Phytomedicine 17 (2010) 698–701

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Short Communication

Effect of isoquinoline alkaloids from two *Hippeastrum* species on *in vitro* acetylcholinesterase activity

L.B. Pagliosa [a], S.C. Monteiro [b], K.B. Silva [a], J.P. de Andrade [a,c,1], J. Dutilh [d], J. Bastida [c], M. Cammarota [b], J.A.S. Zuanazzi [a,*]

[a] *Programa de Pós-graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000 Porto Alegre, RS, Brazil*
[b] *Centro de Memória, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga 6681, Andar 2, 90619-900 Porto Alegre, RS, Brazil*
[c] *Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Catalonia, Spain*
[d] *Instituto de Biologia, Universidade Estadual de Campinas, Rua Monteiro Lobato 255, 13083-862 Campinas, SP, Brazil*

ARTICLE INFO

Keywords:
Acetylcholinesterase
Amaryllidaceae alkaloids
Ellman

ABSTRACT

The treatment of neurological disorders and neurodegenerative diseases is related to the levels of acetylcholine (ACh) through the inhibition of acetylcholinesterase (AChE). Galanthamine, an important alkaloid isolated from the Amaryllidaceae family, is approved for the pharmacological treatment of Alzheimer's disease (AD) and acts by inhibiting the acetylcholinesterase (AChE) activity. In the present study, Ellman's method was used to verify the inhibition of AChE activity of some isoquinolines alkaloids such as galanthamine, montanine, hippeastrine and pretazettine. At the concentrations 1 mM, 500 μm and 100 μm, galanthamine presented an AChE inhibition higher than 90%. Montanine inhibited, in a dose-dependent manner, more than 50% of the enzyme at 1 mM concentration. With the concentrations 500 μm and 100 μm, 30-45% of AChE activity inhibition was detected. The alkaloids hippeastrine and pretazettine presented no significant inhibition of the AChE activity. The results demonstrate that montanine significantly inhibits AChE activity at the tested concentrations, suggesting the necessity of further investigations on this alkaloid use in treating neurological disorders.

Introduction

The structures elucidation, the strategies and developed methodology to synthesize Amaryllidaceae alkaloids have been motivated by their diverse and important pharmacological properties (Magnus et al. 1999), including activities such as anticancer, antiviral, immunostimulatory, antimalarial, and acetylcholinesterase (AChE) inhibition (Manpadi and Kornienko 2005). The varied activities assigned to Amaryllidaceae alkaloids and the degree of these alkaloids biological responses may be related to their biosynthetic origin, where the alkaloid norbelladine suffers conversion into *O*-methylnorbelladine through three different oxidative coupling (*ortho-para', para-para', para-ortho'*), resulting in alkaloids of different skeleton types with different biological features possible (Bastida et al. 2006).

Alzheimer's disease (AD) is a fairly common age-related neurodegenerative disease with many cognitive and neuropsychiatric manifestations that result in progressive disability and eventual incapacitation (Hung et al. 2008). This disease is characterized by a loss of cholinergic neurons in the brain and is associated with decreased levels of acetylcholine (ACh) (Lane et al. 2006). The enzymes AChE and butyrylcholinesterase (BuChE) seem to be simultaneously active in the synaptic hydrolysis of ACh (Lane et al. 2006). AChE contributes to the integrity and permeability of the synaptic membrane during neurotransmission (Grafius et al. 1971). This enzyme has been implicated in cholinergic and non-cholinergic actions, which may play a role in neurodegenerative diseases (Henderson et al. 1996; Cummings 2000; Arendt et al. 1992). Inhibition of AChE is considered a strategy for the treatment of neurological disorders. A potential source of AChE inhibitors is certainly provided by the abundance of plants in nature (Mukherjee et al. 2007). In this context, reversible inhibitors of this enzyme have been used as cognitive enhancers in treatment of patients with Alzheimer's and other neurodegenerative disorders (Lane et al. 2006).

In traditional practices, numerous plants have been used to treat neurodegenerative diseases and different neuropharmacological disorders. Ethnopharmacological approach and bioassay-guided isolation have provided a lead in identifying potential AChE inhibitors from plant sources, including those used for memory dysfunctions (Mukherjee et al. 2007).

* Corresponding author. Tel.: +55 51 33085258.
E-mail address: zuanazzi@farmacia.ufrgs.br (J.A.S. Zuanazzi).
[1] Becario del MAEC – AECID.

