Inhibition of *Ptychopetalum olacoides* on Acetylcholinesterase Isoforms in Brain of Mice

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Abstract: **Objective** To further characterize the acetylcholinesterase inhibitors (AChE-Is) pattern of *Ptychopetalum olacoides* ethanol extract (POEE) on the cytosolic globular monomer (G1) and membrane bound globular tetramer (G4) AChE isoforms in brain areas relevant for cognition. **Methods** The G1 and G4 AChE isoforms were prepared according to the reported methods and the determination of AChE activity used was adapted from colorimetric method. **Results** POEE mostly inhibited G1 in hippocampus (75%), and G4 in frontal cortex (58%) and striatum (75%) ($P < 0.05$). Kinetic analysis indicated that POEE-induced AChE inhibition in hippocampus was of a competitive nature for G1 but uncompetitive for G4. **Conclusion** Considering the high density of cholinergic projection to the cortex and striatum, and the usefulness of conserving cytosolic acetylcholine to replenish synaptic vesicles in a highly active cognition site such as hippocampus, we argue that this could be a desirable profile for a clinically relevant AChE-I.

Key words: acetylcholinesterase; Alzheimer's disease; kinetic analysis; molecular isoforms; *Ptychopetalum olacoides*
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Introduction

Ptychopetalum olacoides Benth. (Olacaceae), known as Marapuama, is a medicinal plant widely used in Amazonian communities and especially favored by the elderly. Coherently with users' claims, this research group has established antidepressive (Piato *et al*, 2009), anti-oxidative (Siqueira *et al*, 2007), neuroprotective (Siqueira *et al*, 2004), promnesic (da Silva *et al*, 2004; 2008), and anti-amnesic properties (da Silva *et al*, 2009) for a standardized *P. olacoides* ethanol extract (POEE).

Cognitive deficits are often associated with aging, and are core symptoms in dementias, both of which are expected to increase in prevalence due to the expanded human life expectancy (Panza *et al*, 2006; Rauk, 2009). Alzheimer's disease (AD) has become the commonest form of dementia in the elderly, currently affecting nearly 2% of population in developed countries (Mattson, 2004). In AD there is a marked acetylcholine (ACh) hypofunction thought to result from decreased levels of choline acetyltransferase and rate-limiting enzyme for ACh synthesis (Lane, Kivipelto, and Greig, 2004). Because the reduction in cholinergic activity is correlated with the degree of cognitive impairment (Francis *et al*, 1999), several drugs have been designed to enhance cognitive function in AD patients by targeting acetylcholinesterase (AChE) in an attempt to increase ACh permanence at the synaptic cleft (Barnes *et al*, 2000). Unfortunately, the success with available therapy, using either acetylcholinesterase inhibitors (AChE-Is), memantine (an uncompetitive *N*-methyl-*D*-aspartate receptor antagonist), or the combination of these drugs, is still far less than the desirable (Giacobini, 2003; Hartmann and Mobius, 2003; Tariot *et al*, 2004; Cummings *et al*, 2006; Pepeu and Giovannini, 2009). In general, AChE-Is have limited success as they only improve memory in mild dementia (Sonkusare, Kaul,

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and Ramarao, 2005). AChE (EC 3.1.1.7) is recognized to occur in different molecular isoforms, distinguishable by their subunit associations and hydrodynamic properties (Brimijoin, 1983; Massoulièe *et al*, 1993). The two major isoforms in the brain are the globular monomer (G1) cytosolic form and the globular tetramer (G4) membrane bound form (Massoulièe *et al*, 1993; Massoulièe, 2002). It has also been found that these isomeric forms had different cellular distribution and functional significance in synaptic transmission (Brimijoin, 1983). In healthy human brain, G1 and G4 AChE isoforms are responsible for 80% of total cholinesterase activities (Atack *et al*, 1986), whereas in AD brain there is a selective loss of G4 and a relative sparing of G1 (Siek *et al*, 1990; Schegg *et al*, 1992). Isoform specificity varies among clinically relevant AChE-Is: Galanthamine does not show selectivity, Donepezil preferentially inhibits G4, and Rivastigmine inhibits G1 (Rakonczay, 2003; Lane, Kivipelto, and Greig, 2004; Lane, Potkin, and Enz, 2006). Isoform specific selectivity may be of relevance to improving therapeutics if appropriate targets could be elected (Zhao and Tang, 2002).

Because anticholinesterasic properties were identified for POEE (Siqueira *et al*, 2003) and likely to underlie its memory enhancing (da Silva *et al*, 2004; 2007) and anti-amnesic effects (da Silva *et al*, 2009), we here characterized the inhibition of POEE in brain areas relevant for cognition of mice (hippocampus, frontal cortex, and striatum), taking into account specificities for G1 and G4 isoforms. Additionally, the nature of inhibition was determined in the hippocampus.

