules derived from secondary metabolism that can act as rich sources of research in biomedicine and drug discovery area (Lu et al., 2012). Thus, several alkaloids isolated from natural herbs exhibit potential activity against cancer. In fact, the first agents to advance into clinical use were indole alkaloids, vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae), used in combination with other chemotherapeutic drugs for the treatment of a variety of cancers including lymphocytic leukemia, testicular cancer, breast and lung cancer (Cragg and Newman, 2005). On the other hand, the diterpene alkaloid paclitaxel isolated from Pacific Yew (*Taxus brevifolia*), inhibits cell proliferation by altering the dynamics of tubulin addition (Mohan et al., 2012), and is used in the treatment of breast, ovarian, and non-small cell lung cancer, and has also shown efficacy against Kaposi sarcoma (Cragg and Newman, 2005). Another important source of important remedies in Oriental medicine is Chinese herbs, on this matter, evodiamine, a quinolone alkaloid, is one of the major bioactive compounds isolated from *Evodia rutaecarpa*. Studies have demonstrated that evodiamine has anti-cancer activities both *in vitro* and *in vivo* by inhibiting proliferation, invasion, metastasis and apoptosis in a variety of cancer cell lines (Jiang and Hu, 2009). On the other hand, berberine, an isoquinoline alkaloid widely distributed in natural herbs including *Rhizoma coptidis* (a prescribed Chinese herb), has shown potential anti-cancer activity in *in vitro* and

in vivo experiments by interfering with the multiple aspects of tumorigenesis and tumor progression (Lu et al., 2012). Furthermore, Amaryllidaceae alkaloids; lycorine, amarbellisine, haemanthamine, and haemanthidine reported by Van Goietsenoven et al. (2010), exert antiproliferative activities toward cancer cell lines that are apoptosis-resistant. Although the source of alkaloids with anti-cancer potentials is very extensive, considerations, such as water solubility, bioavailability and toxicity must be evaluated (Lu et al., 2012).

Malaria is an infectious disease, causing approximately one million deaths annually and 300–400 million infections per annum (Şener et al., 2003; Dua et al., 2013). Protozoan species of the genus *Plasmodium* are responsible for this infection, although the majority of fatal cases are caused by *P. falciparum*. The challenge in malaria chemotherapy is to find safe and selective agents with potency that will not be compromised by plasmodial resistance (Frederich et al., 2008). Literature supports the potential of plant-derived alkaloids as source for anti-malarial drug development (Frederich et al., 2008; Onguéné et al., 2013). In this regard, the study reported by Chierrito et al. (2014) highlights the activity against *P. falciparum* and low cytotoxicity in *in vitro* experiments shown by the monoterpene indole alkaloid, aspidoscarpine, isolated from *Aspidosperma olivaceum*. On the other hand, Dua et al. (2013) reported that the steroidal alkaloid conessine isolated from the bark of *Holarrhena antidysentrica* exhibited substantial anti-malarial activity with slight cytotoxic nature.

Likewise, alkaloids can be used for antiparasitic activity in the control of *Trichomonas vaginalis*, a flagellate protozoan which infects the human urogenital tract and causes vaginitis in women and urethritis in men, the most common non-viral sexually transmitted

disease (He et al., 2015). In relation to this, the reports agree on the potential of lycorine alkaloid and its chemical derivates as anti-protozoal (Vieira et al., 2011; Giordani et al., 2011).

Furthermore, antiviral activities of alkaloids have been described. For example, Özçelik et al. (2011) evaluated the antiviral activity of commercial alkaloids. Atropine, a tropane-type alkaloid showed potent antiviral effect on Herpes Simplex Virus (HSV) and parainfluenza type 3 virus (PI-3). Moreover, Farnsworth et al. (1968) evaluated 36 alkaloids isolated from *Catharanthus roseus* and *Catharanthus lanceus* for their antiviral activity. Nine of these alkaloids were classified with effective antiviral against vaccinia and polio type III viruses, with pericalline being the most effective. Evaluation of lycorine, pseudolycorine and pretazettine exhibited *in vitro* activity against the flaviviruses, such as Japanese encephalitis, yellow fever, and dengue viruses (Gabrielsen et al., 1992). Another important biological property of alkaloids is their antibacterial activity, where it is possible to find literature that supports the broad spectrum of microorganisms Gram positive and Gram negative, on which different type of alkaloids present bacteriostatic and/or bactericidal activity (Karou et al., 2005; Zuo et al., 2011; Cheesman et al., 2012). As it has been demonstrated, alkaloids seem to play a significant role in human diseases showing their potential as natural drugs. Recently, optimization of bioactive alkaloids seems to be performed using QSAR, resulting in new more potent compounds. For instance, Akula et al. (2006) described that three-dimensional quantitative structure–activity relationship (3D-QSAR) studies by molecular docking and comparative molecular field analysis (CoMFA) may serve as a useful tool to gain insight into the mechanism of inhibition and to predict the inhibitory properties of newly designed compounds. QSAR development provides a

powerful tool to correlate the biological activities of compounds to their structural or physicochemical parameters and extends the correlated parameters for the prediction of new active ligands (Viswanadhan et al., 1989). CoMFA, needs the studied molecules to be aligned in the 3D space. In conventional CoMFA studies, as in the first study by Cramer et al. (1988), the molecules are fitted to a reference molecule.

Table1: Summary of the most used currently Protein-ligand complexes.

Compounds	Software	Crystal structure (PDB code)	Resolution (Å)	Post-docking approach	Validation	Reference
Euchrestifoline*	FRED 2.1	1ACL	2.80	Further, 3D-QSAR	Enzymatic bioassay	(Rehman et al., 2013)
Buxamine –B* Buxamine –C*	FlexX	1ACL	2.80	LIGPLOT and WebLab ViewerPro	Yes, by enzymatic bioassay	(Khalid et al., 2005)
82 *N*-benzylpiperidine derivatives		1ACE; 1AMH	2.50; 2.50	3D-QSAR, CoMFA	No	(Bernard et al., 1999)
Hybrid tacrine-donepezil	GOLD 2.1.1	1EVE	2.50	ADMTE	No	(da Silva et al., 2006)
Corydaline*	GOLD 3.0.1	1EVE	2.50		No	(Dan et al., 2007)
Hypol A Hypol B	GOLD	1EVE	2.50		Fischer's randomization test	(Gupta and Mohan, 2014)
1-nitro-2-phenylethane	Molegro Virtual Docker (MVD)	1C2B	4.50	Gaussian 03	Biautogram	(Silva et al., 2014)
eburnamine*, eburnamonine*, eburnamenine* geissoschizol*	CHIMERA	1B41	2.76	Further ADME and 3D-QSAR	No	(Naaz et al., 2013)

***Alkaloid compounds identified from plants**

Table1: Summary of the most used currently Protein-ligand complexes. Continued

Compounds	Software	Crystal structure (PDB code)	Resolution (Å)	Post-docking approach	Validation	Reference
Selection of compounds from Chem Score	GOLD	1EVE; 1B41	2.50 2.76	CoMFA	No	(Guo et al., 2004)
Steroidal Alkaloid*	AUTODOCK 2.4	1ACL	2.80		No	(Zaheer-Ul-Haq et al., 2003)
Bis-tacrina structures	FleXx and FlexiDock	1Q84	2.45	3D-QSAR	No	(Akula et al., 2006)
N-Aryl derivates	AutoDock (3.0.5)	1B41	2.76		Kinetic assays	(Correa-Basurto et al., 2007)
Tacrine-hybrids	CDOCKER	2CKM	2.15	MM/PBSA	Ellman assay	(Chen et al., 2012)
Xanthstigmine derivatives	FleXx	1EVE	2.50	3D-QSAR	No	(Gupta and Mohan, 2011)
Tacrine-hybrids	GOLD v 5.2	4EY7; 2CMF	2.35 2.50		Ellman assay	(Jin, 2014)
Pharmacophore model	GOLD	1EVE	2.50	QSAR	NO	(Tyangi et al., 2010)
Salicylanilide *N*-alkylcarbamates	GOLD v 5.0	2WHQ	2.15		Ellman assay	(Imramovsky et al., 2012)
Genistein derivatives*	MOE 2010	4EY7	2.35	MM/GBSA	Ellman assay	(Fang et al., 2014)
Oliveline*noroliverioline*, liridoline*, isooncodine*, polyfothine*, darienine*	CHIMERA	1B41	2.76		No	(Naaz et al., 2013)
pleiocarpine*, kopsinine*, pleiocarpamine*	CHIMERA	1B41	2.76		No	(Naaz et al., 2013)

***Alkaloid compounds identified from plants**

This reference molecule should be the most rigid of the active molecules. In this case, the molecule is always in its "biologically active" conformation. However, when active molecules are flexible, finding an appropriate alignment becomes a complicated task with high probability of a misleading result. Another example is the study performed by Gupta and Mohan (2011), where a good quality of CoMFA model would suggest the validity of proposed models of inhibitor-enzyme interactions obtained by the automated docking procedure. The applications of QSAR methods for alkaloid discovery are summarized in Table 1.

2.3 Alkaloids and their potential as natural AChEi drugs.

The AD is the most common cause of dementia in our ageing society. Although recent evidence suggests that AD is a heterogeneous disorder comprising several different phenotypic and genotypic expressions, it is characterized by an insidious decline in cognitive and non-cognitive function. Traditionally, short and long-term memory is impaired, while language skills, concentration and attention are often affected (McGleenon et al., 1999). An important approach to treat AD involves the inhibition of the AChE enzyme, which has proven to be the most viable therapeutic target for symptomatic improvement in AD because cholinergic deficit is a consistent and early finding in this disease (Mehta et al., 2012).

Alkaloids have been studied as AChEi with more than 35 alkaloids reported so far. However, a few of them have entered therapeutic use (Mukherjee et al., 2007). Different classes of compounds have been considered, namely indole derivatives (such as

physostigmine and related compounds), isoquinoline and related derivatives (such as galantamine and lycorine-type alkaloids), steroid and terpenoid alkaloids, and many other derivatives that present significant inhibitory effects on AChE (Mukherjee et al., 2007).

Galantamine is a natural plant alkaloid (Ma and Gang, 2008), produced by *Galanthus nivalis* L. and related plants (Amaryllidaceae family) (Lilienfeld, 2002). Huperzine A, is a potent but reversible inhibitor of AChE used in China for treating patients with AD. The source of huperzine A is *Huperzia serrata*, a moss that has been used for treating contusions, strains, hematuria and swelling in Chinese folk medicine (Mukherjee et al., 2007). Furthermore, Montanine (Ortiz et al., 2012) as well as 11β-hydroxygalanthamine, (de Andrade et al., 2011), orydaline and corydine (Adsersen et al., 2007) and stepharanine, cyclanoline and N-methyl stepholidine (Ingkaninan et al., 2006) showed significant acetylcholinesterase inhibitory activity in a dose-dependent way.

2.4 Acetylcholinesterase: 3D structure and its role as target

Acetylcholinesterase is known for carrying out a rapid hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Zaheer-ul-Haq et al., 2010; Hai et al.,2013) (Dvir et al., 2010). Availability of AChE crystal structures from several species with and without ligands provides a solid basis for structure-based design of novel AChE inhibitors (Guo et al., 2004). One main feature that must be known for drug discovery using AChE is its active site. This active site, widely called “binding site or pocket”. For proteins, is mainly composed by five subsites: (1) esterasic (ES), (2) anionic (AS), (3) axyanion hole (AH), (4) acyl poket (AP) and (5) periphericor anionic subsites

(PAS) (da Silva et al., 2006). Thus, the entire active site is buried near the bottom of a deep and narrow gorge that penetrates half way into the enzyme and widens out close to its base. This gorge is named as the “aromatic gorge” since it is lined by 14 conserved ar omatic amino acids (Khalid et al., 2005). It has been reported that this area contains a catalytic triad (ES), composed by Ser203, Glu334 and His447 (human AChE, PDB code: 1B41) (Guo et al., 2004) responsible for hydrolyzing the ester bond in ACh and oxyanion hole forming residues (Gly118, Gly119, and Ala204) (Harel et al., 1995) (Figure 1).

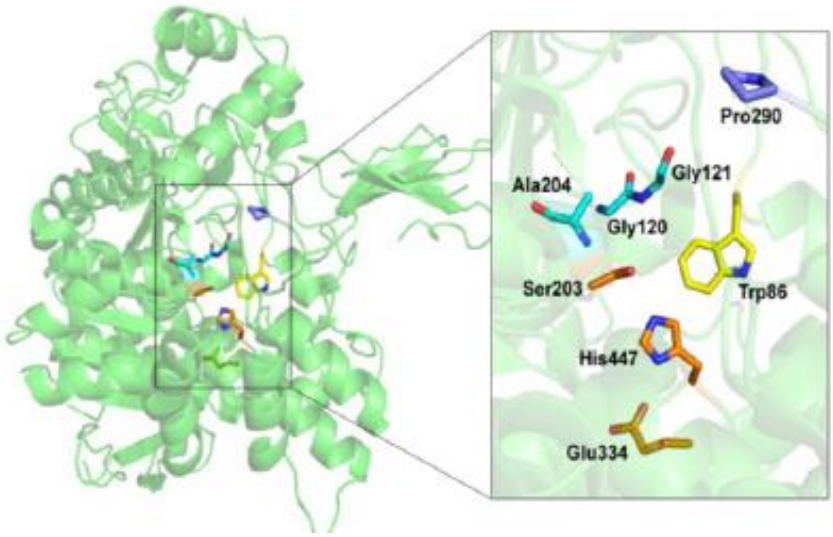

Figure 1. Super-imposed crystal structures of huAChE (PDB: 1B41) (green). Right scheme shows the super-imposed active site.

The anionic subsite (Trp86), also known as quaternary ammonium-binding site, is responsible for binding the quaternary trimethyl ammonium tail group of ACh by cation–electrostatic interaction (Harel et al., 1993). The active site of AChE is characterized by having a highly negative electrostatic potential, which is the binding site for the quaternary nitrogen of their substrates and some ligands (Dvir et al., 2010). It has been proposed that

to be recognized by the active site of AChE, the ligands should have nitrogen atoms, which could carry a positive partial charge generated by resonance effects through an aromatic system (Correa-Basurto et al., 2007). For instance, computational studies on the alkaloid euchrestifoline (Ecf) exhibited considerable inhibitory activity with half maximal inhibitory concentration (IC50: 93.1 μM) against AChE, according to Rehman et al. (2013). Their results showed favorable molecular interactions between aromatic rings of Ecf with His440, Phe330, Gly441 and Ile444, and interactions of heterocyclic oxygen atoms of Ecf with the residue Ser122 of AChE through hydrogen bonds at a distance of 3.49Å. Interestingly, the carbonyl oxygen of Ecf was found to be involved in holding the molecular contact with Tyr121 of PAS in AChE via dipole-dipole electrostatic interactions. Involvement of PAS subsite of AChE in macromolecular complex of inhibitor and enzyme seems to be an additional factor behind considerable inhibitory activity of the compound. Likewise, PAS subsite seems to be involved in favorable molecular contacts between Ecf and AChE. Moreover, for Trp84 residue, it has been reported a parallel conformation to the aromatic frame of Ecf, which ultimately leads to favorable π-π stacking effects with the inhibitor showing favorable hydrophobic interactions. The aromatic rings of Ech were found to be favorably surrounded by His440, Phe330, Gly441 and Ile444, providing support to the macromolecular complex. Overall, compounds with instantaneous and good binding interactions with PAS subsite provided a major clue behind their significant AChE inhibitory activity.

2.5 Molecular docking for alkaloids and its application for natural drug discovery as AChEi.

Currently, it has been proposed that the prediction of protein-ligand interaction could be useful to screen thousands of candidate molecules. Thus, *in silico* approaches could be able to generate suitable conformations of a ligand within a protein-binding site and demand energetic evaluations for the quality of the interaction (Danuello et al., 2012). Taking into account the disadvantages from experimental procedures, *in silico* methods have arisen as a cheaper, faster and safety way to identify potential bioactive drugs. Thus, molecular docking simulations can be used. This method can give information about how to optimize the structure of the target and account for flexibility; how to proceed for the refinement of docked complexes to calculate binding free energies, to provide an accurate ranking of the potential ligands; and in the latest developments, to find the binding site (Alonso et al., 2006).

The development of new technologies has produced not only a general improvement in health from the discovery and manufacture of new and more effective drugs, but also has contributed to the advance of science itself, impelling the development of complex and more accurate tools and techniques for the discovery of new active compounds along with the understanding of their targets (Vyas et al., 2008). When the structure of the target protein is known, drug discovery process follows a well-established procedure. Usually, docking techniques are designed to find the correct conformation of a ligand into its receptor (Khalid et al., 2005). The process of binding a small molecule to its protein target

is not simple; several entropic and enthalpic factors influence the interactions them (Correa-Basurto et al., 2007; Gupta and Mohan, 2014). The mobility of both ligand and receptor, the effect of the protein environment on the charge distribution over the ligand and their interactions with the surrounding water molecules, complicates the quantitative description of the process (Silva et al., 2014). For example, Zaheer-ul-Haq et al. (2010), highlighted that the presence of water molecules played an important role in the accuracy of ligand-protein docking predictions. Water molecules can be involved in protein ligand recognition either by forming mediating hydrogen bonds between the protein and the ligand or by being displaced by the ligand; both mechanisms have been shown to be important for drug discovery. For example, an inhibitory activity of the compound tacrine against the AChE revealed that its binding mode was found to sandwich between the rings of Phe330 and Try84 and its aromatic phenyl and pyridine rings showing parallel π-π bond interaction with the phenyl ring of Phe330 forming average distances of 3.4 and 3.6 Å (Badran et al., 2010), respectively. Likewise, Kapkova et al. (2003) performed docking experiment to explore the binding affinity of several synthetic bispyridinium type-ligands with the homology model of AChE enzyme. They observed many interactions like π-π stacking and cation-π contacts with amino acid residues of the anionic substrate binding site (Trp84, Phe331, and Tyr334) and the peripheral anionic binding site (Trp279). Likewise, docking analysis with two alkaloids, (+)- buxabenzamidienine and (+)-buxamidine isolated from *Buxus sempervirens,* also showed good interactions with the active site of human AChE including several hydrophobic interactions (Orhan et al., 2011). The idea behind this technique is to generate a comprehensive set of conformations of the protein-ligand

complex, and then, to rank them according to their stability and energy. In this sense, the most popular docking software includes FRED 2.1 (Rehman et al., 2013); AutoDock4.2 (Zaheer-Ul-Haq et al., 2003), CDOCK (Kuntz et al., 1982) and FlexX (Gupta and Mohan 2011). Considering that the 3D structure of both ligand and protein are necessary for the application of docking techniques, the 3D structure of several AChE has been solved experimentally by both X-ray diffraction and Nuclear Magnetic Resonance (NMR) of proteins. This last allowing to build homology models of AChE from very specific species. The above provides the essential part for drug discovery and design (Rudnitskaya et al., 2010). Recently, a number of potential candidates have been tested for the development of drugs against AD, which have been also studied using molecular docking and reported targeting AChE (Khalid et al., 2005; Alonso et al., 2006; Danuello et al., 2012). For example, binding and potential inhibitory activity of several alkaloids have been analysed by Naaz et al. (2013). Their results showed pleiocarpine as the best ligand in term of its low binding energy (-12.50 kcal/mol) and *in silico* Ki associated (6.90 × 10-4 μM). Binding energies, such as -11.12 (kcal/mol), -10.84(kcal/mol), -10.77 (kcal/mol), -10.61(kcal/mol), -10.48(kcal/mol), -10.35(kcal/mol), -10.35 (kcal/mol), -8.56 (kcal/mol), -9.36 (kcal/mol), -7.10(kcal/mol), -8.37(kcal/mol) and -6.57(kcal/mol) were obtained for the rest of alkaloids.