0944-7113/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2009.10.003

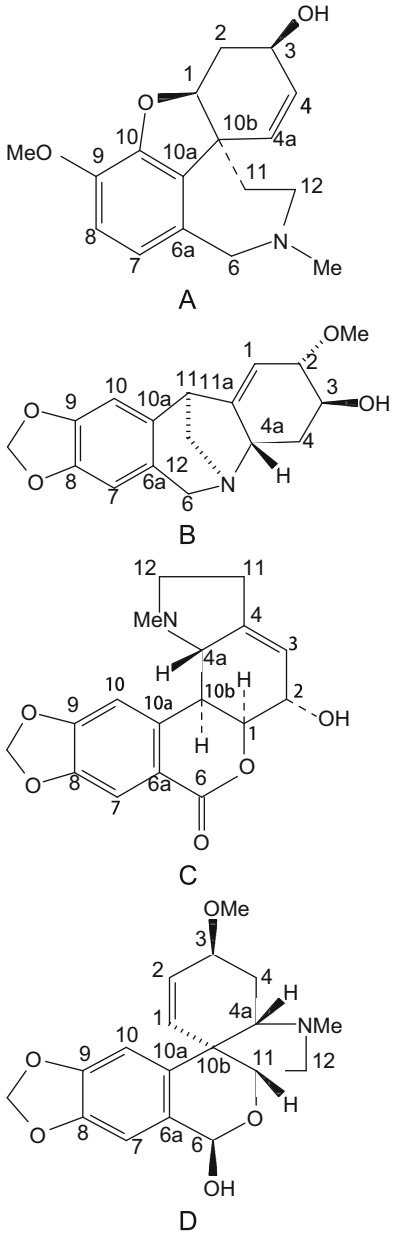

Fig. 1. Chemical structures of galanthamine (A), montanine (B), hippeastrine (C) and pretazettine (D).

The alkaloid galanthamine (Fig. 1), obtained from *Galanthus* and *Narcissus* species (Amaryllidaceae), has a dibenzofuran nucleus and is biosynthetically obtained by *para-ortho'* oxidative coupling (Bastida et al. 2006). Currently this selective, reversible and competitive AChE inhibitor is commercialized to treat AD (Berkov et al. 2008). Montanine *(para-para')*, hippeastrine (*ortho-para'*), and pretazettine (*para-para'*) (Fig. 1) are isoquinolines alkaloids isolated from different species of Amaryllidaceae. Preliminary investigations carried out by our research group with montanine reported significant antitumor activity of this alkaloid for some cell lines (Silva et al. 2008), in addition to psychopharmacological activities including anxiolitic, antidepressive and anticonvulsive effects when intraperitoneally administered (i.p.) (Silva et al. 2006). In the investigation of mitogen-activated protein kinases (MAPKs) signaling pathway, using hippocampal slices treated with montanine and galanthamine, an increase of phosphorylation of MAPKs and CREB factor transcription was observed, indicating the possible role of these alkaloids in the mechanisms related to the memory formation (Silva 2005).

Considering that montanine, hippeastrine, and pretazettine are isoquinolinic alkaloids similar to galanthamine which has been used to AD treatment, and that previous studies of our group demonstrated an increase of MAPKs and CREB phosphorylation in hippocampal slices treated with montanine, we decided to investigate the influence of the alkaloids montanine, hippeastrine, and pretazettine on the *in vitro* AChE activity, an important enzyme related to memory mechanisms.

Material and methods

General and materials

EIMS were obtained on CG-MS Hewlett-Packard 6890+MSD 5975 operating in EI mode at 70 eV. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 500 MHz NMR spectrometer operating at 500 MHz for ^{1}H and 125 for ^{13}C nuclei, respectively. Solvent peaks were used as a reference standard. For chromatographic procedures, silica gel PF 254 and silica gel 230-400 mesh or 60-230 mesh (Merck) were used. All other chemicals were purchased from SigmaChemical Co., St. Louis, MO, USA.

Plant material

H. psittacinum was collected in Atibaia, São Paulo, Brazil, where a voucher specimen is deposited under the number UEC-143513. *H. vittatum* was collected in the South of Brazil (Silva et al. 2008).