Although many comparative molecular docking studies have been reported for AChE (Vigers and Rizzi, 2004; Xie et al., 2006), the reported findings were devoid of the comments regarding conserved water molecules in the active site of AChE, which can mediate the interaction between amino acid side chains and inhibitors (Zaheer-ul-Haq et al.,

2010). Success and failures of molecular docking are probably based on the conformations of Phe330, which was reported to be involved in the recognition of ligands (Dan et al., 2007).

For AChE, there are three orientations of the Phe330 side chain, i.e., open, half open and close gate. The TcAChE-E2020 complex (1EVE) with the open-gate conformation, TcAChE-THA (1ACJ) with closed conformation and TcAChE-TMTFA (1AMN) with the half-open conformation was used separately to determine the importance of side-chain flexibility of Phe330 for ligand traffic (Zaheer-ul-Haq et al., 2010). These methods can be combined to identify a number of new hit compounds with potent inhibitory activity and to understand the main interactions at the binding sites. It is believed that the current use of molecular docking and consensus scoring functions could readily minimize false positive and false negative errors encountered by ligand-based (pharmacophore) virtual screening (Lu et al., 2011). In this sense, Akula et al. (2006) described that the steric contour of compound situated near the peripheral binding site (nitrogen) suggests an addition of bulky groups in this region will cause a reduction in activity. Studies carried out for Khalid et al. (2005) described the importance of amino groups on the inhibitory activities of triterpenoidal alkaloid compounds. Instead, theoretical studies of Correa-Basurto et al. (2007) showed that several functional groups modify the electronic density on the aromatic ring and the nitrogen atom, which might change the affinity between the ligands and the enzymes. In conclusion, although all compounds are able to bind the active side of the gorge, not all of them are able to interact with all the important residues (Lu et al., 2011).

Ligand size, electron density, hydrogen bonding and orientations of the Phe330 may be the reason for some of the inhibitory activities. Thus, it seems that the complement between drug discovery and molecular docking can provide new putative drugs, which is corroborated by 26 studies performed on AChE enzymes using the *in silico* approach. Less research has been carried out on alkaloid discovery, where 9 studies can be highlighted. More detailed information has been summarized in Table 1. Virtual screening has become an important tool for for chemical optimization in drug discovery programs (Dasgupta et al., 2009; Klebe, 2006). When a target of high resolution is available, the most common methodology of virtual screening involves the use of docking algorithms in which conformational sampling methods are used to insert the ligand into the active site of the target macromolecule (Zaheer-ul-Haq et al., 2010). There are two main types of virtual screening: (1) ligand-based and (2) receptor-based. Ligand-based methods are based on finding new ligands similar to existing high affinity ones. On the other hand, receptor-based methods are trying to find molecules that are capable to bind in a receptor binding site (Marsh, 2011). These methods have shown the potential to find completely novel ligands (Katritch et al., 2010; Lu et al., 2011). However, their success has been dependent on the ability to accurately classify virtual ligands (molecules based on AChE inhibition collected from the ChemBank data base (Seniya et al., 2014) and/or National Cancer Institute (NCI) chemical database (Lu et al., 2011), considering whether they have the potential to bind tightly to a binding site or not. It is worth mentioning, that the complexity of the docking problem increases with the size of the ligand and its number of rotatable bonds (Klebe, 2006). The rotations around bonds lead to deviations from ideal geometry that results in a

small energy penalty when compared to deviations from ideality in bond lengths and bond angles (Zaheer-ul-Haq et al., 2010). Likewise, the presence of water molecules plays an important role in the accuracy of ligand-protein docking predictions as it was mentioned before. Both of these mechanisms have been shown to be important for drug discovery (Ladbury, 1996; Graaf et al., 2006). For example, the analysis of several thousand crystal structures of ligand–protein complexes using the waterbase module in Relibase (Hendlich et al., 2003) revealed that, in about two-thirds of all cases, a water molecule is involved in ligand binding, frequently mediating contacts between protein and ligand.

Preliminary studies using virtual screening, resulted in discouraging findings, where no consistency was shown between *in silico* and experimental assays. Thus, Kellenberger et al. (2004) described that failures are often due to insufficient conformational sampling for highly flexible ligands, which means that the ligand could not take all the available conformations and subsequently, the most favourable energies. Nowadays, virtual screening has shown its potential as strong screening method. For example, Carlsson et al. (2011) reported the use of virtual screening of 1.5 million molecules using the D3 receptor as target. Despite authors used both crystal and homology model structures, they could reduce the number of candidates to only 26 molecules. Subsequently, a compound (eticloprida derived) was found to bind strongly to the receptor, which was further optimized to bind even stronger.

As it was listed for molecular docking, virtual screening in AChEi discovery (Dan et al., 2007; Chen et al., 2012) currently uses AutoDock3.0.5 (Goodsell et al., 1996),

FlexX1.10 (Böhm, 1998), MOE2006.08 (Ding et al., 1995), GOLD3.2 (Jones et al., 1997) and FRED2.2.3 (McGann et al., 2003) as docking software.

2.6 Future Perspectives

The development of new drugs is undoubtedly one of the most challenging tasks of today's science. Alkaloids have been shown to be potent source of new drugs. Thus, molecular docking methods have proved to be helpful in understanding the interaction between the AChE with various drug/lead molecules. Considering that, the accurate prediction of protein-ligand interaction suggests how useful can be for the success of the screening approaches employed during structure-based drug descovery. For this approach in silico tools are required, which are able to generate suitable conformations of a ligand within a protein-binding site and demands energetic evaluations for the quality determination of the interaction (Danuello et al., 2012). Taking into account the disadvantages derived from experimental procedures, these in silico methods have arisen as a cheaper, faster and safe way to identify potential bioactive drugs. Thus, molecular docking simulations can be used, which can give information about how to optimize the flexibility of structure target; how to proceed for the refinement of docked complexes, to include solvent effects; to calculate binding free energies, to provide an accurate ranking of the potential ligands; and find the binding site (Alonso et al., 2006).

CHAPTER III

Rhodolirium andicola: a new renewable source of alkaloids with acetylcholinesterase inhibitory activity, a study from nature to molecular docking

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Rhodolirium andicola: A new renewable source of alkaloids with acetylcholinesterase inhibitory activity, a study from nature to molecular docking

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Abstract

Acetylcholinesterase (AChE) is an important target for control of neurodegenerative diseases causing cholinergic signaling deficit. Traditionally, galanthamine has been used as an Amaryllidaceae-derived AChE inhibitor, although new Amaryllidaceae plants could serve as source for better AChE inhibitors. Therefore, the objective of this study was to characterize the alkaloid composition from bulbs of *Rhodolirium andicola*, a native Chilean Amaryllidaceae specie, and assess their inhibitory activity on AChE by *in vitro* and *in silico* methodologies. Alkaloidal extracts from *R. andicola* exhibited an inhibitory activity with IC_{50} values between 11.25 ± 0.04 to 57.78 ± 1.92µg/mL that included isolated alkaloid, galanthamine (2.3 ± 0.18 µg/mL), Additionally, twelve alkaloids were detected using gas chromatography-mass spectrometry (GC-MS) and identified by comparing their mass fragmentation patterns with literature and database NIST vs.2.0. To better understand the bioactivity of isolated compounds and alkaloidal extracts against AChE, a molecular docking approach was performed. Results suggested that alkaloids such as lycoramine, norpluvine diacetate and 6α-deoxy-tazettine expand the list of potential AChE inhibitors to not only galanthamine. The role of *R. andicola* as a source for AChE inhibitors is further discussed in this study.

Keywords: *Rhodolirium andicola*; Alkaloids; Acetylcholinesterase inhibitors; Molecular docking

3.1. Introduction

The enzyme acetylcholinesterase (AChE) is known by its rapid hydrolysis of neurotransmitter acetylcholine (ACh) in the cholinergic synapses (Barnard, 1974; Stryer, 1995). Inhibition of AChE is an important strategy for the treatment of diseases that involve cholinergic transmission deficit such as myasthenia gravis and Alzheimer's disease (AD) (Rahman and Choudhary, 2001; Mehndiratta et al., 2011). AD is the most common form of dementia in our society (World Alzheimer Report, 2015). Worldwide, it is currently estimated that 46 million people have AD or a related dementia, and considering that life expectancy will increase, it is estimated that people with AD will reach to 131.5 million by 2050 (World Alzheimer Report, 2015). These facts make AD one of the most investigated diseases throughout the world (Perry, 1986; Greig et al., 2001). Although AChE inhibition is an established therapeutic strategy to ameliorate cognitive dysfunction and memory loss associated with AD (Rahman and Choudhary, 2001), only a few compounds, such as tacrine, donepezil, physostigmine and galanthamine (Zarotsky et al., 2003) have been approved by the Food and Drug Administration (FDA) in the United States. However, several side-effect such as hepatotoxicity and problems associated with gastrointestinal symptoms, have been reported for the synthetic drugs tacrine and donepezil, respectively (Schulz, 2003; Mehta et al., 2012). In contrast, physostigmine and galanthamine, both from natural origin, have fewer side effects in patients with mild to moderate AD (Mehta et al., 2012). Consequently, many research groups have focused their studies on finding new renewable sources of compounds with acetylcholine esterase inhibitory activity (Mukherjee et al., 2007). In this regard, after the isolation of natural compound galanthamine, a long-acting, selective, reversible and competitive AChE inhibitor, approved in 2001 by FDA (Razadyne®), for clinical treatment of mild and moderate AD, several Amaryllidaceae species have been evaluated as new sources of galanthamine or other alkaloids with

potential AChE inhibitory activity (López et al., 2002; Rhee et al., 2004; Ortiz et al., 2012). Although the chemical synthesis of galanthamine is available (Marco and Carreiras, 2006; Bulger et al., 2008), current pharmaceutical production of this compound is mainly limited to the extraction of natural populations of the Amaryllidaceae *Leucojum aestivum* and *Narcissus* spp. (Heinrich and Teoh, 2004).

In Chile, around 35 species of the Amaryllidaceae family are present covering a wide variety of 11 genera (Ravena, 2003). Particularly, *Rhodolirium andicola*, part of endemic Amaryllidaceae species growing in Chile, represents a potential source of alkaloids with AChE inhibitory activity. In this study, we describe the alkaloidal composition of *R. andicola* for the first time. We isolated three well known alkaloids and evaluated their AChE inhibitory by the Ellman method (Ellman et al., 1961) as a first approach to probe *R. andicola* as a source of alkaloids. Additionally, we tested twelve other alkaloids identified by gas chromatography-mass spectrometry (GC-MS) by molecular docking using a crystal structure of AChE to propose new alkaloids as potential AChE inhibitors that could be used in further assays and likely treatments of neurodegenerative diseases.

3.2. Materials and Methods

3.2.1. Chemicals

Silica gel 60 Merck, (70-230 mesh) was used for Column chromatography (CC) and silica gel 60 F_{254} for Thin Layer Chromatography (TLC) analytical and preparative. MeOH and water (HPLC grade), $CHCl_3$, H_2SO_4, Et_2O, NH_4OH, *n*-hexane, BuOH, NH_3, EtOAc (analytical grade) were purchased from J.T. Baker (México). Acethyltiocholine iodide (ATCI), Acetylcholinesterase (AChE) from *Electrophorus electricus* (type VI-S lyophilized powder), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) and hydrocarbon mixture (C_6-C_{26}) (chemical purity > 99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA)

whereas, Reminyl® (Galanthamine hydrobromide salt) were purchased from JANSSEN-CILAG (Spain).

3.2.2. Plant material

The *Rhodolirium andicola* bulbs were collected during the flowering season in December 2016 from National park Conguillio, Araucanía Region, Chile (S 38°44,426' WO 72°38,887 height: 1389 m.a.s.l.). The plant was identified by Dr. Marcelo Baeza and deposited at the herbarium of Universidad de Concepción, Concepción, Chile (voucher N° CONC 182466).

3.2.3. Alkaloid extraction and fractionation

Dried bulbs (2.5 Kg) were extracted three times with MeOH (1 g of dry sample by 10 mL of solvent) at room temperature for one week (Rhee et al., 2004**)**. The solution was filtered and the solvent was evaporated under reduced pressure on a rotary evaporator (40°C). The residue (150 g) was dissolved in water (250 mL) and acidified to pH 2 with H_2SO_4 (2% v/v). The acid solution was defatted with Et_2O (5 x 100 mL) and $CHCl_3$ (5 x 100 mL). Then, the acid solution was basified with 25% ammonia solution up to pH = 9-10 and the alkaloids were extracted with *n*-hexane (5 x 100 mL)**,** $CHCl_3$ (5 x 100 mL) and BuOH (5 x 80 mL), to obtain the hexanic (0.27 g), chloroformic (1.3 g), and buthanolic alkaloidal extracts (2.5 g) respectively (Ortiz et al., 2012; Sheng-Dian et al., 2013; de Andrade et al., 2016). The hexanic alkaloidal extract was roughly separated by column chromatography on 10 g of silica gel 60 (Merck, 70-230 mesh) using a gradient with *n*-hexane (100%), gradually enriching with $CHCl_3$ (0→100%) and subsequently increasing the polarity with EtOAc, and finally increasing it with MeOH (0→50%) (de Andrade et al., 2014; Ortiz et al., 2016) to give five fractions (I-V). Column fractions were monitored by TLC, and similar ones were combined and evaporated to dryness. Fractions I and II were

combined and subjected to preparative TLC, (silica gel 60 F_{254} with $CHCl_3$/*n*-hexane/MeOH, 5:2:3, in NH_3 atmosphere) to give the compound-A (10 mg). Column chromatographic on Sephadex LH-20 of fractions III to V in MeOH gave three subfractions. The second subfraction, positive to Dragendorff reagent, was subjected to preparative TLC, (silica gel 60 F_{254} with $CHCl_3$/MeOH, 9:1, in NH_3 atmosphere) to give the compound-B (15 mg). Whereas, the separation of compounds from chloroformic alkaloidal extract (1.3 g) was performed by preparative column chromatography on 50 g of silica gel 60 (Merck, 70-230 mesh), as stationary phase (Ortiz et al., 2012). The elution started with chloroform increasing the polarity with methanol, enriched gradually with 10% methanol up to 100% methanol (Elisha et al., 2013) to give one hundred fractions of 10 mL. Fractions with similar profiles based on visualized under ultraviolet light (254 nm), and analysis by Dragendorff reagent were combined and evaporated to dryness. Column chromatographic on Sephadex LH-20 of fractions 60-100 in MeOH gave four subfractions. The third subfraction, positive to Dragendorff reagent, was subjected to preparative TLC, (silica gel 60 F_{254} with $CHCl_3$/MeOH, 9:1, in NH_3 atmosphere) to give the compound-C (20 mg) and compound-D (40 mg) respectively.

3.2.4. GC/MS analysis

The extracts were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (70 eV) using an Agilent, model 7890A chromatograph equipped with a HP-5ms capillary column (30 m by 0.25 mm by 0.25μm; J&W Scientific) with helium carrier gas. The GC oven was programmed to ramp from 100℃ (for three minute) to 280 ℃ at 10 ℃/min and held for 19 min. The injector and transfer line temperatures were 250℃ and 285℃ respectively. The alkaloid compounds were identified by comparing their GC mass spectra with data from the NIST MS Search 2.0 library, Kovats indices (RI) and mass spectra reported in the literature (Mukherjee et al.,

2007; Ortiz et al., 2016). The Kovats retention indexes of the compounds were recorded with standard of an *n*-hydrocarbon mixture (C_9-C_{26}). The proportion of each alkaloid in the basic extracts is expressed as a percentage of ion current (TIC).

3.2.5. Acetylcholinesterase inhibitory activity

Inhibition of AChE by alkaloidal extracts and isolated compounds was determined using the spectrophotometric method according to Ellman et al. (1961) and modified by Ortiz et al. (2012). Fifty microliters of AChE (0.25 U/mL) in phosphate buffer saline (8 mM KH_2PO_4, 2.3 mM Na_2HPO_4 , 0.15 M NaCl, pH 7.5) and 50 µL of the samples dissolved in the same buffer were added to the wells. The plates were incubated for 30 minutes at 25°C before the addition of 100 µL of the substrate solution (0.04 M Na_2HPO_4, 0.2 mM, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 0.24 nM acethyltiocholine iodide (ATCI) in HPLC grade water). The absorbance was read in a microplate reader (Varioskan™ Flash) at 405 nm after 5 min. Inhibition of enzyme was calculated as a percentage compared with an assay using a buffer without any inhibitor. The IC_{50} values were the means ± SD of three determinations. Reminyl® was used as positive control.

3.2.6. AChE refinement and molecular docking

Binding affinities of alkaloids were evaluated through molecular docking against a refined 3D structure of AChE 1C2B.pdb and the human AChE 4PQE.pdb. For 1C2B, 5 ns of molecular dynamics were run to refine the crystal structure of AChE. The AChE structure was refined using molecular dynamics with NAMD v2.9 and CHARMM36 force field. The protein was solvated with water (TIP3P model) in a cubic box with a minimum distance of 15 Å between the protein and the edge of the box. Likewise, the system was neutralized by adding Na^+ or Cl^- randomly placed in the box. All protein preparation was carried out using Visual Molecular Dynamics software (VMD). Configuration files were

prepared in order that the system was simulated under periodic boundary conditions with a cutoff radius of 12 Å for non-bonded interactions and a time step of 2 fs. Extensive energy minimizations (50000 steps) were performed followed by heating through short simulations of 1 ps at 50, 100, 150, 200, 250 and 300 K. Long simulations were kept at 298 K and 1 bar pressure in the NTP (referred to a constant number of particles, temperature and pressure) during 5 ns. Root-mean-square deviation (RMSD) trajectory tool in VMD was used to calculate the RMSD with reference to the starting structure. When the plotted RMSD did not showed any big changes, coordinates were analyzed every 50 frames to obtain the best representative structure (lowest energy, kcal mol^{-1}). When the representative structure of the enzyme was selected, molecular docking was performed using Autodock 4.2 (Morris et al., 1998). Thus, ligands were prepared as PDBQT files including torsional bonds when corresponding using Autodock Tools (ADT). Two hundred runs of Lamarckian genetic algorithm (GA) as the best method to find the lowest energy structures were used (Morris et al., 1998). Likewise, a grid box with dimension 50x50x50 and orientations 27.05 (x-center), 77.14 (y-center) and 20.109 (z-center) with default space of 0.375 Å using Autogrid, was set for 1C2B. Moreover, dimensions of 50x50x50 and orientations -25.3 (x-center), 24.623 (y-center) and -6.754 (z-center) were set for the human AChE (4PQE). Ligands were optimized using SPARTAN Software with Hartree-Fock with basis set 6-31G* and water environment, considered flexible while the protein was rigid. Every docked conformation and clusters were analyzed by ADT and the best binding modes were selected according to the lowest binding energy.

3.3. Results and Discussion

Spectroscopic analysis by GC-MS is a valuable tool for the detection, identification and quantification of alkaloids in Amaryllidaceae plants (Cortes et al., 2015). For this study, the technique was used to detect thirteen alkaloids from extracts of bulbs from *R.*

andicola, six of them identified from their mass spectra and retention index (Table.1). Galanthamine was found in hexanic and chloroformic alkaloidal extracts and ranged from 1.64 to 4.96 % of total ion current (TIC), respectively. Furthermore, galanthamine-type alkaloids such as lycoramine, galanthaminon and 3-O-acetyl-1,2-dihidro-galanthamine were detected in hexanic alkaloidal extract. The isolated and purified compounds were identified as galanthamine (A), haemanthamine (B, C) and tazettine (D) from their mass spectra and retention index.