Extraction and isolation of alkaloids

Fresh bulbs (3.37 kg) of *H. psittacinum* were triturated and macerated with EtOH. The procedure was repeated until negative test against Bertrand reagent. The EtOH extracts were dried under vacuum, and the residue was partitioned in light petroleum and HCl (10%). The HCl phases were washed with CH_2Cl_2. The remaining acid phase was basified with NH_4OH (pH 9) and the extract with CH_2Cl_2. The residue obtained by drying under vacuum yielded 3.78 g of CH_2Cl_2 extracts. 1.6 g of this extract was submitted to silica gel (60-220 mesh) vacuum liquid chromatography using different solvents in increasing order of polarity (hexane, ethyl ether, dichloromethane, *n*-butanol, ethyl acetate, acetone and methanol) to afford seven fractions. The *n*-butanol and ethyl acetate fractions provided a pure product, pretazettine (70 mg), and the methanol fraction afforded hippeastrine (115 mg). These alkaloids were identified by spectroscopic methods and are in agreement with those reported in the literature (Zhang et al. 2006; Evidente et al. 2004).

The isolation and identification of the alkaloid montanine employed in this work was described by Silva et al. (2006). The isolation of galanthamine was performed through an acid-base extraction of galanthamine commercial tablets (Remynil®).

AChE activity

Rats were killed at the age of 90 days. Their brains were removed and the hippocampus was dissected out. AChE activity was determined using a standard spectrophotometric method according to Ellman et al. (1961) and modified by Lassiter et al. (2003). Hydrolysis rates were measured at an acetylthiocholine concentration of 0.8 mM in 1 ml assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-Dithiobis-(2-nitrobenzoic Acid) (DTNB) at 25 °C. About 50 ml of rat hippocampus supernatant was added to the reaction mixture and preincubated

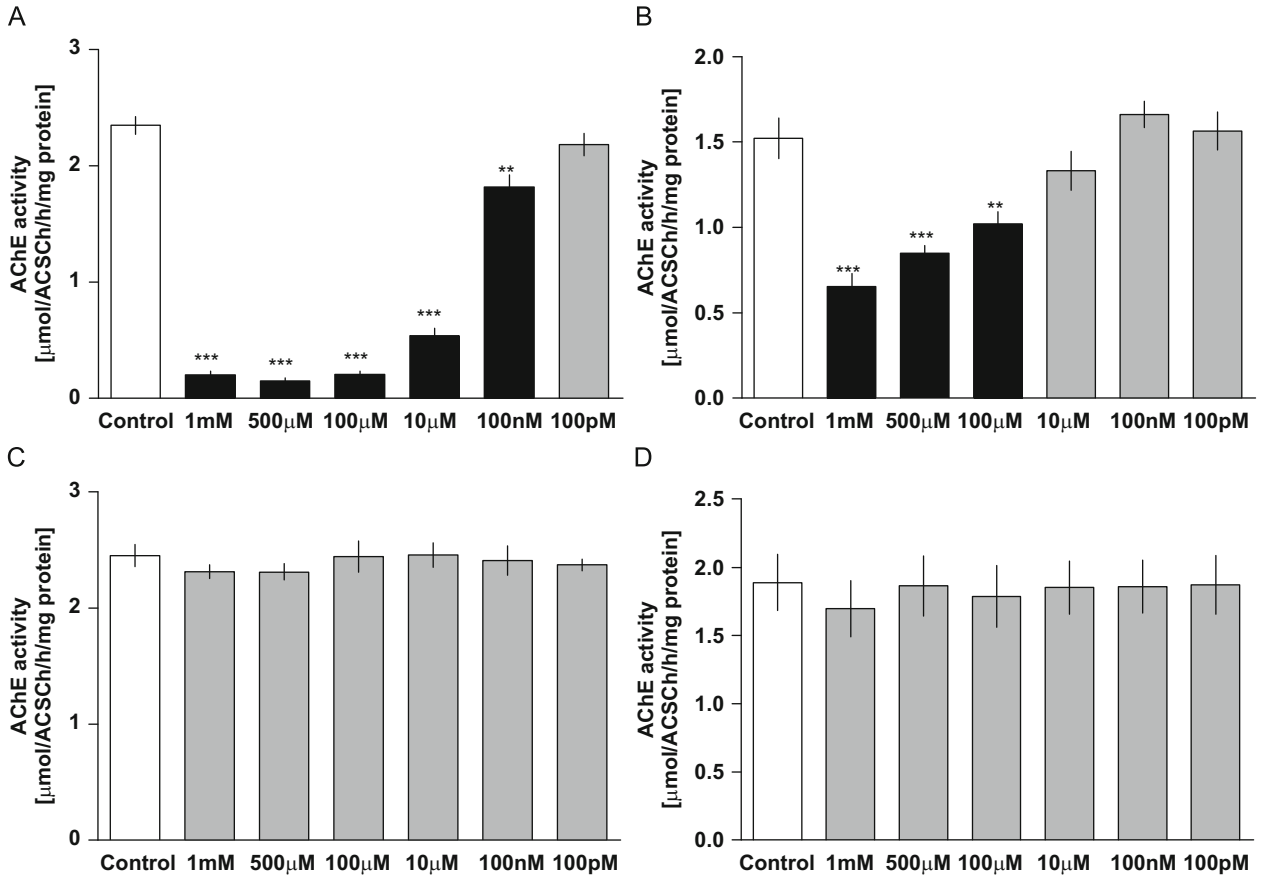

Fig. 2. *In vitro* effect of the alkaloids galanthamine (A), montanine (B), hippeastrine (C) and pretazettine (D) on AChE activity from hippocampus of adult wistar rats. Results are expressed as mean±S.E. for five independent experiments performed in duplicate. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared to control (Dunnett's Mulptiple Range Test).

for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nM for 3 min with 30 s intervals. Specific enzyme activity was expressed as μmol ASCh per hour per mg of protein. All samples were run in duplicate. The alkaloids montanine, hippeastrine, pretazettine and galanthamine were added to the experimental assays at the range of 1 mM, 500 μm, 100 μm, 10 μm, 100 nm, and 100 pm. Control did not contain alkaloids in the incubation medium.

Protein determination

Protein determination was performed by the method of Bradford (1976) using bovine albumin serum as standard.

Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by ANOVA followed by the Duncan multiple range test when the *F* test was significant. Pearson linear regression coupled to ANOVA was also used to verify dose-dependent effects. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

Results

The alkaloids obtained in the crystalline form by vacuum liquid chromatography were submitted to CG-MS analyses. The resulting products of *n*-butanol/ethyl acetate and methanol fractions presented fragmentation pattern in mass spectra of the alkaloids pretazettine ($C_{18}H_{21}NO_5$, m/z=331 [M^+]) and hippeastrine ($C_{17}H_{17}NO_5$,m/z=315 [M^+]), respectively, when compared with the literature data (Zhang et al. 2006; Evidente et al. 2004). Spectroscopic analyses by ^1H and ^{13}C NMR confirmed these hypotheses.

Fig. 2 shows the percentage of inhibition of the alkaloids galanthamine (A), montanine (B), hippeastrine (C), and pretazettine (D) compared to control. The Fig. 2A shows that the galanthamine displayed more than 90% inhibition of AChE $F[(6,35)=215.5;\ ^{***}p < 0.001]$ at concentrations 1 mM, 500 μm, and 100 μm. As can be seen in Fig. 2B, the montanine inhibited in a dose-dependent manner more than 50% of the enzyme $F[(6,35)=18.34;\ ^{***}p < 0.001]$ at 1 mM concentration, and at the concentrations 500 μm and 100 μm the inhibition was in the range of 30-45%. The Figs. 2C and D displayed that hippeastrine and pretazettine, respectively, showed no significant inhibition of the AChE.

Discussion

Amaryllidaceae alkaloids represent a kind of phenylalanine and tyrosine derivates restricted to Amarylidaceae family only. These compounds exhibited several types of pharmacological activities including on central-nervous system (Mroczek and Mazurek 2009). Synthetic medicines, such as tacrine and donepezil, are used for treatment of cognitive dysfunction and memory loss associated with AD. However, some compounds have been reported to have adverse effects including gastro-intestinal disturbances and problems with bioavailability (Oh

et al. 2004; Schulz 2003). After the discovery that the alkaloid galanthamine is a potent acetylcholinesterase inhibitor and, consequently, very important for the symptomatic treatment of AD (Liu et al. 2004), the isolation and characterization of alkaloids from Amaryllidaceae has exponentially increased with the interest in finding better AChE inhibitors from natural resources.

It has been reported that the damage caused by reactive oxygen species is considered a contributing factor to several diseases including AD (Giordani et al. 2008). In this context, a recently study showed that the treatment with vitamins E plus C significantly reversed the action of ovariectomy on Na^+, K^+ - ATPase and AChE activities in hippocampus of female adult rats (Monteiro et al. 2007). A preliminary study of our group demonstrated a promising antioxidant and anticholinesterase activity of three *Hippeastrum* species (Amaryllidaceae) by bioautography method (Giordani et al. 2008).