The alkaloidal extracts and purified compounds were tested to evaluate their AChE inhibitory activity, using Reminyl® as positive control. The results expressed as half-maximal inhibitory concentration (IC_{50}**)** values, are showed in Table 2**.** The hexanic and chloroformic alkaloidal extracts showed a notable AChE inhibitory effect, considering that they are fractions and not pure compounds, with IC_{50} values of 11.25 ± 0.04 and 17.34 ± 1.13 μg/mL respectively, compared with IC_{50} value of 0.17 ± 0.15 μg/mL for Reminyl® (positive control). On the other hand, buthanolic alkaloidal extract showed a low activity against AChE with an IC_{50} value of 57.78 ± 1.92 μg/mL which was three hundred-fold less than that of Reminyl®. The results expressed as IC_{50} values can be compared with values obtained by Cortes et al. (2015), where AChE inhibitory activity of alkaloidal extracts from five Amaryllidaceae plants was evaluated obtaining IC_{50} values between 5.97 ± 0.24 and 70.22 ± 0.24 μg/mL, compared with IC_{50} value of 1.55 μg/mL for galanthamine (reference compound). Cortes and his colleagues suggested that the notable activity showed by alkaloidal extract from *Zephyranthes carinata* (5.97 ± 0.24 μg/mL) could be specifically related to the presence of lycoramine, galanthamine and lycorine. In this regard, Ortiz et al. (2012) evaluated six alkaloidal extracts from different Amaryllidaceae species that grow in Argentina against AChE-activity by spectrophotometric Ellman assay (Ellman et al., 1961), obtaining a high AChE inhibitory activity with IC_{50} values between 1.0 ± 0.01 to 2.0 ± 0.20 μg/mL, compared with the IC_{50} value 0.29 ± 0.07 μg/mL reported for galanthamine. The

authors describing that the highest AChE inhibitory activity showed by alkaloid extract from *Habranthus jamesonii*, could be related to the high content of galanthamine and galanthamine-type alkaloids showed by GC-MS analysis. On the other hand, Elisha et al. (2013) showed both a high and low AChE inhibitory activity for buthanolic and ethyl acetate alkaloidal extracts from bulbs of *Ammocharis coranica* with IC_{50} values of 0.05 ± 0.02 and 43.1 ± 1.22 μg/mL respectively, compared with IC_{50} value of reference compound physostigmine (1.51 μg/mL). In our study, the notable AChE inhibitory activity showed by hexanic and chloroformic alkaloidal extracts could be associated with the content of galanthamine-type alkaloids showed by GC-MS analysis. For the isolated compounds, galanthamine showed an IC_{50} value (2.3 ± 0.18 μg/mL) lower than all alkaloidal extracts and other isolated compounds (Table 2), which corroborates its selective, competitive and reversible affinity for AChE (López et al., 2002). In contrast, haemanthamine showed a weak AChE inhibitory activity with a IC_{50} value of 287.32 ± 1.82 μg/mL, similar to reported in literature (López et al., 2002; Houghton et al., 2006). The other major isolated alkaloid, tazettine, did not show AChE inhibitory activity (López et al., 2002).

In general, it is difficult to compare the results obtained from different studies in relation to AChE inhibitory activities for alkaloids isolated from Amaryllidaceae plants. The possibility of false-positive results in the AChE inhibitory activity values due to chemical inhibition (Rhee et al., 2003) should not be ruled out; however, different authors have showed that members of the galanthamine and lycorine group have notable AChE inhibitory activities (López et al., 2002; Elisha et al., 2013) . Recently, a series of lycorine derivatives were synthesized and evaluated for anti-cholinesterase activity; in fact the lycorine derivate compound, 2-O-tert-butyldimethylsilyl-1-O-(methylthio)methyl lycorine, showed dual cholinesterase inhibitory activities of human AChE and butyrylcholinesterase with IC_{50} values of 11.40 ± 0.66 μM and 4.17 ± 0.29 μM, respectively (Wang et al., 2012). On the other hand, galanthamine-type alkaloid such as sanguinine and N-(14-methylallyl)

norgalanthamine have showed an IC_{50} value lower than galanthamine (0.10 against 1.07 μM and 0.16 vs 1.82 μM respectively) (López et al., 2002 ; Berkov et al., 2008).

Table 2. Enzymatic inhibition activity of alkaloidal extracts and isolated compounds from bulbs of *Rhodolirium andicola* expressed as IC_{50} values.

Sample	**IC_{50} (μg/mL)***
hexanic alkaloidal extract	11.25 ± 0.04
chloroformic alkaloidal extract	17.34 ± 1.13
buthanolic alkaloidal extract	57.78 ± 1.92
tazettine	441.04 ± 1.67
hemanthamine	287.32 ± 1.82
galanthamine	2.3 ± 0.18
**galanthamine (Reminyl ®)	0.17± 0.15

*Expressed as mean ± standard error mean (SEM). **Reference compound.

The present study demonstrates that that *R. andicola* serves as source of alkaloids with AChE inhibitory activity, containing thirteen alkaloid compounds, including galanthamine and other galanthamine–type alkaloids. Our efforts that led to the isolation of three alkaloids (galanthamine, haemanthamine and tazettine) allowed us to corroborate *R. andicola* as a rich source of AChE inhibitors, mainly based on successful inhibition assays with galanthamine. However, in an attempt to explain the bioactivity of hexanic and chloroformic alkaloidal extracts and considering the difficulties to obtain enough plant material for the isolation of the other ten compounds, molecular docking was conducted to

simulate the interactions of the entire profile of alkaloidal compounds in the catalytic site of a crystal structure of *Electrophorus electricus* AChE (PDB: 1C2B), same enzyme as the one used in inhibitory assays. Additionally, we performed molecular docking studies on the human AChE 4PQE.pdb and compared the resulting binding energies between the top scoring alkaloids.

Molecular docking is a useful tool to predict the binding mode of small molecules to target proteins as well as giving an approximation of the binding affinity of small molecules. Table 3 shows the results of binding interactions with the active site of the crystal structure of AChE from *T. californica* (1C2B) with alkaloids identified in extracts from *R. andicola* bulbs as ligands. In order to extrapolate potential human activity, the top four scoring ligands at 1C2B were carried forward for docking calculations at the human AChE with scores shown in table 4. The 1C2B AChE active-site gorge is shown as a schematic in figure 1. The bottom of the gorge is characterized by several subsites: the 'anionic' site (Trp86, Phe338 and Glu202), in which the choline moiety of ACh interacts by π-cation interactions; the 'catalytic' site, which contains the three residues of the catalytic triad (Ser203, Glu334 and His447); the 'oxyanion' hole (Gly121, Gly122, Ala204), and the "acyl pocket" (Phe295, Phe297), which confers substrate specificity (Houghton et al., 2006).

Table 1. Alkaloid composition of *Rhodolirium andicola*

Alkaloid	Area % in alkaloidal extract		$[M^+]$ and characteristic ions (%)	RI*	RI reference	MS reference
	hexanic	chloroformic				
Galanthamine	4.96	1.64	287(90), 286(99); 244(27)	2433	[1]	[2] [3] [4]
Lycoramine	1.05	1.53	289 (62),288 (99), 274 (10)	2449	[1]	[1]
Galanthaminon	0.70	-	284(99), 285 (93), 216(42)	2508	[1]	[1]
6α-deoxy-tazettine	0.45	0.43	315(31), 300(36), 231(71)	2516	[NIST]	[4]
norpluviine diacetate	6.49	-	357 (46), 296(13), 270(14)	2554		[NIST]
3-O-acetyl-1,2-dihidro- galanthamine	40.77	-	330(77), 270(21), 213(14)	2577		[NIST]
Heamanthamine	17.60	3.58	301 (77), 272 (99), 257(53)	2595	[6]	[2] [4]
undulatine diol	19.25	-	349(38), 247(99), 318(78)	2594		NIST
Tazettine	-	90.21	331(19), 247(71), 240 (20)	2593	[7] [NIST]	[2] [4]
Acetylnatalensine	0.51	-	343(76), 250 (28), 225(25)	2592		[NIST]
undulatine	0.27	-	331(99), 258(35), 205(69)	2587		[5]
3-epi-macronine	7.92	-	314(19), 245(65), 201(83)	2583		[2]
crinan-3-one	-	2.23	271(99), 270 (48), 238(16)	2580		[4]

(-) Not found in extract; *Experimental Kovats index; [NIST]; NIST reference; [1] Berkov et al. (2012); [2] de Andrade et al (2016); [3] Ortiz et al. (2016); [4] Berkov et al. (2004); [5] Tram et al. (2014); [6] de Andrade et al. (2014); [7] Gotti et al. (2006).

The molecular docking calculations from 1C2B predict that the binding energy of galanthamine is −8.0 kcal/mol (1.38 μM of predicted K_i), whereas the binding energies for compounds lycoramine, norpluvine diacetate and 6α-deoxy-tazettine are -9.07 (0.23 μM of predicted K_i), -8.94 (0.28 μM of predicted K_i) and -8.12 kcal/mol (1.13 μM of predicted K_i), respectively. In human AChE (4PQE), alkaloids galanthamine, lycoramine, norpluvine diacetate and 6α-deoxy-tazettine had comparable binding affinities as shown in table 4. For the complexes lycoramine, norpluvine diacetate and 6α-deoxy-tazettine with 1C2B, the amino acids Glu202 (anionic site), Ser203 and His447 (catalytic site) have a structural arrangement similar to that observed in the AChE-galatanthamine complex (Bartolucci et al., 2001). The similar binding affinities observed for all ligands and side chain conformations of amino acids are unsurprising considering the 88.52 % global sequence identity and near complete conservation of residues at the level of the active site between both models (Edgar, 2004; Waterhouse et al., 2009) (figure 3). In AChE-lycoramine complex, the inhibitor binds at the base of the active site gorge. The hydroxyl oxygen of lycoramine forms a closed hydrogen bond with Ser203 (2.0Å), similar to that observed in AChE-galanthamine complex (Ortiz et al., 2016), where the hydroxyl oxygen of galanthamine forms a closed hydrogen bond with the charged Glu202 (2.1Å) and methoxy group with Ser203 (3.2Å). There may also be hydrogen bonding interactions between the N-methyl group of the inhibitor lycoramine and amino acid residues Tyr449 and Tyr337. It must be assumed that the binding energy for lycoramine and galanthamine comes from a number of smaller enthalpic contributions, coupled to an unusually

small entropic penalty (Lee et al., 2007; Cortes et al., 2015). This latter point arises from the rigidity of the molecule, which allows the numerous interactions to occur with minimal loss of entropy (Greenblatt et al., 1999). The theoretical representation presented in Figure 2 provides information about how the galanthamine inhibitor is stabilized by the AChE enzyme. These same interactions occurred with lycoramine, norpluvine diacetate and 6α-deoxy-tazettine. The binding poses for these molecules suggest that the hydroxyl functional group could play a key role to stabilizing these alkaloids through hydrogen bonds with Ser203 and Glu202 (Figure 2A and 2B). Likewise, epoxides present in 6α-deoxy tazzetine could form hydrogen bonds with close residues (Figure 2C). For norpluvine diacetate, residues such as Ser125 and Ser203 are candidates to establish hydrogen bonds due to their proximity to alcohol groups. Moreover, hydrophobic interactions seem to predominate in the stabilization of norpluvine diacetate with the participation of Tyr and Phe residues (Figure 2D). The most stable conformation of this complex shows that the Ser203 and His447 residue are close to the inhibitor. Besides, the amino acid residues Tyr86 (involved in π – cation interactions with the protonated head of ACh) and Glu202 are in a close conformation to the inhibitor molecule, similar to the AChE-galanthamine complex (Atanasova et al., 2005). Therefore, the amino acid Glu202 (anionic site) is implicated in AChE inhibitory mechanism.

Table 3. Estimated binding energies of inhibition for alkaloid compounds from *Rhodolirium andicola* in the active site of AChE (PDB: 1C2B)

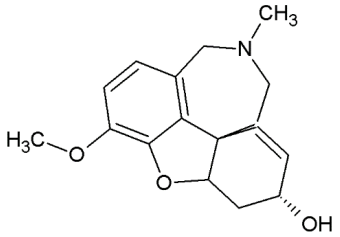
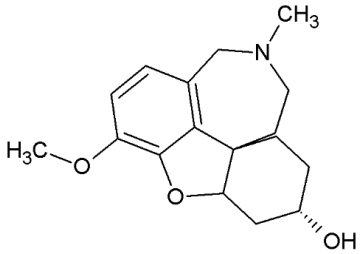
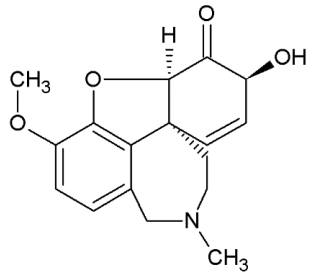
Alkaloid	Type	Chemical structure	Free binding energy (kcal mol^{-1})	Closest residues	Hydrogen bonds
galanthamine	galanthamine		-8.00	Trp86, Gly120, Gly121, Gly122, Tyr124, Glu202, Ser203, Ala204, Phe297, Tyr337, Phe338, Tyr341	Glu202
lycoramine	galanthamine		-9.07	Trp86, Gly121, Gly122, Tyr124, Glu202, Ser203, Phe297, Tyr337, His447, Tyr449	Ser203, Tyr337
galanthaminon	galanthamine		-8.09	Trp86, Gly120, Gly121, Gly122, Gly126, Tyr133, Glu202, Tyr337	Tyr133, Glu202

Table 3. Estimated binding energies of inhibition for alkaloid compounds from *Rhodolirium andicola* in the active site of AChE (PDB: 1C2B) Continuation

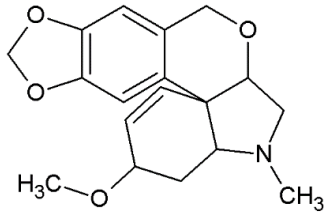
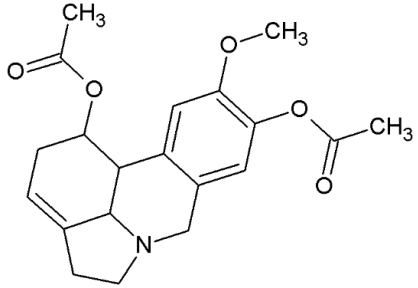
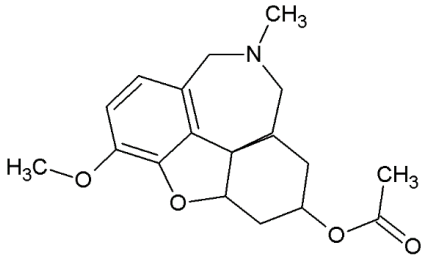
Alkaloid	Type	Chemical structure	Free binding energy (kcal mol^{-1})	Closest residues	Hydrogen bonds
6α-deoxy-tazettine	tazettine		-8.12	Trp86, Gly121, Gly122, Tyr124, Ser125, Glu202, Ser203, Phe297, Tyr337, Phe338, Tyr341, His447	Tyr341
norpluvine diacetate	lycorine		-8.94	Gly121, Gly122, Tyr124, Ser125, Glu202, Ser203, Ala204, Trp236, Phe295, Phe297, Tyr337, Phe338, Tyr341, His447	
3-O-acetyl-1,2-dihidro-galanthamine	galanthamine		-8.09	Trp86, Gly121, Tyr124, Ser125, Glu202, Phe295, Phe297, Phe338, Tyr341, Tyr337	Glu202

Table 3. Estimated binding energies of inhibition for alkaloid compounds from *Rhodolirium andicola* in the active site of AChE (PDB: 1C2B) Continuation

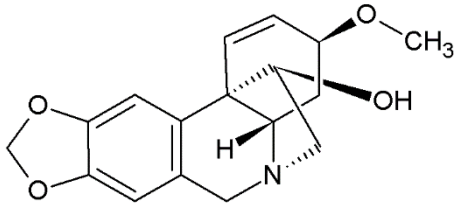
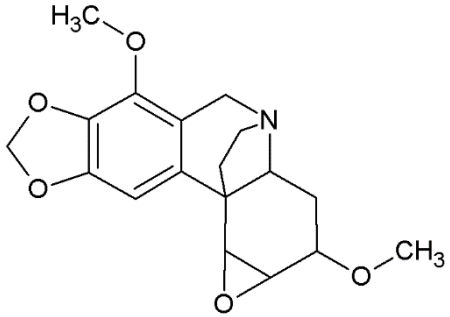
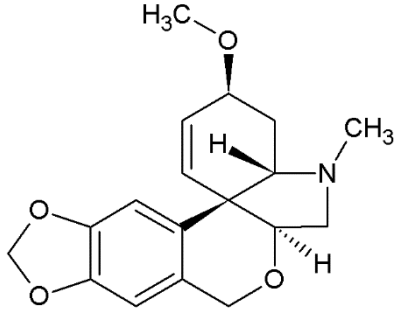
Alkaloid	Type	Chemical structure	Free binding energy (kcal mol^{-1})	Closest residues	Hydrogen bonds
haemanthamine	haemanthamine		-7.22	Gly121, Gly122, Tyr124, Ser125, Glu202, Ser203, Ala204, Trp236, Phe297, Tyr337, Phe338, His447	Gly122, Tyr337
undulatiane diol	crinine		-6.61	Trp86, Gly121, Gly122, Tyr124, Ser125, Glu202, Ser203, Ala204, Tyr337, Phe338, His447	Gly122, Glu202
tazettine	tazettine		-8.08	Trp86, Gly121, Gly122, Ser125, Ser203 Ala204, Phe297, Tyr337, Phe338, Tyr341, His447	

Table 3. Estimated binding energies of inhibition for alkaloid compounds from *Rhodolirium andicola* in the active site of AChE (PDB: 1C2B) Continuation

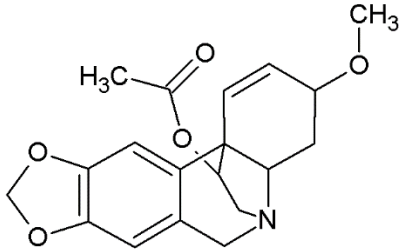
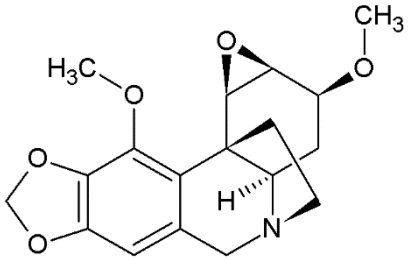
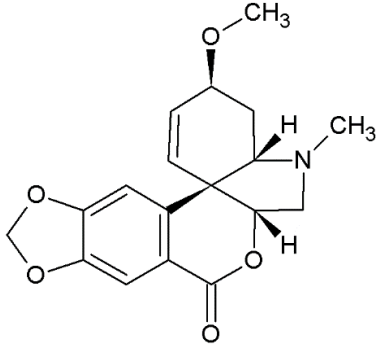
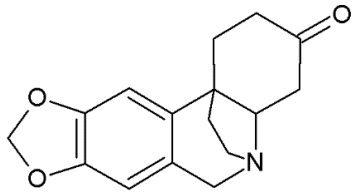
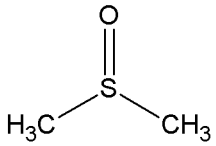
Alkaloid	Type	Chemical structure	Free binding energy (kcal mol^{-1})	Closest residues	Hydrogen bonds
acetylnatalensine	crinine		-8.08	Gly121, Gly122, Tyr124, Ser125, Glu202, Ser203, Trp236, Tyr337, Phe338, Phe297, His447	Gly122
undulatine	crinine		-8.09	Trp86, Gly121, Gly122, Tyr124, Ser125, Ser203, Ala204, Trp236, Phe297, Tyr337, Phe338, His447	Gly122
3-epi-macronine	tazettine		-7.97	Trp86, Gly121, Gly122, Try124, Ser125, Phe297, Tyr337, Phe338, Try341, His447	

Table 3. Estimated binding energies of inhibition for alkaloid compounds from *Rhodolirium andicola* in the active site of AChE (PDB: 1C2B) Continuation

Alkaloid	Type	Chemical structure	Free binding energy (kcal mol^{-1})	Closest residues	Hydrogen bonds
crinan-3-one	crinine		-7.55	Trp86, Gly121, Tyr124, Gly126, Glu202, Typ337, Phe338	
dimethyl sulfoxide*	-		-3.32	Gly120, Gly121, Gly122, Ser203, Ala204, Phe297, His447	Gly122, Ala204

*Ligand considered as negative control for molecular docking.

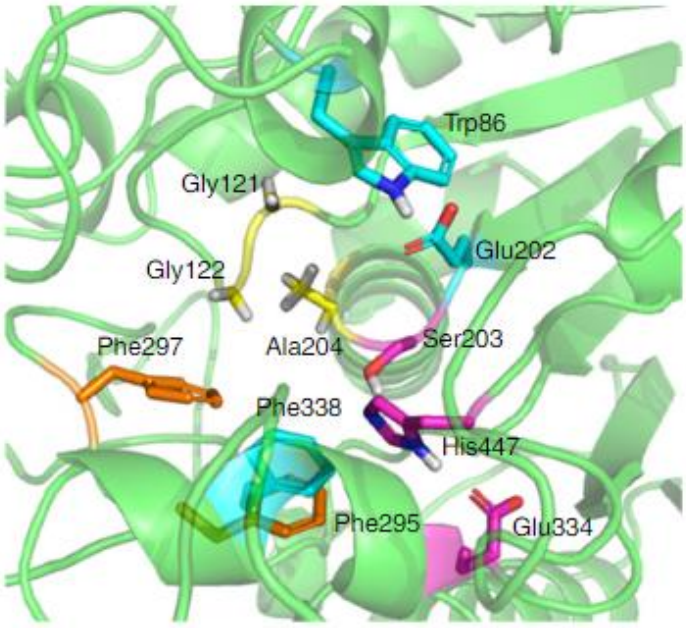


Figure. 1. Schematic view of the active-site gorge of AChE from *T. californica* (1C2B) 'anionic' site (Trp86, Phe338 and Glu202), 'catalytic' (Ser203, Glu334 and His447) 'oxyanion' hole (Gly121, Gly122, Ala204) "acyl pocket" (Phe295, Phe297).

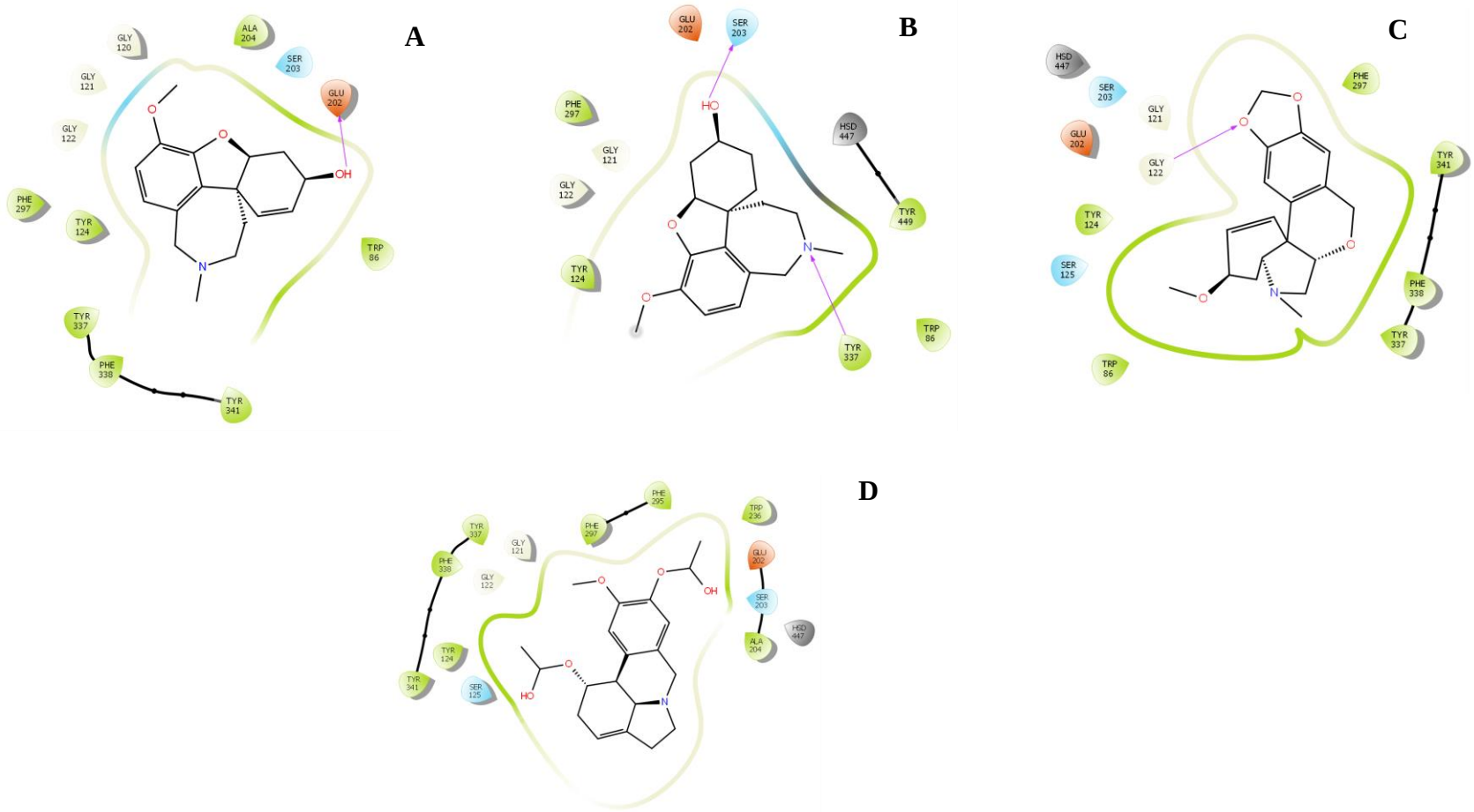


Figure 2. Schematic representation of main interactions of galanthamine (A), lycoramine (B), tazettine, 6a-deoxy (C) and norpluvine diacetate (D) with AChE- catalytic site (Ser203, Glu334 and His447) and 'anionic' site (Trp86, Phe338 and Glu202). The schematic representations of protein-ligand interactions were created with Maestro software. Green color represents hydrophobic interactions, light blue represents polar interactions, blue represents positively charged residues, red represents negatively charged residues, arrows represent the presence of hydrogen bonds and connected residues by lines represent pi-pi interactions.

Figure 3. Sequence alignment of active site regions of 1C2B and 4PQE generated in MUSCLE (Edgar, 2004) and displayed in Jalview (Waterhouse et al., 2009). Active site residues Glutamate, Serine and Histidine are highlighted by arrows in positions 233, 260 and 478 respectively.

Considering the results from molecular docking and the presence of lycoramine, norpluvine diacetate, galanthamine and 6α-deoxy-tazettine in hexanic and chloroformic alkaloidal extracts, we propose that these compounds should be considered for further AChE inhibitory activity assays.

Table 4. Estimated binding energies between four best alkaloids and the human acetylcholinesterase (PDB:4PQE)

Alkaloid	Free binding energy (kcal mol^{-1})	Closest residues	Hydrogen bonds
galanthamine	-8.58	Tyr72, Trp86, Thr83, Asn87, Gly121, Tyr124, Ser125, Tyr337,	Tyr72
lycoramine	-8.83	Gln71, Val73, Asp74, Thr83, Trp86, Asn87, Gly121, Tyr124, Ser125, Tyr337, Tyr341	
6α-deoxy-tazettine	-8.57	Arg18, Gly19, Ile20, Val59, Thr63	
norpluvine diacetate	-8.92	Ser203, Ser125, Phe295	Ser125, Phe295

3.4 Conclusions

The findings of the present study integrated *in vitro* and *in silico* methodologies, which demonstrated the potential of a wild Chilean Amaryllidaceae plant, *R. andicola*, as a new renewable source of galanthamine and other alkaloids with potential use as AChE inhibitors. Thus, molecular docking approaches suggested that lycoramine, 6α-deoxy-tazettine and norpluvine diacetate are interesting AChE-inhibitory alkaloids based on their presence in active hexanic and chloroformic extracts. Although galanthamine is known for its use in treating neurodegenerative diseases, the tazettine-type alkaloids should be evaluated in the search for more selective compounds with potential AChE inhibitory activity, though more experimental evidence is required.

3.5 Acknowledgments

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CHAPTER IV

Galanthamine and other Amaryllidaceae related alkaloids are inhibitors of α7, α4β2 and α3β4 nicotinic acetylcholine receptors

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Galanthamine and other Amaryllidaceae related alkaloids are inhibitors of α7, α4β2 and α3β4 nicotinic acetylcholine receptors

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Abstract

Galanthamine is an Amaryllidaceae-derived acetylcholinesterase inhibitor used to treat memory impairment in Alzheimer's disease and vascular dementia. There is evidence that galanthamine, in addition to its effects on acetylcholinesterase, may enhance or inhibit brain nicotinic acetylcholine receptors, which could increase or decrease the therapeutic efficacy of galanthamine, respectively. Here, we evaluated the effects of galanthamine and two others Amaryllidaceae acetylcholinesterase inhibitors (haemanthamine and tazettine) analysed by gas chromatography–mass spectrometry and identified by comparing their mass fragmentation patterns with literature and database NIST vs.2.0 on the agonist responses of brain nicotinic acetylcholine receptors α7, α3β4, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$. Using nicotinic acetylcholine receptors expressed heterologously in *Xenopus* oocytes, in conjunction with two-electrode voltage clamping, we found that galanthamine inhibits the function of nicotinic acetylcholine receptors assayed through a mix competitive and non-competitevely. Nicotinic acetylcholine receptor α7 was significantly more sensitive to inhibition (17±0.6μM) than the heteromeric receptor, α3β4 (90±3.4μM). Neither haemanthamine nor tazettine were more potent than galanthamine.

Keywords: Isoquinolinic alkaloids, Nicotinic Acetylcholine Receptors, *Xenopus* oocytes

4.1 Introduction

Galanthamine is a competitive and reversible inhibitor of the enzyme acetylcholinesterase (AChE), used for the treatment of neurodegenerative Alzheimer's disease (AD) (Zarotsky et al. 2003). Traditionally, pharmacological treatments for AD, or similar diseases, related to a deficit of the neurotransmitter acetylcholine (ACh) are aimed to acetylcholinesterase inhibitors (Zarotsky et al. 2003). However, the putative roles for nicotinic acetylcholine receptors (nAChR) in AD has led to search of new candidate AD drugs targeting nAChR from natural or synthetic origin (Arneric et al., 2007). In this regard, in addition to its effects on AChE, electrophysiological studies have suggested that the Amaryllidaceae alkaloid, galanthamine, allosterically enhances the function of a number of nAChR providing therapeutic benefits in the areas of cognition, attention and antineurodegenerative activity (Samochocki et al., 2003). However, other studies using slightly different experimental procedures have found that galanthamine inhibits nAChR in a non-competitive manner (Smulders et al., 2005). The aim of this study was to examine the effects of galanthamine and other Amaryllidaceae alkaloids on the function of $\alpha7$, $\alpha3\beta4$ and the alternate forms of the $\alpha4\beta2$ [$(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$] nAChR expressed heterologously in oocytes using electrophysiological.

4.2 Material and Methods

4.2.1 Chemicals

Galanthamine, haemanthamine and tazettine were previously isolated from of *Rhodolirium andicola (Poepp.) Traub,* Amaryllidaceae bulbs (Moraga-Nicolás et al., 2018). The plant was identified by Dr. Marcelo Baeza and deposited at the herbarium of Universidad de Concepción, Concepción, Chile (voucher no. CONC 182466). Other chemicals were purchased from Sigma Chemical (Poole, Dorset, UK). Fresh ACh stock solutions were made daily in a oocyte perfusion solution (OPS) containing 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4.

4.2.2 Gas Chromatography–Mass Spectrometry (GC/MS) analysis of Galanthamine, Haemanthamine and Tazettine

The well-known Amaryllidaceae alkaloids galanthamine, haemanthamine and tazettine were analyzed by coupled GC-MS with electron impact ionization (70 eV) using an Agilent, model 7890A chromatograph equipped with a HP-5ms capillary column (30 m by 0.25 mm by 0.25μm; J&W Scientific) with helium carrier gas. The GC oven was programmed to ramp from 100°C (for three minute) to 280 °C at 10 °C/min and held for 19 min. The injector and transfer line temperatures were 250°C and 285°C respectively. The alkaloid compounds were identified by comparing their gas chromatography mass spectra with data from the NIST mass spectrometry Search 2.0 library, Kovats indices (RI) and mass spectra reported in the literature (Mukherjee et al., 2007; Ortiz et al., 2016). The Kovats retention indexes of the compounds were recorded with standard of an *n*-hydrocarbon mixture (C_9-C_{26}). The proportion of each alkaloid in the basic extracts is expressed as a percentage of ion current (TIC).

4.2.3 Nicotinic acetylcholine receptors expression in *Xenopus laevis* oocytes

The α7, α3β4, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nicotinic acetylcholine receptors (wild type) were expressed heterologously in defolliculated oocytes from *Xenopus laevis*, which were dissected from adult female *X. laevis* frogs (Nasco, USA). The care and use of *X. laevis* frogs in this study was approved by the Oxford Brookes University Animal Research Committee, in accordance with the guidelines of the 1986 Scientific Procedures Act of the United Kingdom. Human α7 cDNA or a mixture of α4 and β2 or α3 and β4 subunit cDNAs were injected into the nuclei of oocytes in a volume of 23 nl/oocyte by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA). For expression of α3β4 receptors the ratio of α3 and β4 cDNAs injected was 1:1, whereas for the expression of $(\alpha4)_2(\beta2)_3$ the ratio was 1 α4 to 10 β2 (Moroni and Bermudez, 2006). For expression of $(\alpha4)_3(\beta2)_2$ the ratio used was 10 α4 to 1 β2 (Moroni and Bermudez, 2006). After injection, oocytes were incubated at 17°C in OPS supplemented with a mixture of penicillin-streptomycin-anphotericin-B (10,000 penicillin, 10 mg streptomycin and 25 µg amphotericin-B/ml) and amikacin (100 µg/ml). Experiments were performed on oocytes 2 to 6 days after injection.

4.2.4 Electrophysiological Recordings

Electrophysiological recording from oocyte post-injection was made at room temperature using a standard two electrode voltage clamp technique with an automatic multichannel system (HiClamp, Multichannel Systems, Germany). Oocytes were impaled by two borosilicate capillary glass (Harvard Instrument: 150 TF GC) microelectrodes filled with 3 M KCl (0.3–2.0 MΩ) and voltage-clamped at -60 mV. During recording, oocyte

were perfused OPS, as described in the manual of HiClamp. The sensitivity of the receptors to inhibition by antagonists was tested by first immersing the oocyte into the antagonist for 5 s and then coupling it with an EC_{50} concentration of ACh (100µM) 10 s. Antagonist concentration-response data were normalized to the appropriate ACh EC_{50}. Between each successive ACh and/or compound application, the cell was perfused with OPS solution for 3 min to allow drug clearance and prevent receptor desensitization. To construct antagonist concentration-effect curves, the responses elicited by co-application of an EC_{50} ACh concentration and increasing concentrations of compound were normalized to the response elicited by an EC_{50} concentration of ACh alone.

4.2.5 Data Analyses

Concentration-response data for antagonists were fitted using a nonlinear regression (Prism 5.0; GraphPad, USA). The data were fitted to the logistic equation Y=Bottom+Top-Bottom)/(1+$10^{((LogEC50-X)*HillSlope)}$), where X is the logarithm of concentration of the antagonist. Y is the response; Y starts at the bottom and goes to Top with sigmoidal shape. Results are presented as mean ± S.E.M. of at least six separate experiments from at least two different batches of oocytes.

4.2.6 Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al. 2015). Data for wild type receptor studied were obtained from oocytes from at least three different donors. Statistical and non-linear regression analyses of the data from concentration response were performed using Prism 5 (GraphPad, San Diego, CA). Unpaired Student's t-tests were used for comparison

between two groups (control and test). Values are presented as arithmetic mean ± SEM. Statistical tests with $p < 0.05$ were considered significant.

4.3 Results and Discussion

Spectroscopic analysis by GC-MS is a valuable tool for the detection, identification and quantification of alkaloids in Amaryllidaceae plants (Cortes et al., 2015). For this study, the technique was used to identify the well-known Amaryllidaceae alkaloids galanthamine, haemanthamine and tazettine comparing their mass spectra with data from the NIST mass spectrometry Search 2.0 library, literature and retention index (Table 1). The effects of galanthamine and two others related Amaryllidaceae alkaloids, tazettine and haemanthamine, on oocytes expressing heterelogously α7, α3β4, $(\alpha4)_2(\beta2)_3$ or $(\alpha4)_3(\beta2)_2$ were assayed using two electrode voltage clamp recording. Galanthamine inhibited all receptors tested in a concentration-dependent manner with an inhibitory potency (IC_{50}) that was receptor-dependent (Table 2). The rank order of sensitivity to inhibition by galanthamine was: $\alpha7 > (\alpha4)_3(\beta2)_2 \cong (\alpha4)_2(\beta2)_3 >> \alpha3\beta4$ (see Table 2).

Haemanthamine and tazettine inhibited all nAChR tested but with low potency, compared to galanthamine (Table 2). Galanthamine has been reported to enhance the agonist responses of α4β2, α3β4, α6β4 and α7 nAChRs (Samochocki et al., 2003). However, we did not find any potentiating effects for galanthamine, even at concentrations reported to potentiate nAChRs. To determine if the inhibitory effects of galanthamine were competitive or non-competitive, we examined the effect of galanthamine on the ACh concentration-response of the receptors α7 and $(\alpha4)_3(\beta2)_2$. As shown Table 3, galanthamine decreased the maximal responses to ACh (Imax, which was accompanied by a decrease in

ACh potency (EC_{50}). This pattern of inhibition is consistent with a mixed competitive and non-competitive mode of inhibition. This is in contrast to studies that have found that galanthamine enhances agonist responses of α7 and $(\alpha4)_3(\beta2)_2$ receptors through an allosteric mechanism (Maelicke et al., 2001; Texidó et al., 2005). Interestingly, these studies reported that galanthamine enhanced the agonist responses of nAChR at concentrations ranging from nM (100 nM; Texidó et al., 2005) to µM (0.1-1 µM; Maelicke et al., 2001; Samochocki et al., 2003), concentrations at which we observed inhibition.