In this work, the activity of four isoquinolines alkaloids isolated from *Hippeastrum* species was investigated through the assay of AChE activity developed by Ellman et al. (1961). The pretazettine and hippeastrine, alkaloids isolated from *Hippeastrum psittacinum*, did not show anticholinesterase activity by the proposed method.

The alkaloid montanine significantly inhibited AChE activity at concentrations 1 mM, 500 μm, 100 μm by the method from Ellman et al. (1961). In previous investigations, this alkaloid also showed inhibitory activity of AChE by bioautography method (Silva, 2005). Although montanine has decreased the AChE activity at higher concentrations than galanthamine, this alkaloid requires further investigations regarding its structure-activity relationship, interaction with AchE, as well as investigations that relate the AChE activity and cognition at *in vivo* memory studies.

The interest in products for AD treatment, providing desired therapeutic effects and less adverse effects, is based on studies that showed that galanthamine is a less potent alkaloid than physostigmine and tacrine, yet less toxic (Mary et al. 1998). A variety of synthetic galanthamine derivates has been previously described including C-ring derivates (Han et al. 1992), quaternary ammonium derivatives (Han et al. 1992), or other carbamates of 6-*O*-demethylgalanthamine (Bores and Kosley 1996), which have been more active than galanthamine (Jia et al. 2009).

Considerable research efforts have been devoted to elucidate the molecular, biochemical, and cellular mechanisms of AD in the past decades (Sheng et al. 2009). Our results with montanine can contribute to the research of alternative products for the treatment of neurological disorders as well as neurodegenerative diseases. Therefore, further investigations on the structure of montanine may result in a more active compound, at lower concentrations.

Acknowledgments

This investigation was supported by grants of CAPES, FAPERGS and CNPq (Brazil). J.A.S.Z. acknowledge CNPq for researcher fellowship.

References

Arendt, T., Bruckner, M.K., Lange, M., Bigl, V., 1992. Chances in acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease resemble ambryonic development a study of molecular forms. Neurochem. Int. 21, 381–396.

Bastida, J, Lavilla, R, Viladomat, F., 2006. Chemical and biological aspects of *Narcissus* alkaloids. In: Cordell, GA (Ed.), The Alkaloids, Chemistry and Biology, Vol.. 63. Elsevier Academic Press, New York, pp. 87–179.

Berkov, S., Codina, C., Viladomat, F., Bastida, J., 2008. *N*- alkylated galanthamine derivates: potent acetylcholinesterase inhibitors from *Leucojum aestivum*. Bioorg. Med. Chem. Lett. 115, 2263–2266.

Bores, G.M., Kosley Jr, R.W., 1996. Drugs Future 21, 621–635.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248–254.

Cummings, J.L., 2000. The role of cholinergic agents in the management of behavioral disturbances in Alzheimer's disease. Int J Neuropsychopharmacol. 3, 21–29.

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

Evidente, A., Andolfi, A., Abou-Donia, A.H., Touema, S.M., Hammoda, H.M., Shawky, E., Motta, A., 2004. Amarbellisine, a lycorine-type alkaloid from *Amaryllis belladona* L. growing in Egypt. Phytochemistry. 64, 2113–2118.

Giordani, R.B., Pagliosa, L.B., Henriques, A.T., Zuanazzi, J.A.S., 2008. Investigação do Potencial Antioxidante e anticolinesterásico de *Hippeastrum* (Amaryllidaceae). Quim. Nova 31, 2042–2046.

Grafius, M.A., Bond, H.E., Millar, D.B., 1971. Acetylcholinesterase interaction with a lipoprotein matrix. Eur. J. Biochem. 22, 382–390.

Han, S.Y., Sweeney, J.E., Bachaman, E.S., Schweiger, E.J., Forloni, G., Coyle, J.T., Davis, B.M., Joullié, M.M., 1992. Chemical and pharmacological characterization of galanthamine, and acetylcholinesterase inhibitor, and its derivates. A potential application in Alzheimer's disease?. Eur. J. Med. Chem. 27, 673–687.

Henderson, V.W., Watt, L., Buckwalter, J.G., 1996. Cognitive skills associated with estrogen replacement in women with Alzeimer's disease. Psychoneuroendocrinology 21, 421–430.

Hung, T.M., Na, M., Dat, N.T., Ngoc, T.M., Youn, U., Kim, H.J., Min, B., Lee, J., Bae, K., 2008. Cholinesterase inhibitory and anti-amnesic activity of alkaloids from *Corydalis turtschanimovii*. J Ethnopharmacol. 119, 74–80.