Table 1. GC-MS analysis of isolated alkaloids from *R. andicola* bulbs

Alkaloid	$[M^+]$ and characteristic ions (%)	RI*	RI reference	MS reference
galanthamine	287(90); 286(99); 244(27)	2433	2406 [1]	[2][3][4]
heamanthamine	301 (77), 272 (99), 257(53)	2595	2441 [5]	[2] [4]
tazettine	331(19), 247(71), 240 (20)	2593	2585 [6]	[2] [4]

*Experimental Kovats index; [NIST]; NIST reference; [1] Berkov et al., (2012); [2] de Andrade et al., (2016); [3] Ortiz et al., (2016); [4] Berkov et al., (2004); [5] de Andrade et al., (2014); [6] Gotti et al., (2006).

Table 2. Antagonism of galantahmine and other Amaryllidaceae alkaloids on nicotinic acetylcholine receptors.

	IC_{50} ± SEM (µM)			
Compound	*α7*	*α3β4*	*$(α4)_2(β2)_3$*	*$(α4)_3(β2)_2$*
Galanthamine	$17±0.6^{***}$	$90±3.4^{***}$	$24±3.7^{***,a}$	$22±0.6^{***,a}$
Hemanthamine	$97±1.6^{***}$	$216±32^{***}$	$349±7.9^{***,b}$	$246±18^{***,b}$
Tazettine	276±39	$169±12^{*}$	$305±48^{*}$	$367±49^{*}$

Data represent the mean ± S.E.M. of five experiments. Asterisks indicate levels of significances. Letter a indicate IC_{50} values are not significantly different; *** denotes that the IC_{50} values are all significantly different from each other ($p< 0.0001$); letter b, indicate that IC_{50} values between $(α4)_3(β2)_2$ and $(α4)_2(β2)_3$ are not significantly different; * Effects of tazettine on heteromeric receptors are significantly different from each other but they are not different from α7 ($p< 0.05$).

Table 3. Effects of galanthamine on the concentration response curve evoked by acetylcholine on α7 and $(\alpha4)_3(\beta2)_2$ nicotinic receptors.

	Galanthamine–concentration (μM)							
	0		20		40		100	
Receptors	EC_{50}	Imax	EC_{50}	Imax	EC_{50}	Imax	EC_{50}	Imax
α7	122±6.2*	0.9848***	129±12*	0.8511***	132±6.7*	0.6773***	180±14*	0.6404***
$(\alpha4)_3(\beta2)_2$	94±1.3*	1.107***	123±19*	0.8313***	136±7.1*	0.5963***	164±3.5*	0.3824***

*, $p<0.05$; ***, $p< 0.0001$, relative to the effects of alkaloid galanthamine

The EC_{50} values estimated from the concentration-response curve data are shown as means ± S.E.M.

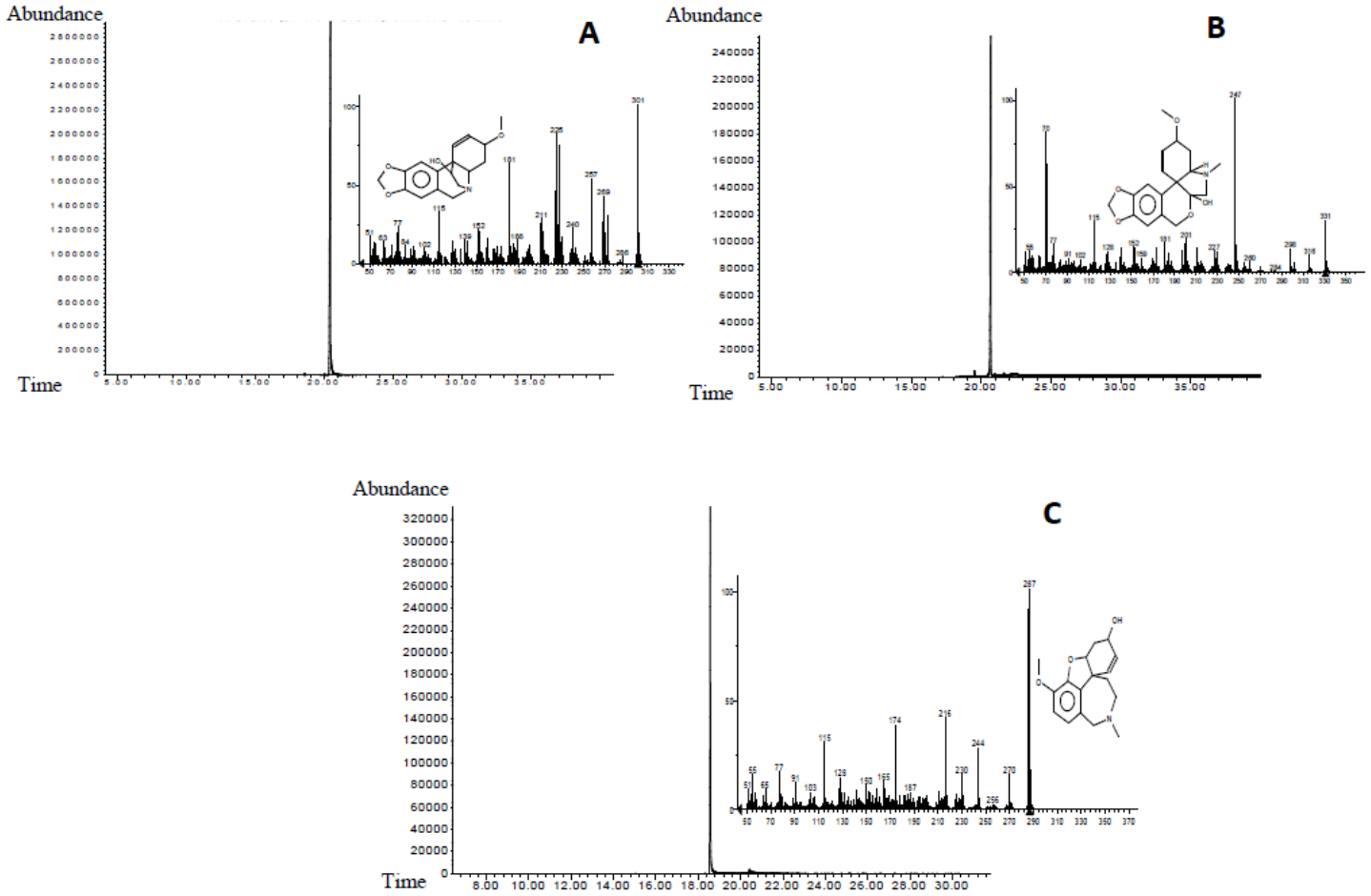

Figure 1. Chromatograms from GC-MS analisys of haemanthamine (A), tazettine (B) and galanthamine (C), shown estimated purity of isolated compounds.

In agreement with our findings, Smulders et al. (2005) reported that galanthamine inhibits α4β2 nAChRs. Furthermore, Kuryatov et al. (2008) found no significant potentiating effects of galanthamine on $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ receptors, although they reported that galanthamine appeared to be a specific allosteric modulator of α5α4β2 nAChR. These discrepancies may well reflect differences in experimental conditions. For

example, Texidó et al. (2005) tested the effects of galanthamine on the responses of α7 receptors to 500 μM ACh, a concentration that is well above the ACh EC_{50} for human α7 nAChRs expressed heterologously in *Xenopus* oocytes (Chavez-Noriega et al., 1997, see also, Table 2) and, critically, observed significant potentiation at only one concentration of galanthamine. Further studies have to be carried out to resolve these discrepancies, however, it is important to note that our studies and those of Smulders et al (2005) and Kuryatov et al. (2008) were carried out using a wide range of concentrations of galanthamine and that our findings showed that the effects of galanthamine were clearly graded over a wide concentration range.

Does the inhibitory effect of galanthamine on nAChRs offset its effects on cholinergic signaling through its effects on AChE? This is unlikely. The IC_{50} for inhibition of AChE by galanthamine is around 0.1-1 μM, whereas inhibition of nAChRs occurs at concentrations higher than 10 μM. Indeed, the plasma concentration of galanthamine needed for therapeutic efficacy is of 0.163 ± 0.073, 0.261 ± 0.105 and 0.368 ± 0.145 μmol/L, for different daily doses of 8 mg, 16 mg or 24 mg, respectively, a concentration ten times lower than the concentration of galanthamine required for maximal inhibition of α4β2 nAChRs (Wattmo et al., 2013). From this, it is clear that the mild cognitive benefits for patients with Alzheimer's disease are exerted by enhancing cholinergic signaling through inhibition of the enzyme AChE. The cognitive effects of galanthamine are similar to those other cholinesterase inhibitors including donepezil, rivastigmine, and tacrine. In addition, safety galanthamine profile is similar to that of other cholinesterase inhibitors with regard to cholinergically mediated gastrointestinal symptoms. Indeed, the use of galanthamine in Alzheimer's disease has been approved in several countries, including Argentina, Australia,

Canada, Czechia, the European Union (except for The Netherlands), Iceland, Korea, Mexico, Norway, Poland, Singapore, South Africa, Switzerland, Thailand, and the United States (Loy and Schneider, 2006).

Allosteric modulation of α5α4β2 nAChRs could still be a therapeutic target for galanthamine, as reported by Kuryatov et al. (2008), making galanthamine a multi-target therapeutic tool.

4.4 Conclusions

In conclusion, the results demonstrate that galanthamine and related Amaryllidaceae alkaloids inhibit neuronal nicotinic acetylcholine receptor function. The α7 receptor was significantly more sensitive to inhibition than the heteromeric receptors. We found that galanthamine behaves as a mixed competitive and non-competitive inhibitor of α7 and $(\alpha4)_3(\beta2)_2$ receptors. Although several authors have suggested that galanthamine allosterically modulate neuronal nicotinic receptors, our findings revealed that neither galanthamine nor tazettine or haemanthamine allosterically enhanced the function of the nicotinic receptors.

4.5 Acknowledgements

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CHAPTER V

General discussion, concluding remarks and future directions

5.1. General discussion

Secondary metabolites contained in plants have been a source of novel drugs that have served as an inspiration for the synthesis of non-natural molecules (Houghton, 2001). Among these, alkaloids constitute an important class of secondary metabolites with a wide range of pharmacological (Orhana et al., 2007; Kosalec et al., 2009; Kaur and Arora, 2015) and biological properties (Macel et al., 2005; Bustamante et al., 2006; Domínguez et al., 2012).

Amaryllidaceae family species from Chile and Argentina have gained importance in recent years due to the presence of isoquinoline alkaloids and related biological activities (Ortiz et al., 2012; Ortiz et al., 2016; Tallini et al., 2018). Particularly, *Rhodolirium andicola*, part of endemic Amaryllidaceae species growing in Chile, represents a potential source of alkaloids with AChE inhibitory activity from which there are no reports on the chemistry and biological activity For this reason, we have considered the study of chemical profile of alkaloids present in *Rhodolirium andicola* and evaluate their biological activity against AChE and modulator effect on nAChRs.

In the present thesis the chemical composition and biological activity of alkaloidal extracts and isolated compounds from *R. andicola* were evaluated for potencial use as AChE inhibitors and enhance the agonist responses of nAChRs. This study was carried out following three approaches: (1) To evaluate the AChE inhibitory activity of pure alkaloids isolated from *R. andicola*, by enzymatic assays; (2) To determine the interactions between bioactive alkaloids and AChE previously identified, by molecular docking and (3) To evaluate the pharmacological response of pure alkaloids on nAChRs.

Thus, the chemical analysis in bulbs of *R. andicola* collected from National Park Conguillio showed different types of isoquinoline alkaloids as expected from a member of Amaryllidaceae family. Spectroscopic analysis by GC-MS is a valuable tool for the detection, identification and quantification of alkaloids in Amaryllidaceae plants (Cortes et al., 2015). For this study, the technique was used to detect thirteen alkaloids from extracts of bulbs from *R. andicola,* six of them identified from their mass spectra and retention index. Thus, galanthamine was found in hexanic and chloroformic alkaloidal extracts and ranged from 1.64 to 4.96% of total ion current (TIC), respectively. Furthermore, galanthamine-type alkaloids, such as lycoramine, galanthaminon and 3-O-acetyl-1,2-dihidro-galanthamine were detected in hexanic alkaloidal extracts. In this sense, similar alkaloidal profile was observed in Chilean representatives of *Rhodophiala* genus in which the biosynthesis of alkaloids is specific to an organ of the plant (Tallini et al., 2018).

In an attempt to evaluate the biological activity of pure compounds and alkaloidal extracts *in vitro* AChE inhibitory and effect assays on nAChRs, were performed. In this way, the alkaloidal extracts and purified compounds were tested, using Reminyl® as positive control by Ellman method (Ellman et al., 1961). The hexanic and chloroformic alkaloidal extracts showed a notable AChE inhibitory effect, considering that they are fractions and not pure compounds, with IC_{50} values of 11.25 ± 0.04 and 17.34 ± 1.13 μg/mL respectively, compared with IC_{50} value of 0.17 ± 0.15 μg/mL for Reminyl® (positive control). On the other hand, buthanolic alkaloidal extract showed a low activity against AChE with an IC_{50} value of 57.78 ± 1.92 μg/mL which was three hundred-fold less than that of Reminyl®. The results expressed as IC_{50} values can be compared with values obtained by Cortes et al. (2015), where AChE inhibitory activity of alkaloidal extracts from five Amaryllidaceae plants was evaluated obtaining IC_{50} values between 5.97 ± 0.24 and

70.22 ± 0.24 μg/mL, compared with IC_{50} value of 1.55 μg/mL for galanthamine (reference compound). Cortes and his colleagues suggested that the notable activity showed by alkaloidal extract from *Zephyranthes carinata* (5.97 ± 0.24 μg/mL) could be specifically related to the presence of lycoramine, galanthamine and lycorine. Likewise, Ortiz et al. (2012) evaluated six alkaloidal extracts from different Amaryllidaceae species that grow in Argentina against AChE-activity by spectrophotometric Ellman assay (Ellman et al., 1961), obtaining a high AChE inhibitory activity with IC_{50} values between 1.0 ± 0.01 to 2.0 ± 0.20 μg/mL, compared with the IC_{50} value 0.29 ± 0.07 μg/mL reported for galanthamine. The authors described that the highest AChE inhibitory activity showed by alkaloid extract from *Habranthus jamesonii*, could be related to the high content of galanthamine and galanthamine-type alkaloids showed by GC-MS analysis. On the other hand, Elisha et al. (2013) showed both a high and low AChE inhibitory activity for buthanolic and ethyl acetate alkaloidal extracts from bulbs of *Ammocharis coranica* with IC_{50} values of 0.05 ± 0.02 and 43.1 ± 1.22 μg/mL, respectively, compared with IC_{50} value of reference compound physostigmine (1.51 μg/mL). In this way, the notable AChE inhibitory activity showed by hexanic and chloroformic alkaloidal extracts could be associated with the content of galanthamine-type alkaloids showed by GC-MS analysis. For the isolated compounds, galanthamine showed an IC_{50} value (2.3 ± 0.18 μg/mL) lower than all alkaloidal extracts and other isolated compounds, which corroborates its selective, competitive and reversible affinity for AChE (López et al., 2002). In contrast, haemanthamine showed a weak AChE inhibitory activity with an IC_{50} value of 287.32 ± 1.82 μg/mL, similar to reported in literature (López et al., 2002; Houghton et al., 2006). The other major isolated alkaloid, tazettine, did not show AChE inhibitory activity. Furthermore, in an attempt to explain the bioactivity of hexanic and chloroformic alkaloidal

extracts and considering the difficulties to obtain enough plant material for the isolation of the other ten compounds, molecular docking was conducted to simulate the interactions of the entire profile of alkaloidal compounds in the catalytic site of a crystal structure of *Electrophorus electricus* AChE (PDB: 1C2B), same enzyme as the one used in inhibitory assays. In addition, we performed molecular docking studies on the human AChE 4PQE.pdb and compared the resulting binding energies between the top scoring alkaloids.

Molecular docking is a useful tool to predict the binding mode of small molecules to target proteins as well as giving an approximation of the binding affinity of small molecules. In order to extrapolate potential human activity, the top four scoring ligands at 1C2B were carried forward for docking calculations at the human AChE with scores. The molecular docking calculations predict that the most favourable binding energies are showed by galanthamine −8.0 kcal/mol (1.38 μM of predicted K_i), lycoramine, norpluvine diacetate and 6α-deoxy-tazettine are -9.07 (0.23 μM of predicted K_i), -8.94 (0.28 μM of predicted K_i) and -8.12 kcal/mol (1.13 μM of predicted K_i), respectively. In human AChE (4PQE), alkaloids galanthamine, lycoramine, norpluvine diacetate and 6α-deoxy-tazettine had comparable binding affinities. For the complexes lycoramine, norpluvine diacetate and 6α-deoxy-tazettine with 1C2B, the amino acids Glu202 (anionic site), Ser203 and His447 (catalytic site) have a structural arrangement similar to that observed in the AChE-galatanthamine complex (Bartolucci et al., 2001). The similar binding affinities observed for all ligands and side chain conformations of amino acids are unsurprising considering the 88.52% of global sequence identity and near complete conservation of residues at the level of the active site between both enzymes (i.e., *E. electricus* and human AChE) (Edgar, 2004; Waterhouse et al., 2009; Moraga-Nicolás et al., 2018). In AChE-lycoramine complex, the inhibitor binds at the base of the active site gorge. Thus, the hydroxyl oxygen of lycoramine

forms a closed (2.0 Å) hydrogen bond with Ser203, similar to that observed in AChE-galanthamine complex (Ortiz et al., 2016), where the hydroxyl oxygen of galanthamine forms a closed hydrogen bond with the charged Glu202 (2.1 Å) and methoxy group with Ser203 (3.2 Å). There may also be hydrogen bonding interactions between the N-methyl group of the inhibitor lycoramine and amino acid residues Tyr449 and Tyr337. Besides the above, we can assume that the binding energy for lycoramine and galanthamine comes from a number of smaller enthalpic contributions, coupled to an unusually small entropic penalty (Lee et al., 2007; Cortes et al., 2015). This latter point arises from the rigidity of the molecule, which allows the numerous interactions to occur with minimal loss of entropy (Greenblatt et al., 1999). The binding poses for these molecules suggest that the hydroxyl group could play a key role to stabilize these alkaloids through hydrogen bonds with Ser203 and Glu202. Likewise, epoxides present in 6α-deoxy tazzetine could form hydrogen bonds with close residues (Moraga-Nicolás et al., 2018). For norpluvine diacetate, residues such as Ser125 and Ser203 are candidates to establish hydrogen bonds due to their proximity to alcohol groups. Moreover, hydrophobic interactions seem to predominate in the stabilization of norpluvine diacetate with the participation of Tyr and Phe residues. The most stable conformation of this complex shows that the Ser203 and His447 residue are close to the inhibitor. Besides, the amino acid residues Tyr86 (involved in π – cation interactions with the protonated head of ACh) and Glu202 are in a close conformation to the inhibitor molecule, similar to the AChE-galanthamine complex (Atanasova et al., 2005). Therefore, the amino acid Glu202 is implicated in AChE inhibitory mechanism.