Jia, P., Sheng, R., Zhang, J., Fang, L., He, Q., Yang, B., Hu, Y., 2009. Design, synthesis and evaluation og galanthamine derivates as acetylcholinesterase inhibitors. Eur. J. Med. Chem. 44, 772–784.

Lane, R.M., Potkin, S.G., Enz, A., 2006. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int. J. Neuropsychopharmacol. 9, 101–124.

Lassiter, T.L., Marshall, R.S., Jackson, L.C., Hunter, D.L., Vu, J.T., Padilla, S., 2003. Automated measurement of acetylcholinesterase activity in rat peripheral tissues. Toxicology 186, 241–253.

Liu, J., Hu, W.X., He, L.F., Ye, M., Li, Y., 2004. Effects of lycoryne on HL-60 cells via arresting cell cycle and inducing apoptosis. FEBS Lett. 578, 245–250.

Magnus, P., Bailey, J.M., Porter, M., 1999. Application of the β-azidonation reaction to the enantioselective synthesis of the lycorane Amaryllidaceae alkaloids. Tetrahedron 55, 13927–13936.

Manpadi, M., Kornienko, A., 2005. Highly anti-selective conjugate addition of arylcuprates to a γ-alkixy-α, β-enoate. A new method to address stereochemical challenges presented by Amaryllidaceae alkaloids. Tetrahedron Lett. 46, 4433–4437.

Mary, A., Renko, D.Z., Guillou, C., Thal, C., 1998. Potent acetylcholinesterase inhibitors: design, synthesis, and structure-activity relationships of bis-interacting ligands in the galanthamine series. Bioorg. Med. Chem. 6, 1835–1850.

Monteiro, S.C., Mattos, C.B., Scherer, E.B.S., Wyse, A.T.S., 2007. Supplementation with vitamins E plus C or soy isoflavones in ovariectomized rats: effect on the activities of Na^+, K^+ -ATPase and cholinesterases. Metab. Brain Dis. 22, 156–171.

Mroczek, T., Mazurek, J., 2009. Pressurized liquid extraction and anticholinesterase activity-based thin-layer cromatografy with bioautography of Amaryllidaceae alkaloids. Anal. Chim. Acta 633, 188–196.

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

Oh, M.H., Houghton, P.J., Whang, W.K., Cho, J.H., 2004. Screening of Korean herbal medicines used to improve cognitive function for anti-cholinesteraseactivity. Phytomedicine 11, 544–548.

Sheng, R., Xu, Y., Hu, C., Zhang, J., Lin, X., Li, J., Yang, B., He, Q., Hu, Y., 2009. Design synthesis and AchE inhibitory activity of indanone and aurone derivates. Eur. J. Med. Chem. 44, 7–17.

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

Silva, A.F.S., Andrade, J.P., Machado, K.R.B., Rocha, A.B., Apel, M.A., Sobral, M.E.G., Henriques, A.T., Zuanazzi, J.A.S., 2008. Screening for cytotoxic activity of extracts and isolated alkaloids from bulbs of *Hippeastrum vittatum*. Phytomedicine 15, 882–885.

Silva, A.F.S., Andrade, J.P., Bevilaqua, L.R.M., Souza, M,M., Izquierdo, I., Henriques, A.T., Zuanazzi, J.A.S., 2006. Anxiolitytic-, antidepressant-and anticonvulsant-like effects of the alkaloid montanine isolated from *Hippeastrum vittatum*. Pharmacol. Biochem. Behav. 85, 148–154.

Silva, A.F.S., 2005. *Hippeastrum vittatum* (L'Hér) Herbert e *Hippeastrum striatum* (Lam.)Moore: análise química e avaliação biológica dos alcalóides isolados (*Hippeastrum vittatum* (L'Hér) Herbert and *Hippeastrum striatum* (Lam.) Moore: chemical analysis and biological evaluation of the isolated alkaloids). Thesis (Ph.D.). Universidade Federal do Rio Grande do Sul-UFRGS, Brazil.

Zhang, F., Tu, Y., Liu, J., Fan, X., Shi, L., Hu, X., Wang, S., Zhang, Y., 2006. A general approach to crinine-type of (±)-haemanthidine, (±)-pretazettine, (±)-tazettine and (±)-crinamine. Thetrahedron 62, 9446–9455.