Finally, the effects of isolated compounds galanthamine and two other related Amaryllidaceae alkaloids, tazettine and haemanthamine, on oocytes expressing heterelogously α7, α3β4, $(\alpha4)_2(\beta2)_3$ or $(\alpha4)_3(\beta2)_2$ were assayed using two electrode voltage

clamp recording. We showed that galanthamine inhibited all tested receptors in a concentration-dependent manner with an inhibitory potency (IC_{50}) that was receptor-dependent ($\alpha7 > (\alpha4)_3(\beta2)_2 \cong (\alpha4)_2(\beta2)_3 >> \alpha3\beta4$). Additionally, haemanthamine and tazettine inhibited all tested nAChRs but with low potency, compared with galanthamine. In this sense, galanthamine has been reported to enhance the agonist responses of α4β2, α3β4, α6β4 and α7 nAChRs (Samochocki et al., 2003). However, we did not find any potentiating effects for galanthamine, even at concentrations reported to potentiate nAChRs This is in contrast to studies that have found that galanthamine enhances agonist responses of α7 and $(\alpha4)_3(\beta2)_2$ receptors through an allosteric mechanism (Maelicke et al., 2001; Texidó et al., 2005). Interestingly, these studies reported that galanthamine enhanced the agonist responses of nAChR at concentrations ranging from nM (100 nM; Texidó et al., 2005) to µM (0.1-1 µM; Maelicke et al., 2001; Samochocki et al., 2003), concentrations at which we observed inhibition. In agreement with our findings, Smulders et al., (2005) reported that galanthamine inhibits α4β2 nAChRs. Furthermore, Kuryatov et al. (2008) found no significant potentiating effects of galanthamine on $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ receptors, although they reported that galanthamine appeared to be a specific allosteric modulator of α5α4β2 nAChR. These discrepancies may well reflect differences in experimental conditions. For example, Texidó et al. (2005) tested the effects of galanthamine on the responses of α7 receptors to 500 µM ACh, a concentration that is well above the ACh EC_{50} for human α7 nAChRs expressed heterologously in *Xenopus* oocytes (Chavez-Noriega et al., 1997) and, critically, observed significant potentiation at only one concentration of galanthamine. Further studies have to be carried out to resolve these discrepancies, however, it is important to note that our studies and those of Smulders et al.

(2005) and Kuryatov et al. (2008) were carried out using a wide range of concentrations of galanthamine and that our findings showed that the effects of galanthamine were clearly graded over a wide concentration range.

5.2. Concluding remarks

Alkaloid composition of *R. andicola* bulbs were determined and the effects of alkaloid extracts and pure compounds against AChE and nAChRs α7; $(\alpha4)_3(\beta2)_2$; $(\alpha4)_2(\beta2)_3$; α3β4 were evaluated. Thus, isoquinoline alkaloids were identified in bulb extracts of *R. andicola* where the type and amount of alkaloids depend on the organ of the plant and the geographical conditions. In this regards, tazettine was the more abundant alkaloid isolated and identified from *R. andicola* bulbs.

In relation with AChE inhibitory activity of alkaloid extracts and isolated compounds by *in vitro* and *in silico* methodologies, we demonstrated the potential of a wild Chilean Amaryllidaceae plant, *R. andicola*, as a new renewable source of well-known AChE inhibitor galanthamine and other alkaloids, with low inhibitory activity against AChE. Although, molecular docking approaches suggested that lycoramine, 6α-deoxy-tazettine and norpluvine diacetate bind to the catalytic site of AChE with good binding energies, these results must to be confirmed with future *in vitro* experiments.

On the other hand, galanthamine inhibit neuronal nAChR function, whereas related Amaryllidaceae alkaloids haemanthamine and tazettine inhibit neuronal nAChR function with low potency. The α7 receptor was significantly more sensitive to inhibition than the heteromeric receptors. We found that galanthamine behaves as a mixed competitive and non-competitive inhibitor of α7 and $(\alpha4)_3(\beta2)_2$ receptors. Although several authors have

suggested that galanthamine allosterically modulate neuronal nicotinic receptors, our findings revealed that neither galanthamine nor tazettine or haemanthamine allosterically enhanced the function of the nicotinic receptors.

The obtained results in this research confirm that galanthamine has inhibitory effect on AChE and acts as competitive and selective antagonist on nAChR a4b2 subtype, though at high concentrations galanthamine blocked the stimulated currents by ACh on nAChR.

On the other hand, the isolated alkaloids tazettine and haemanthamine are not potent AChE inhibitors, and do not has antagonist effects on nAChR. However there is few knowledge about the others alkaloids presents in this plant, which could has better activities against biological systems assayed in this research, which leaves open the possibility of continuing with the study and isolation of other secondary metabolites from this plant.

5.3. Future directions

Alkaloids are widespread in nature showing a wide range of pharmacological activities. Many of the plants that contain alkaloids from the native flora of Chile have been used in traditional medicine, though chemical and biological studies have been scarce.

In this sense, our results reveal that *R. andicola* extracts are a rich source of isoquinoline alkaloids with promising AChE inhibitory activity. Additionally, molecular docking approaches suggested that isoquinolinic alkaloids found in *R. andicola* bulbs, lycoramine, 6α-deoxy-tazettine and norpluvine diacetate are interesting AChE-inhibitory alkaloids based on their presence in active hexanic and chloroformic extracts. Although galanthamine is known for its use in treating neurodegenerative diseases, the tazettine-type alkaloids should be evaluated in the search for more selective compounds with potential AChE inhibitory activity, though more experimental evidence is required. In this regard, in vitro assays with tazettine-type alkaloids are necessary for confirm the activity showed by *in silico* methods. Futhermore, our findings revealed that neither galanthamine nor tazettine or haemanthamine allosterically enhanced the function of the nicotinic receptors. Further studies have to be carried out to resolve these discrepancies.

REFERENCES

Adsersen, A., Kjølbye, A., Dall, O., Jäger, A.K. 2007. "Acetylcholinesterase and Butyrylcholinesterase Inhibitory Compounds from *Corydalis Cava* Schweigg & Kort." J Ethnopharmacol. 113, 179–82.

Akrami H., Mirjalili, B.F., Khoobi, M., Nadri, H., Moradi, A., Sakhteman, A., Emami A., Foroumadi, A., Shafiee, A. 2014. "Indolinone-Based Acetylcholinesterase Inhibitors: Synthesis, Biological Activity and Molecular Modeling." Eur J Med Chem. 84, 375–81.

Akula, N., Lecanu, L., Greeson, J., Papadopoulos, V. 2006. 3D QSAR Studies of AChE Inhibitors Based on Molecular Docking Scores and CoMFA. Bioorg Med Chem Lett. 16, 6277–80.

Albuquerque, E.X., Pereira, E.F., Alkondon, M., Rogers, S.W. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev. 89(1), 73-120.

Alkondon, M., Albuquerque, E.X. 1991. Initial characterization of the nicotinic acetylcholine receptors in rat hippocampal neurons. J Recept Res. 11, 1001–1022.

Alonso, H., Bliznyuk, A.A., Gready, J.E. 2006. Combining Docking and Molecular Dynamic Simulations in Drug Design. Med Res Rev. med. 20067.

Arneric, S.P., Holladay, M., Williams, M., 2007. Neuronal nicotinic receptors: a perspective on two decades of drug discovery research. Biochem.Pharmacol. 74, 1092-1101

Atanasova, M., Yordanov, N., Dimitrov, I., Berkov, S., Doytchinova, I. 2005. Molecular docking study on galantamine derivatives as cholinesterase inhibitors. Mol. Inf. 34, 394 – 403.

Aubert, I., Araujo, D.M., Cecyre, D., Robitaille, Y., Gauthier, S., Quirion, R. 1992. Comparative alterations of nicotinic and muscarinic binding sites in Alzheimer's and Parkinson's diseases. J Neurochem. 58, 529- 541.

Auld, D.S., Kornecook, T.J., Bastianetto, S., Quirion, R. 2002. Alzheimer's Disease and the Basal Forebrain Cholinergic System : Relations to Beta -Amyloid Peptides, Cognition, and Treatment Strategies". Prog Neurobiol. 68, 209-245.

Badran, M.M., Hakeem, M.A., Abuel-Maaty, S.M. 2010. Design, Synthesis, and molecular- modeling study of aminothienopyridine analogues of tacrine for Alzheimer's disease. Arch Pharm. 343, 590-601.

Bahr, B.A, Parsons, S.M. 1986. Acetylcholine transport and drug inhibition kinetics in Torpedo synaptic vesicles. J Neurochem. 46(4), 1214-1218.

Barnard, E.A. 1974. Neuromuscular transmission—enzymatic destruction of acetylcholine, in: J.I. Hubbard (Ed.), The Peripheral Nervous System, Plenum Press, New York. pp. 201–224.

Bartolucci, C., Perola E., Pilger, C., Fels, G., Lamba, D. 2001. Three-dimensional structure of a complex of galanthamine (Nivalin) with acetylcholinesterase from Torpedo californica: implications for the design of new anti-Alzheimer drugs. Proteins. 42, 182–191.

Bastida, J., Berkov, S., Torras-Claveria, L., Pigni, N.B., de Andrade J.P, Martínez, V., Codina, C., Viladomat, F. 2011. Chemical and biological aspects of Amaryllidaceae alkaloids. Transworld Research Network, Kerala, India

Bastida, J., Lavilla, R., Viladomat, F. 2006. Chemical and biological aspects of Narcissus Alkaloids. In: Cordell, G.A. (Ed.), Alkaloids vol. 63. Elsevier Scientific Publishing, Amsterdam, pp. 87-179.

Berkov, S., Evstatieva, L., Popov, S. 2004. Alkaloids in Bulgarian *Pancratinum maritinum* L. Z. Naturforsch. 59c, 65-69.

Berkov, S., Codina, C., Viladomat, F., Bastida, J. 2008. N-Alkylated galanthamine derivatives: Potent acetylcholinesterase inhibitors from *Leucojum aestivum*". Bioorg. Med Chem Lett. 18, 2263–2266.

Berkov, S., Bastida, J., Sidjimova, B., Viladomat. F., Codina, C. 2011. Alkaloid Diversity in *Galanthus* elwesii and *Galanthus* nivalis. Chem. Biodivers. 8, 115–130.

Berkov, S., Viladomat, F., Codina, C., Suárez, S., Ravelo, A., Bastida, J. 2012. GC-MS of amaryllidaceous galanthamine-type alkaloids. J. Mass. Spectrom. 47, 1065-1073.

Bernard, P., Kireev, D.B., Chrétien, J.R., Fortier, P.R., Coppet, L. 1999. "Automated Docking of 82 N-Benzylpiperidine Derivatives to Mouse Acetylcholinesterase and Comparative Molecular Field Analysis with 'Natural' Alignment." J Comput Aided Mol Des. 13, 355–71.

Bianchi, E., Cole, J.R. 1969. Antitumor Agents from Agave Schotti (Amaryllidaceae). J Pharm Sci. 58, 589–591.

Blanton, M.P., Cohen, J.B. 1994. Identifying the lipid-protein interface of the Torpedo nicotinic acetylcholine-receptor - secondary structure implications. Biochem. 33(10), 2859-2872.

Blennow K, de Leon, M J, Zetter- berg, H. 2006. Alzheimer's disease. Lancet 368, 387–403.

Böhm, H.J. 1998. Prediction of binding constants of protein ligands: a fast method for the prioritization of hits obtained from de novo design or 3D database search programs, J Comput-Aided Mol Des. 12, 309–323.

Bon, S., Vigny, M., Massoulié, J. 1979. Asymmetric and globular forms of Acetylcholinesterase in mammals and birds, Proc Natl Acad Sci. 76, 2546–2550

Bourin, M, Ripoll N, Dailly E. 2003. Nicotinic receptors and Alzheimer's disease. Curr Med Res Opin 19(3):169-77.

Bschleipfer, T., Schukowski, K,, Weidnerm W,, Grandom S,A,, Schwantesm U., Kummer, W., Lips, K.S. 2008. The non neuronal cholinergic system of the human urothelium - Is there a contribution to the pathogenesis of detrusor overactivity? Eur Urol Suppl. 7(3), 184-184.

Bulger, P.G., Bagal, S.K., Marquez, R., 2008. Recent advances in biomimetic natural product synthesis. Nat Prod Rep. 25, 254–297.

Bustamante, R.O., Chacón, P., Niemeyer, H.M. 2006. Patterns of chemical defences in plants: an analysis of the vascular flora of Chile. Chemoecology 16(3), 145-151.

Byamukama, R., Jordheim, M., Kiremire, B., Namukobe, J., Andersen, Ø.M. 2006. Anthocyanins from flowers of Hippeastrum cultivars. Sci Hortic. 109, 262–266.

Carlsson, J., Coleman, R.J., Setola,V., Irwin, J.J., Fan, H., Schlessinger, A., Sali, A., Roth, B.L., Shoichet, B.K. 2011. Ligand Discovery from a Dopamine D3 Receptor Homology Model and Crystal Structure. Nat Chem Biol. 7(11), 769–778.

Cassels, B.K., Bermúdez, I., Dajas, F., Abin-Carriquiry, J.A., Wonnacott, S. 2005. From ligand design to therapeutic efficacy: the challenge for nicotinic receptor research. Drug Discov Today. 10(23-24), 1657-1665.

Castilhos, T., Giordani, R., Dutilh, J., Bastida, J., De Carvalho, J., Henriques, A., Zuanazzi, J. 2002. "Chemical and Biological Investigation of the Rhodophiala Bifida (Herb .) Traub (Amaryllidaceae) Alkaloids from." 24ª Reunião Anual da Sociedade Brasileira de Química - SBQ - 28 a 31 de Maio de 2001

Celie, P.H.N., van Rossum-Fikkert, S.E., van Dijk, W.J., Brejc, K., Smit, A.B., Sixma, T.K. 2004. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. Neuron, 41(6), 907-914.

Chase, M.W., Reveal, J.L., Fay, M.F. 2009. A subfamilial classification for the expanded asparagelean families *Amaryllidaceae, Asparagaceae and Xanthorrhoeaceae*. Bot J Linn Soc. 161, 131-136.

Chavez-Noriega, L.E., Crona, J.H., Washburn, M.S., Urrutia, A., Elliott, K.J., Johnson, E.C. 1997. Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 expressed in *Xenopus* oocytes, J Pharmacol Exp Ther. 280(1), 346-356.

Cheesman, L., Nair, J., Van Staden, J. 2012. "Antibacterial Activity of Crinane Alkaloids from Boophone Disticha (Amaryllidaceae)." J Ethnopharmacol 140 (2), 405–408.

Chen, Y., Fang, L., Peng, S., Liao, H., Lehmann J., Zhang, Y. 2012. Discovery of a Novel Acetylcholinesterase Inhibitor by Structure-Based Virtual Screening Techniques. Bioorg Med Chem Lett 22 (9), 3181–87.

Chierrito, T.P.C., Aguiar, A.C.C., de Andrade, I.M., Ceravolo, I.P., Gonçalves, R.A.C., de Oliveira, A.J.B., Krettli, A.U. 2014. Anti-malarial activity of indole alkaloids isolated from Aspidosperma olivaceum. Malar J 13, 142.

Chu, T.K., Ng, T.B. 2004. First report of a glutamine-rich antifungal peptide with immunomodulatory and antiproliferative activities from family Amaryllidaceae, Biochem Biophys Res Commun 325,167–173.

Čolović, M.B., Krstić, D.Z., Lazarević-Pašti, T.D., Bondžić, A.M., Vasić, V.M. 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology, Curr Neuropharmacol.11(3), 315-335.

Corbin, J., Methot, N., Wang, H.H., Baenziger, J.E., Blanton, M.P. 1998. Secondary structure analysis of individual transmembrane segments of the nicotinic acetylcholine receptor by circular dichroism and Fourier transform infrared spectroscopy, J Biol Chem 273(2), 771-777.

Correa-Basurto, J., Flores-Sandoval, C., Marín-Cruz, J., Rojo-Domínguez, A., Espinoza-Fonseca, L.M., Trujillo-Ferrara, J.G. 2007. Docking and Quantum Mechanic Studies on Cholinesterases and Their Inhibitors, Eur J Med Chem 42, 10–19.

Cortes, N., Alvarez, R., Osorio, E.H., Alzate, F., Berkov, S., Osorio, E. 2015. Alkaloids metabolite profiles by GC-MS and Acetylcholinesterase inhibitory activities with bending-mode predictions of five Amaryllidaceae plants, J Pharm Biomed Anal. 102, 222-228.

Court, J.A., Martin-Ruiz, C., Graham, A., Perry, E. 2000. Nicotinic receptors in human brain: topography and pathology, J Chem Neuroanat. 20 (3-4), 281-298.

Coyle, J.T., Price, D.L., DeLong, M.R. 1983. Alzheimer's disease: a disorder of cortical cholinergic innervation, Sci. 4589, 1184-1190.

Cragg, G.M., Newman, D.J. 2005. Plants as a source of anti-cancer agents, J Ethnopharmacol. 100, 72-73.

Cramer, R.D., Patterson, D.E., Bunce, J.D. 1988. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins, J Am Chem Soc. 110(18), 5959–5967.

Curtis, M.J., Bond, R.A., Spina, D., Ahluwalia, A., Alexander, S.P., Giembycz, M.A., Gilchrist, A., Hoyer, D., Insel, P.A., Izzo, A.A., Lawrence, A.J., MacEwan, D.J., Moon, L.D., Wonnacott, S., Weston, A.H., McGrath, J.C. 2015. Experimental design and analysis and their reporting: new guidance for publication in BJP, Br J Pharmacol. 172(14), 3461-71.

da Silva, C.H., Campo, T.P., Carvalho, V.L., Taft, C. 2006. “Molecular Modeling, Docking and ADMET Studies Applied to the Design of a Novel Hybrid for Treatment of Alzheimer’s Disease, J Mol Graph Model. 25, 169–175.

Daly, J.W. 2005. Nicotinic agonists, antagonists, and modulators from natural sources, Cell Mol Neurobiol. 25(3-4), 513-552.

Dan, C., Ya-fei, P., Chuan-jun, L., Yun-feng, X and Yu-ren, J. 2007. Virtual Screening of Acetylcholinesterase Inhibitors, Open access peer-reviewed chapter.

Dani. J.A., Balfour, D.K.F. 2011. Historical and Current Perspective on Tobacco use and Nicotine Addiction, Trends Neurosci. 34(7), 383–392.

Dani, J.A. 2015. Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine, Int Rev Neurobiol. 124, 3–19.

Danuello, A., Romeiro, N.C., Giesel, G.M., Pivatto, M., Viegas, C., Verli, H., Barreiro, E.J., Fraga, C.A.M., Castro, N.G., Bolzani, V.S. 2012. “Molecular Docking and Molecular Dynamic Studies of Semi-Synthetic Piperidine Alkaloids as Acetylcholinesterase Inhibitors.” J Brazil Chem Soc 23, 163–170.

Dasgupta, P., Rizwani, W., Pillai, S., Kovacs, K., Rastogi, M., Banerjee, S., Carless, M., Kim, M., Coppola, E., Haura, D., Chellappan, E. 2009. "Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines, Int J Cancer. 124(1), 36–45.

de Andrade, J.P., Berkov, S., Viladomat, F., Codina, C., Zuanazzi, J.A.S., Bastida, J. 2011. Alkaloids from Hippeastrum Papilio, Mol 16, 7097–7104.

de Andrade J.P., Guo, Y., Font-Bardia, M., Calvet ,T., Dutilh, J., Villadomat, F., Codina, C., Nair, J.J., Zuanazzi, J.A.S., Bastida. J. 2014. Crinine-type alkaloids from *Hippeastrum alicum* and *H. calyptratum*, Phytochem. 103, 188-195.

de Andrade, J.P, Giordani, R.B., Torras-Claviera, L., Pigni, N.B., Berkov, S., Font-Bardia, M., Calvet, T., Konrath, E., Bueno, K., Sachett, L.G., Dutil, J.H., de Sousa Borgues, W., Villadomat, F., Henriques, A.T., Nair, J.J., Zuanazzi, J.A.S., Bastidas, J. 2016. The Brazilian Amaryllidaceae as source of Acetylcholinesterase inhibitory alkaloids, Phytochem Rev. 15, 147-160.

Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.Y., Lazure, C., Chretien, M., Changeux, J.P. 1988. Amino acids of the Torpedo marmorata acetylcholine receptor a subunit labeled by a photoaffinity ligand for the acetylcholine binding site, Biochem. 27, 2346-2358.

Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P.A.J., Hughes, S.H., Arnold, E. 1995. Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors, Nat Struct Biol 2, 407–415.

Ding, Y., Qu, D., Zhang, K.M., Cang, X.X., Kou, Z.N., Xiao, W., Zhu, J.B. 2017. Phytochemical and biological investigations of Amaryllidaceae alkaloids: a review, J Asian Nat Prod Res. 19(1), 53-100.

Domínguez, E., Aguilera, O., Villa-Martínez, R., Aravena, J.C., Henríquez, J.M. 2012. Ethnobotanical suvey of Kalau Island, ancestral Kawesqar territory, Magallanes Región, Chile, Inst Patagon Ann. 40,19-35.

Dua, V.K., Verma, G., Singh, B., Rajan, A., Bagai, U., Agarwal, D.D., Gupta, N.C., Kumar, S., Rastogi, A. 2013. Anti-malarial property of steroidal alkaloid conessine isolated from the bark of Holarrhena antidysenterica, Malaria J. 12, 194.

Dvir, H., Silmanb, I., Harel, M., Rosenberry, T.L., Sussmana, J.L. 2010. Acetylcholinesterase: From 3D structure to function, Chem Biol Interac. 187, 10-22.

Edgar, R.C., 2004. MUSCLE*:* multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32(5), 1792-1797.

Elgorashi, E., Van Staden, J. 2004. Pharmacological Screening of Six Amaryllidaceae Species, J Ethnopharmacol. 90, 27–32.

Elisha, I.L., Elgorashi, E.E., Hussein, A.A., Duncan, G., Eloff, J.N., 2013. Acetylcholinesterase inhibitory effects of the bulb of *Ammocharis coranica* (Amaryllidaceae) and its active constituent lycorine, S Afr J Bot. 85, 44–47.

Ellman, G.L., Courtney, D.K., Valentino, A. Jr., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem Pharmacol. 7, 88 –95.

Evidente, A., Kornienko, A. 2009. Anticancer Evaluation of Structurally Diverse Amaryllidaceae Alkaloids and Their Synthetic Derivatives, Phytochem Rev. 8, 449–459.

Fang, J., Wu, P., Yang, R., Gao, L., Li, C., Wang, D., Wu, S., Liu, A.L., Du, G.H. 2014. "Inhibition of Acetylcholinesterase by Two Genistein Derivatives: Kinetic Analysis, Molecular Docking and Molecular Dynamics Simulation, Acta Pharm Sin B B. 4(6), 430–437.

Farnsworth, N.R., Svoboda G.H., Blomster, R.N. 1968. Antiviral activity of selected Catharanthus alkaloids. J Pharm Sci 57: 2174–2175.

Ferris, S.H., Farlow, M. 2013. Language Impairment in Alzheimer's Disease and Benefits of Acetylcholinesterase Inhibitors, Clin Interv Aging. 8, 1007-1014.

Flores, C.M., Rogers, S.W., Pabreza, L.A., Wolfe B.B., Kellar, K.J. 1992. A subtype of nicotinic cholinergic receptor in rat-brain is composed of alpha-4-subunit and beta-2-subunit and is up-regulated by chronic nicotine treatment, Mol Pharmacol. 41(1), 31-37.

Frederich, M., Tits, M., Angenot, L. 2008. Potential antimalarial activity of indole alkaloids, Trans R Soc Trop Med Hyg. 102, 11-19.

Gabrielle, P., Jeana, M., Lorenza, E.C. 2003. Cytosolic choline acetyltransferase binds specifically to cholinergic plasma membrane of rat brain synaptosomes to generate membrane-bound enzyme, Neurochem Res. 28, 543-549.

Gabrielsen, B.T.P., Huggins, J.W., Kefauver, D.F., Pettit, G.R., Groszek, G., Hollingshead, M., Kirsi, J.J., Shannon, W.M. and Schubert, E.M. 1992. Antiviral (RNA) Activity of Selected Amaryllidaceae Isoquinoline Constituents and Synthesis of Related Substances, J Nat Prod. 55(11), 1569–1581.

Gahring, L.C., Rogers, S.W. 2005. Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells, AAPS Journal. 7(4), 885-894.

Geromichalos, G.D., Lamari, F.N., Papandreou, M.A, Trafalis, D.T., Marigoula Margarity, Papageorgiou, A., Sinakos, Z. 2012. Saffron as a Source of Novel Acetylcholinesterase Inhibitors: Molecular Docking and in Vitro Enzymatic Studies, J Agric Food Chem. 60, 6131–6138.

Giacobini, E. 1990. Cholinergic receptors in human brain - effects of aging and Alzheimer-disease, J Neurosci Res. 27(4), 548-560.

Gilson, M.K., Straatsma, T.P., McCammon J.A., Ripoll, D.R., Faerman, C.H., Axelsen, P.H., Silman, I., Sussman, J.L. 1994. Open "back door" in a molecular dynamics simulation of Acetylcholinesterase, Sci. 263, 1276–1278.

Giordani, R.B., Vieira, P.B., Weizenmann, M., Rosember, D.B., Souza, A.P., Bonorino, C,, de Carli, G.A., Bogo, M.R., Zuanazzi, J.A.S., Tasca, T. 2011. Lycorine induces cell death in the amitochondriate parasite, Trichomonas vaginalis, via an alternative non-apoptotic death pathway, Phytochem 72, 645-650.

Goodsell, D.S., Morris, G.M., Olson, A.J. 1996. Automated docking of flexible ligands: applications of AutoDock, J Mol Recog. 9, 1–5.

Gotti, C., Fornasari, D., Clementi, F. 1997. Human neuronal nicotinic receptors, Prog Neurobiol. 53(2), 199-237.

Gotti. C., Clementi, F. 2004. Neuronal nicotinic receptors: from structure to pathology, Prog Neurobiol. 74(6), 363-396.

Gotti, R., Fiori, J., Bartolini, M., Cavrini, V. 2006. Analysis of Amaryllidaceae alkaloids from Narcissus by GC-MS and capillary electrophoresis, J Pharm Biomed. Anal 42, 17-24.

Graaf, C.D., Oostenbrink, C., Keizers, P.H.J., Wijst, T., Jongejan, A., Vermeulen, N.P.E. 2006. Catalytic site prediction and virtual screening of cytochrome P450 2D6 sub- strates by consideration of water and rescoring in automated docking, J Med Chem. 49, 2417–2430.

Greenblatt, H.M., Kryger, G., Lewis, T., Silman, I., Sussman, J.L. 1999. Structure of acetylcholinesterase complexed with (-)-galanthamine at 2.3 Å resolution, FEBS Letters. 463, 321-326.

Greig, N.H., Utsuki, T., Yu, Q., Zhu1, X., Holloway, H.W., Perry, T., Lee, B., Ingram, D.K., Lahir, D.K. 2001. A new therapeutic target in Alzheimer's disease treatment: attention to butyrylcholinesterase, Curr Med Res Opin. 17, 159-165.

Guo, J., Hurley, M.M., Wright, J.B., Lushington, G.H. 2004. A Docking Score Function for Estimating Ligand-Protein Interactions: Application to Acetylcholinesterase Inhibition, J Med Chem. 47, 5492–5500.

Gupta, S, Mohan, C.G. 2011. 3D-Pharmacophore Model Based Virtual Screening to Identify Dual-Binding Site and Selective Acetylcholinesterase Inhibitors, Med Chem Res. 20, 1422–1430.

Gupta, S., Mohan, G. 2014. Dual Binding Site and Selective Acetylcholinesterase Inhibitors Derived from Integrated Pharmacophore Models and Sequential Virtual Screening, BioMed Res In, 2014.

Gurdon, J.B., Lane, C.D., Woodland, H.R., Marbaix, G. 1971. Use of frog eggs and oocytes for study of messenger rna and its translation in living cells, Nature. 233, (5316) 177-&.

Hai, A., Kizilbash, N.A., Zaidi, S.H., Alruwaili, J. 2013. Porphyrin Derivatives as Inhibitors for Acetylcholinesterase from Drosophila Melanogaster, Bioinformation. 9(12), 645–649.

Halliwell, J.V., Adams, P.R. 1982. Voltage-clamp analysis of muscarinic excitation in hippocampal neurons, Brain Res. 250(1), 71-92.

Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P.H., Silman, I., Sussman, J.L. 1993. Quaternary ligand binding to aromatic residues in the active- site gorge of Acetylcholinesterase, Proc Natl Acad Sci. U.S.A. 90, 9031-9035.

Harel, M., Kleywegt, G.J., Ravelli, R.B., Silman, I., Sussman, J.L. 1995. Crystal structure of an acetylcholinesterase-fasciculin complex: interaction of a threefingered toxin from snake venom with its target, Structure. 3, 1355–1366.

Hasan, A.A., Mohammad, M.A. 2013. “Molecular Docking Studies of Ginkgo Biloba against Acetylcholinesterase Enzyme, Int J Pharm Sci Rev Res. 21(2), 321-324.

He, M., Qu, C., Gao, O., Hu, X., Hong, X. 2015. Biological and Pharmacological Activities of Amaryllidaceae Alkaloids, RSC Adv. 5. RSC Adv: 16562–16574.

Heinrich, M., Teoh, H.L., 2004. Galanthamine from snowdrop—the development of a modern drug against Alzheimer's disease from local caucasian knowledge, J Ethnopharmacol. 92, 147–162.

Hendlich, M., Bergner, A., Gunther, J., Klebe, G. 2003. Relibase: design and development of a database for comprehensive analysis of protein–ligand interactions, J Mol Biol. 326, 607–620.

Heywood, V.H. 1985. The plants with flowers. Barcelona, Spain, Ed. Reverté.

Houghton, P.J. 2001. Old Yet New—Pharmaceuticals from Plants, J Chem Ed. 78(2), 175-184.

Houghton, P.J., Ren, Y., Howes, M.J. 2006. Acetylcholinesterase inhibitors from plants and fungi, Nat Prod Rep. 23, 181–199.

Imramovsky, A., Stepankova, S., Vanco, J., Pauk, K., Monreal-Ferriz, J., Vinsova, J., and Jampilek, J. 2012. Acetylcholinesterase-Inhibiting Activity of Salicylanilide N-Alkylcarbamates and Their Molecular Docking, Mol. 17, 10142–10158.

Ingkaninan, K., Phengpa, P., Yuenyongsawad, S., Khorana, N. 2006. Acetylcholinesterase Inhibitors from Stephania Venosa Tuber, J Pharm Pharmacol 58, 695–700.

Ingrassia, L., Lefranck, L,, Mathieu, V,, Darro, F., Kiss, R. 2008. Amaryllidaceae Isocarbostyril Alkaloids and Their Derivatives as Promising Antitumor Agents, Transl Oncol. 1(1), 1–13.

Jiang, J., Hu, C. 2009. Evodiamine: a novel anti-cancer alkaloid from evodia rutaecarpa, Molecules. 14(5), 1852– 1859.

Jimenez, A., Santos, A., Alonso, G., Vazquez, D. 1976. "Inhibitors of protein synthesis in eukarytic cells. Comparative effects of some amaryllidaceae alkaloids, Biochim Biophys Acta. 425(3), 342-348.

Jin, Z. 2011. "Amaryllidaceae and Sceletium Alkaloids, Nat Prod Rep. 28 (00): 1126–42.

Jin, H. 2014. Acetylcholinesterase and Butyrylcholinesterase Inhibitory Properties of Functionalized Tetrahydroacridines and Related Analogs, Med Chem. 4(10),688–96.

Johnson, J.L., Cusack, B., Davies, M.P., Fauq, A., Rosenberry, T.L. 2003. Unmasking tandemsite interaction in human acetylcholinesterase. Substrate activation with a cationic acetanilide substrate, Biochem. 42, 5438–5452.

Jones, G., Willett P., Glen, R.C., Leach A.R., Taylor, R. 1997. Development and validation of a genetic algorithm for flexible docking, J Mol Biol. 267, 727–748.

Kapkova, P., Stiefl. N., Sürig, U. 2003. Synthesis, biological activity, and docking studies of new acetylcholinesterase inhibitors of the bispyridinium type, Arch Pharm. 336, 523-540.

Karou, D., Savadogo, A., Canini, A., Yameogo, S., Montesano, C., Simpore, J., Colizzi, V., Traore A.S. 2005. Antibacterial activity of alkaloids from Sida acuta, African J Biotechnol. 4(12), 1452-1457.

Katritch, V., Jaakola, V.P., Lane, J.R., Lin, J., Ijzerman, A.P. 2010. Structure- based discovery of novel chemotypes for adenosine A (2A) receptor antagonists, J Med Chem 53, 1799–1809.

Kaur, R., Arora, S. 2015. Alkaloids-important therapeutic secondary metabolites of plant origin, J Crit Rev. 2(3), 1-8.

Kellenberger, E., Rodrigo, J., Muller, P., Rognan, D. 2004. Comparative Evaluation of Eight Docking Tools for Docking and Virtual Screening Accuracy, Proteins: Struct, Funct and Gen, 57, 225–242.

Khalid, A., Azim, M.K., Shehnaz Parveen, Rahman, U., Choudhary, M. 2005. Structural Basis of Acetylcholinesterase Inhibition by Triterpenoidal Alkaloids, Biochem Biophys Res Commun. 333, 1528–1532.

Kihara, T., Sawada, H., Nakamizo, T., Kanki, R., Yamashita, H., Maelicke, A., Shimohama, S. 2004. Galantamine modulates nicotinic receptor and blocks Ab-enhanced glutamate toxicity, Biochem Biophys Res Commun. 325(3), 976-82.

Klebe, G. 2006. Virtual ligand screening: strategies, perspectives and limitations, Drug Discov Today (13-14), 580-94.

Kosalec, I., Gregurek, B., Kremer, D., Zovko, M., Sanković, K., Karlović, K. 2009. Croatian barberry (*Berberis croatica* Horvat): a new source of berberine analysis and antimicrobial activity, World J Microb Biot. 25(1), 145-150.

Kua, J., Zhang, Y., McCammon, J.A. 2002. Studying Enzyme Binding Specificity in Acetylcholinesterase Using a Combined Molecular Dynamics and Multiple Docking Approach, J Am Chem Soc. 124, 10572–10577.

Kuntz, I.D., Blaney, J.M., Oatley, S.J., Langridge, R., Ferrin, T.E. 1982. A geometric approach to macromolecule-ligand interactions, J Mol Biol. 161(2), 269–288.

Kuryatov, A., Onksen, J., Lindstrom, J. 2008. Roles of accessory subunits in alpha4beta2(*) nicotinic receptors, Mol Pharmacol. 74(1), 132-43.

Ladbury, J.E. 1996. Just add water! The effect of water on the specificity of protein- ligand binding sites and its potential application to drug design, Chem Biol. 3, 973–980.

Lee, S.S., Venkatesham, U., Rao, C.P., Lam, S.H., Lin, H.M., 2007. Preparation of secolycorines against Acetylcholinesterase, Bioorg. Med Chem. 15, 1034−1043.

Lilienfeld, S. 2002. Galantamine - a Novel Cholinergic Drug with a Unique Dual Mode of Action for the Treatment of Patients with Alzheimer's Disease, Cns Drug Reviews. 8 (2), 159–76.

Lindstrom, J., Anand, R., Peng, X., Gerzanich, V., Wang, F., Li, Y. 1995. Neuronal nicotinic receptor subtypes, Ann N Y Acad Sci. 757, 100-116.

Lizama-Bizama, I., Pérez, C., Baeza, C.M., Uriarte, E., Becerra, J. 2018. Alkaloids from Chilean species of the genus Rhodophiala C. Presl (Amaryllidaceae) and their chemotaxonomic importance, Gayana Bot. 75(1), 459-465.

Llona, I. 1995. Synaptic like microvesicles: do they participate in regulated exocytosis? Neurochem Int. 27(3):219-26.

López, S., Bastida, J., Viladomat, F., Codina, C., 2002. Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts, Life Sci. 71, 2521 – 2529.

Loy, C., Schneider, L. 2006. Galantamine for Alzheimer's disease and mild cognitive impairment, Cochrane Database Syst Rev. DOI: 10.1002/14651858.CD001747.pub3

Lu, S.H., Wu, J.W., Liu, H.J., Zhao, J.H., Liu, K.T., Chuang, C.K., Lin, H.Y., Tsai, W.B., Ho, Y. 2011. "The Discovery of Potential Acetylcholinesterase Inhibitors: A Combination of Pharmacophore Modeling, Virtual Screening, and Molecular Docking Studies, J Biomed Sci. 18, 8.

Lu, J.J., Bao, J.J., Chen, X.P., Huang, M., Wang, Y.T. 2012. "Alkaloids Isolated from Natural Herbs as the Anticancer Agents." Evidence-Based Complementary and Alternative Medicine determined with fluorescent probes, Biochemi. 13, 2299–2307.

Lubbe, A., Verpoorte, R. 2011. "Cultivation of Medicinal and Aromatic Plants for Specialty Industrial Materials, Ind Crops Prod. 34 (1), 785–801.

Ma, X., Gang, D.R. 2008. In Vitro Production of Huperzine A, a Promising Drug Candidate for Alzheimer's Disease, Phytochem. 69, 2022–2028.

Macel, M., Bruinsma, M., Dijkstra, S.M., Ooijendijk, T., Niemeyer, H.M., Klinkhamer, P.G. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species, J Chem Ecol. 31(7), 1493-1508.

Machocho, A.K., 2000. Alkaloids from Kenyan Amaryllidaceae. Faculty of Pharmacy, vol. Dorctor. University of Barcelona, Barcelona.

Maelicke, A., Samochocki, M., Jostock, R., Fehrenbacher, A., Ludwig, J., Albuquerque, E.X., Zerlin, M. 2001. Allosteric Sensitization of Nicotinic Receptors by Galantamine, a New Treatment Strategy for Alzheimer's Disease, Biol Psychiatry. 49(3), 279-288.

Magazanik, L.G., Fedorov, V.V., Snetkov, V.A. 1984. Factors determining the duration of a single postsynaptic response in neuromuscular junctions, Neirofiziologiia. 16(5), 590-602.

Marco, L., Carreiras, M.D., 2006. Galanthamine, a natural product for the treatment of Alzheimer's disease, Recent Pat CNS Drug. Discov. 1, 105–111.

Marsh, L. 2011. "Prediction of Ligand Binding Using an Approach Designed to Accommodate Diversity in Protein-Ligand Interactions." PLoS ONE 6 (8).

McGleenon, B.M., Dynan, K.B., Passmore A.P. 1999. Acetylcholinesterase inhibitors in Alzheimer's disease, Br J Clin Pharmacol. 48, 471- 480.

McGann, M.R., Almond, H.R., Nicholls, A., Grant, J.A., Brown, F.K. 2003. Gaussian docking functions, Biopolymers. 68, 76–90.

McGleen, B.M., Dynan, K.B., Passmore, A.P. 1999. Acetylcholinesterase inhibitors in Alzheimer's disease, Br J Clin Pharmacol. 48(4):471-80.

Meerrow, A.W., Snijman, D.A. 1998. Amaryllidaceae. In Kubitzki K (Ed.), The family an genera of vascular plants. Berlin, Germany: Springer-Verlag, 83-110.

Mehndiratta, M.M., Pandey S., Kuntzer, T. 2011. Acetylcholinesterase inhibitor treatment for myasthenia gravis, Cochrane Database Syst. Rev. 2, 1-20.

Mehta, M., Adem, A., Sabbagh, M., 2012. New Acetylcholinesterase Inhibitors for Alzheimer's Disease. Int. J. Alzheimers. Dis. 1-8.

Methfessel, C., Witzemann. V., Takahashi, T., Mishina, M., Numa, S., Sakmann, B. 1986. Patch clamp measurements on Xenopus laevis oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels, Pflugers Arch 1 de diciembre. 407 (6), 577-88.

Middleton, R.E., Cohen, J.B. 1991. Mapping of the acetylcholine binding-site of the nicotinic acetylcholine-receptor - [h-3] nicotine as an agonist photoaffinity label, Biochem. 30(28), 6987-6997.

Miksátková, P., Lanková, P., Huml, L., Lapcík, O. 2014. Isoflavonoids in the Amaryllidaceae family, Nat Prod Res. (28)10, 690–697.

Mohan, K., Jeyachandran, R., Deepa. 2012. Alkaloids as anticancer agents. Ann Phytomed. 1, 46-53.

Mooser, G., Sigman, D.S. 1974. Ligand binding properties of acetylcholinesterase determined with fluorescent probes, Biochem. 13 (11), 2299–2307.

Moraga-Nicolás, F., Jara, C., Godoy, R., Iturriaga-Vásquez, P., Venthur, H., Quiroz, A., Becerra, J., Mutis, A., Hormazábal, E. 2018. *Rhodolirium andicola*: A new renewable source of alkaloids with acetylcholinesterase inhibitory activity, a study from nature to molecular docking, Rev Bras Farmacogn. 28, 34-43.

Moroni, M., Bermudez, I. 2006. Stoichiometry and pharmacology of two human alpha4beta2 nicotinic receptor types, J Mol Neurosci. 30(1-2), 95-6.

Morris, G.M., Goodsell, S.D., Halliday, R.S., Huey, R.S., Hart, R., Belew, R.K., Olson, J., 1998. Automated docking using a lamarckian genetic algorithm and an empirical binding free energy function, J Compt Chem. 19, 1639–1662.

Mukherjee, P.K., Kumar, V., Mal, M., Houghton, P.J. 2007. Acetylcholinesterase inhibitors from plants, Phytomed. 14, 289–300.

Naaz, H., Singh, S., Pandey, V.P., Singh, P., Dwivedi, U.P. 2013. "Anti-Cholinergic Alkaloids as Potential Therapeutic Agents for Alzheimer's Disease: An *in silico* Approach. Indian J Biochem Biophys. 50, 120–125.

Nachmansohn D., Wilson IB. 1951. The enzymic hydrolysis and synthesis of acetylcholine. Adv. Enzymol (12) 259–339.

Neher, E., Sakmann, B. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres, Nature 260(5554), 799-802.

Newhouse, P.A., Potter, A., Lenox, R.H. 1993. Effects of nicotinic agents on human cognition: Possible therapeutic applications in Alzheimer's and Parkinson's diseases, Med Chem Res. 2, 628–642.

Oda, Y., Nakanishi, I. 2000. The distribution of cholinergic neurons in the human central nervous system, Histol Histopathol. 15(3), 825-834.

Onguéné, P.A., Ntie-Kang, F., Lifongo, L.L., Ndom, J.C., Sippl, W., Meva'a Mbaze, L. 2013. The potential of anti-malarial compounds derived from African medicinal plants, part I: a pharmacological evaluation of alkaloids and terpenoids, Malaria J. 12, 449.

Orhan, I., Ozçelik, B., Karaoğlu, T., Sener, B. 2007. Antiviral and antimicrobial profiles of selected isoquinoline alkaloids from Fumaria and *Corydalis* species, Z Naturforsch C. 62(1-2), 19-26.

Orhan, I.E., Orhan, G., Gurkas, E. 2011. An overview on natural cholinesterase inhibitors--a multi-targeted drug class--and their mass production, Mini Rev Med Chem. (10),836-842.

Ortiz, J.E., Berkov, S., Pigni, N.B., Theoduloz, C., Roitman, G., Tapia, A., Bastida, J., Feresin, G. 2012. Wild Argentinian Amaryllidaceae, a new renewable source of the acetylcholinesterase inhibitor galanthamine and other alkaloids, Mol. 17, 13473-13482.

Ortiz, J.E., Pigni, N.B., Andujar, S.A., Roitman, G., Suvire, F.D., Enriz, R.D., Tapia, A., Bastida, J., Feresin, G.E. 2016. Alkaloids from *Hippeastrum argentinum* and their Cholinesterase-Inhibitory Activities: An in Vitro and in Silico Study, J Nat Prod. 79 (5), 1241–1248.

Özçelik, B., Kartal, M., Orhan, I. 2011. Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids, Pharma Biol. 49(4), 396–402.

Pacheco, P., Silva, M., Steglich, W., Watson, W.H. 1978. Alkaloids of Chilean Amaryllidaceae hippeastidine and epi-homolycorine, Rev Latinoam Quím. 9, 28-32.

Pacheco, P., Silva M, Kimura, M., Watson, W.H. 1981. Proanthocyanidins of 43 Bibliografía General *Hippeastrum ananuca* I. Rev Latinoam Quím. 12(1), 30-32.

Pacheco, P., Silva M., Sammes, P.G., Watson, W.H. 1982. Estudio químico en Amaryllidaceae chilenas II. Nuevos alcaloides de Hippeastrum ananuca Phil, Boletín de la Sociedad Chilena de Química. 27(2), 289-290.

Pacheco, P., Silva, M. 1992. Amaryllidaceae. En: Muñoz, O (Ed.). Química de la flora de Chile. Departamento Técnico de Investigación, Universidad de Chile. Serie de programas de desarrollo. 1, 351.

Parsons, S.M., Prior, C., Marshall, I.G. 1993. Acetylcholine Transport, Storage, And Release, In Rev Neurobiol. 35, 279-390.

Pereira, E.F., Hilmas, C., Santos, M.D., Alkondon, M., Maelicke, A., Albuquerque, E.X. 2002. Unconventional ligands and modulators of nicotinic receptors, J Neurobiol. 53(4), 479-500.

Pérez, G. 2008. Síntesis y actividad biológica de posibles moduladores del receptor nicotínico de acetilcolina. Tesis entregada a la Universidad de Chile como requisito parcial para optar al título de Doctor en Química.

Perry, E.K. 1986. The cholinergic hypothesis, ten years on, Br Med Bull. 42, 63–69.

Pohanka, M. 2012. "Acetylcholinesterase Inhibitors : A Patent Review (2008 - Present)," Expert Opin Ther Pat. 22:8, 871-886.

Potter, A., Corwin, J., Lang, J., Piasecki, M., Lenox, R., Newhouse, P.A. 1999. Acute effects of the selective cholinergic channel activator (nicotinic agonist) ABT-418 in Alzheimer's disease, Psychopharmacol. 142, 334–342.

Quinn, D.M. 1987. Acetylcholinesterase: Enzyme Structure, Reaction Dynamics, and Virtual Transition States, Chem Rev. 87, 955-979.

Rahman, A.U., Choudhary, M.I., 2001. Bioactive natural products as a potential source of new pharmacophores a theory of memory, Pure Appl Chem. 73, 555–560.

Ravena, P., 2003. Elucidation and systematics of the Chilean genera of Amaryllidaceae, Bot Australis. 2, 1-21.

Rehman, F.U., Khan, M. Khan, I., Roohullah. 2013. "Molecular Interactions of an Alkaloid Euchrestifoline as a New Acetylcholinesterase Inhibitor, Bangladesh J Pharmacol. 8, 361–64.

Reiner, E., Aldridge, N., Simeon, V., Radic, Z, Taylor P. 1991. Mechanism of substrate inhibition of acetylcholinesterase, in: J. Massoulié, F. Bacou, E. Barnard, A. Chatonnet, B.P. Doctor, D.M. Quinn (Eds.), Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology, American Chemical Society, Washington, DC, 227–228.

Rhee, I.K., van Rijn, R.M., Verpoorte, R. 2003. Qualitative determination of false-positive effects in the Acetylcholinesterase assays using thin layer chromatography, Phytochem Anal. 14, 127–131.

Rhee, I.K., Appels, N., Hofte, B., Karabatak, B., Erkelens, C., Stark, L.M., Flippin, L.A., Verpoorte, R. 2004. Isolation of the acetylcholinesterase inhibitor ungeremine from *Nerine bowdenii* by Preparative HPLC Coupled On-Line to a Flow Assay System, Biol Pharm Bull. 27(11), 1804 -1809.

Roberts, B.C., Mancera, R.L. 2008. Ligand-protein docking with water molecules, J Chem Inf Model. 48, 397–408.

Rudnitskaya, A., Török, B., Török, M. 2010. Molecular Docking of Enzyme Inhibitors: a computational tool for structure-based drug design. Biochem Mol Bio Edu. 38 (4), 261–65.

Samochocki, M., Höffle, A., Fehrenbacher, A., Jostock, R., Ludwig, J., Christner, C., Radina, M., Zerlin, M., Ullmer, C., Pereira, E.F., Lübbert, H., Albuquerque, E.X., Maelicke, A. 2003. Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors, J Pharmacol Exp Ther. 305(3),1024-36.

Sargent, P.B. 1993. The diversity of neuronal nicotinic acetylcholine-receptors, Annual Rev Neurosce 16, 403-443.

Schmidt, B.M., Rylett, R.J. 1993. Basal synthesis of acetylcholine in hippocampal synaptosomes is not dependent upon membrane-bound choline acetyltransferase activity, Neurosci. 54(3), 649-56.

Schulz, V. 2003. Ginkgo extract or cholinesterase inhibitors in patients with dementia: what clinical trial and guidelines fail to consider, Phytomed. 10, 74–79.

Séguéla, P., Wadiche, J., Dineley-Miller, K., Dani, J.A., Patrick, J.W. 1993. Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium, J Neurosci. 13(2), 596-604.

Şener, B., Orhan, I., Satayavivad, J. 2003. Antimalarial Activity Screening of Some Alkaloids and the Plant Extracts from Amaryllidaceae, Phytotherapy Res. 17, 1220–1223.

Seniya, C., Khan, G.J., Uchadia, K. 2014. "Identification of Potential Herbal Inhibitor of Acetylcholinesterase Associated Alzheimer's Disorders Using Molecular Docking and Molecular Dynamics Simulation." Biochem Res Int. 2014, Article ID 705451, 7 pages

Sepúlveda, B.A, Pacheco, P, Silva, M.J., Zemelman, R. 1982. Alcaloides de Amarillidaceas chilensis III. Estudio químico y actividad biológica en Hippeastrum bicolor (R. et P.) Baker, Bol. Soc. Chil. Quim. 27(2), 178-180.

Sheng-Dian, H., Yul , Z., Hong-Ping, H., Shi-Fei, L., Gui-Hua, T., Duo-Zhi, Ch., Ming-Ming, C., Ying-Tong, D., Xiao-Jiang, H., 2013. A new Amarylliceae alkalolid from the bulbs of *Lycoris radiate*, Chin J Nat Med. 11(4), 406−410.

Shimohama, S., Akaike, A., Kimura, J. 1996. Nicotine-induced protection against glutamate cytotoxicity - Nicotinic cholinergic receptor-mediated inhibition of nitric oxide formation, Neurobiol AD. 777, 356-361.

Silman, I., Futherman, A.H. 1987. Modes of attachment of acetylcholinesterase to the surface membrane, Eur J Biochem.170(1-2), 11-22

Silman, I., Sussman, J.L. 2005. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology, Curr Opin Pharmacol. 5, 293–302.

Silva, N.N.S., Silva, J., Alves, C.N., Andrade,E.H., Da Silva, J.K.R., Maia, J.G.S. 2014. Acetylcholinesterase Inhibitory Activity and Molecular Docking Study of 1-Nitro-2-Phenylethane, the Main Constituent of Aniba Canelilla Essential Oil, Chem Biol Drug Design. 84, 192–98.

Smulders, C.J., Zwart, R., Bermudez, I., van Kleef, R.G., Groot-Kormelink, P.J., Vijverberg, H.P. 2005. Cholinergic drugs potentiate human nicotinic α4β2 acetylcholine receptors by a competitive mechanism, Eur J Pharmacol.509(2-3), 97-108.

Song, P., Spindel, E.R. 2008. Basic and clinical aspects of non-neuronal acetylcholine: Expression of non-neuronal acetylcholine in lung cancer provides a new target for cancer therapy, J Pharmacol Sci. 106(2), 180-185.

Steitz, T.A., Shulman, R.G. 1982. Crystallographic and NMR studies of the serine proteases, Ann. Rev. Biophys. Bioeng. 11, 419–444.

Stryer, L. 1995. Biochemistry, fourth ed. W.H. Freeman & Co, New York.

Stühmer W. 1998. Electrophysiologic recordings from *Xenopus oocytes*. Methods Enzymol. 293:280-300.

Szegletes, T., Mallender, W.D., Thomas, P.J., Rosenberry, T.L. 1999 Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis. Substrate inhibition arises as a secondary effect, Biochem. 38, 122–133.

Tallini, L.R., Bastida, J., Cortes, N., Osorio, E.H., Theoduloz, C., Schmeda-Hirschmann, G. 2018. Cholinesterase Inhibition Activity, Alkaloid Profiling and Molecular Docking of Chilean *Rhodophiala* (Amaryllidaceae), Mol. 23,1532-1559.

Taly, A., Corringer, P.J., Guedin, D., Lestage, P., Changeux, J.P. 2009. Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system, Nat Rev Drug Discov. 8, 733–75

Taylor, P., Lappi, S. 1975. Interaction of fluorescence probes with acetylcholinesterase. The site and specificity of propidium binding, Biochem. 14, 1989–1997.

Texidó, L., Ros, E., Martín-Satué, M., López, S., Aleu, J., Marsa, J., Solsona, C. 2005. Effect of galantamine on the human α7 neuronal nicotinic acetylcholine receptor, the Torpedo nicotinic acetylcholine receptor and spontaneous cholinergic synaptic activity, Br J Pharmacol.145(5), 672-678.

Tram, N., Mitova, M., Bankova, V., Handjieva, N., Popov, S.S., 2014. GC-MS of *Crinum Latifolium* L. Alkaloids, Z Naturforsch C. 57, 239-242.

Unwin, N. 2005. Refined structure of the nicotinic acetylcholine receptor at 4 angstrom resolution, J Mol Bio 346, (4), 967-989.

Van Goietsenoven, G., Andolfi, A., Lallemand, B., Cimmino, A., Lamoral-Theys, D., Gras,T., Abou-Donia, A. 2010. Amaryllidaceae Alkaloids Belonging to Different Structural Subgroups Display Activity against Apoptosis-Resistant Cancer Cells, J Nat Prod. 73, 1223–1227.

Varadaraju, K.R., Kumar, J.R., Mallesha, L., Muruli, A., Mohana, K.N.S., Mukunda, C.K., Sharanaiah, U. 2013. "Virtual Screening and Biological Evaluation of Piperazine Derivatives as Human Acetylcholinesterase Inhibitors. Int J Alzheimers Dis. Volume 2013, Article ID 653962, 13 pages.

Vieira, P.B., Giordani, R.B., De Carli, G.A., Zuanazzi, J.A.S., Tasca, T. 2011. Screening and bioguided fractionation of Amaryllidaceae species with anti-Trychomonas vaginalis activity, Planta Med. 77, 1054-1059.

Vigers, G.P.A., Rizzi, J.P. 2004. Multiple active site corrections for docking and virtual screening, J Med Chem. 47, 80–89.

Viswanadhan, V.N., Ghose, A.K., Revankar, G.R., Robins, R.K. 1989. Atomic physicochemical parameters for three dimensional structure directed quantitative structure-activity relationships. 4. Additional parameters for hydrophobic and dispersive interactions and their application for an automated superposition of certain naturally occurring nucleoside antibiotics, J Chem Inf Comput Sci. 29(3), 163–172.

Vyas, V., Jain, A., Jain, A., Gupta, A. 2008. “Virtual Screening: A Fast Tool for Drug Design, Sci. Pharm. 76(3), 333-360.

Wang, Y.H., Wan, Q.L., Gu, Ch. D., Luo, H.R., Long, Ch.L., 2012. Synthesis and biological evaluation of lycorine derivatives as dual inhibitors of human acetylcholinesterase and butyrylcholinesterase, Chem Cent J. 6-96.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., Barton, G J., 2009. Jalview Version 2-a multiple sequence alignment editor and analysis workbench, Bioinformatics. 25, 1189-1191.

Wattmo, C., Jedenius, E., Blennow, K., Wallin, A.K. 2013. Dose and plasma concentration of galantamine in Alzheimer’s disease - clinical application. Alzheimers Res Ther. 5(1), 2. doi: 10.1186/alzrt156. eCollection 2013

Wilson, I.B., Quan, C. 1958. Acetylcholinesterase: Studies on molecular complementariness, Arch. Biochem. Biophys. 73, 131–143.

Wonnacott, S. 1997. Presynaptic nicotinic ACh receptors, Trends Neurosci. 20(2), 92-98.

Woolf, N.J., Hernit, M.C., Butcher, L.L. 1986. Cholinergic and non-cholinergic projections from the rat basal forebrain revealed by combined choline acetyltransferase and *Phaseolus vulgaris* leucoagglutinin immunohistochemistry, Neurosci Lett. 66(3), 281-6.

Woolf , N.J. 1991. Cholinergic systems in mammalian brain and spinal cord, Prog Neurobiol. 37(6), 475-524.

World Alzheimer Report, 2015. The Global Impact of Dementia. http://www.worldalzreport2015.org/ (accessed 11.08.17).

Xie, Q., Tang, Y., Li, W., Wang, X.H., Qiu, Z.B. 2006. Investigation of the binding mode of (−)-meptazinol and bis-meptazinol derivatives on acetylcholinesterase using amolecular docking method, J Mol Model. 12, 390–397.

Zaheer-Ul-Haq, Wellenzohn, B., Liedl, K.R., Rode, B.M. 2003. "Molecular Docking Studies of Natural Cholinesterase-Inhibiting Steroidal Alkaloids from *Sarcococca Saligna*, J Med Chem. 46, 5087–5090.

Zaheer-ul-Haq, S.A.H., Uddin, R., Madura., J.D. 2010. "Benchmarking Docking and Scoring Protocol for the Identification of Potential Acetylcholinesterase Inhibitors, J Mol Graph Model. 28(8), 870–82.

Zarotsky, V., Sramek, J.J., Cutler, N.R. 2003. Galantamine hydrobromide: an agent for Alzheimer's disease, Am J Health Syst Pharm. 60(5), 446-452.

Zhang, H., Karlin, A. 1997. Identification of acetylcholine receptor channel-lining residues in the M1 segment of the beta-subunit, Biochem. 36(50), 15856- 15864.

Zuo, G.Y., Li, Y., Wang T., Han J., Wang G.C., Zhang, Y.L. 2011. Synergistic antibacterial and antibiotic effects of bisbenzylisoquinoline alkaloids on clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), Mol. 16(12), 9819-9826