Materials and methods

Animals

Male (CF1) adult (two months old, 35－45 g) albino mice were obtained from Fundação Estadual de Produção e Pesquisa em Saúde, and maintained at our own animal facility under controlled environmental conditions [(22 ± 1) ℃, 12 h-light/dark cycle, free access to food and water] for at least two weeks before experiments. The project was approved by the University Ethics Committee (approval # 2007834).

Sample preparation

The roots of *Ptychopetalum olacoides* Benth. (Olacaceae) were collected in Pará (Brazil) (voucher MPEG 108.036, Goeldi Museum, identified by Nelson Rosa), in accordance with national guidelines as they related to UN Convention on Biodiversity. POEE preparation and its fingerprint of HPLC [HP 1100/Photodiode Array Detector, Agilent Technologies, with a Zorbax extended C_{18} column (250 mm × 4.6 mm, MeOH-H_2O gradient 10:90→100:0)] are detailed by Siqueira *et al* (2007). Due to patent PI0307647-4 INPI/Br and US61/297,442 issues, the nature of active compounds and/or detailed extract composition cannot be currently disclosed.

Drugs

Acetylthiocholine (ATC) iodide, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA), Tris (hydroxymethyl-d_3) amino-d_2-methane, tetraisopropyl pyrophosphoramide (iso-OMPA), and eserine were obtained from Sigma Chemicals Co. (MO, USA); K_2HPO_4, KH_2PO_4, and DMSO were obtained from Quimibrás Reagen (Rio de Janeiro, Brazil). POEE was dissolved in 20% DMSO.

Enzymatic materials

AChE isoform sources Mice were sacrificed and the brains were quickly removed, cleaned with chilled NaCl 0.9% solution, and the cerebral structures were dissected out over ice. The hippocampus, frontal cortex, and striatum were homogenized in 20, 10, and 20 volumes of buffer (0.01 mol/L Tris-HCl, pH 7.2, and 0.16 mol/L sucrose), respectively, and centrifuged at 5000 × *g* at 4 ℃ for 15 min (Eppendorf Centrifuge 5415R). The resulting supernatant was used as the G1 source. The pellet was suspended in 1% Triton-X 100 (1% in 0.5 mol/L potassium phosphate buffer, pH 7.5), and centrifuged at 100 000 × *g* at 4 ℃ in a Hitachi Refrigerated Centrifuge for 60 min. The supernatant was collected and used as the G4 source (Das, Dikshit, and Nath, 2001).

AChE activity The determination of AChE activity used was adapted from the colorimetric method originally described by Ellman *et al* (1961). Briefly, 33 μL of 10 mmol/L DTNB, 68 μL of Tris-HCl buffer, 100 μL of POEE (0－1000 μg/mL), and 33 μL of enzymatic material (3 μg/μL of protein for G1 or G4 AChE) were added to microplates followed by 33 μL of a final concentration of 0.8 mmol/L ATC. The incubation solution contained the butyrylcholinesterase inhibitor iso-OMPA at a final concentration of 100 μmol/L in

order to specifically investigate AChE activity. The microplate was read at 415 nm every 30 s for 2.5 min (Microplate Reader Model 680, Bio-Rad Laboratories, UK). Experiments were performed in triplicate. AChE activities were expressed as μmol of ATC hydrolyzed/ hour/milligram of protein (μmol ATC/h/mg protein).

Kinetic analysis To determine the type of enzyme inhibition, Lineweaver-Burk double reciprocal plots were produced by varying the concentration of substrate ATC from 0.01 to 0.075 mmol/L in hippocampus. Plots were used to determine K_m and V_{max} for POEE at the concentration of 0, 30, 100, 300, and 1000 μg/mL. Specific activities are expressed as μmol ATC/h/mg protein.

Protein assay

The protein content was determined as described by Lowry *et al* (1951), using BSA as standard.

Statistical analysis

The AChE activity and kinetic analysis are expressed as $\bar{X} \pm s$. One-way ANOVA followed by Duncan multiple group comparison was used ($P < 0.05$).

Results

Effects of POEE on G1 and G4 AChE isoforms

Figs. 1—3 show the effects of POEE (0—1000 μg/mL) on G1 and G4 isoforms from hippocampus, frontal cortex, and striatum, respectively. The experimental conditions were validated with eserine (Ese, 27.5 μg/mL) which inhibited G1 and G4 in all tissues ($P < 0.05$); DMSO was devoid of effect. POEE significantly inhibited G1 in hippocampus (100 and 300 μg/mL, $F_{(6,28)} = 59.2$; Fig. 1A), frontal cortex (30—1000 μg/mL, $F_{(6,28)} = 32.5$; Fig. 2A), and striatum (1000 μg/mL, $F_{(6,28)} = 30.6$; Fig. 3A) ($P < 0.05$). The activity of G4 was also significantly reduced in hippocampus (100 μg/mL, $F_{(6,28)} = 114.3$; Fig. 1B), frontal cortex (30—1000 μg/mL, $F_{(6,28)} = 174.0$; Fig. 2B), and striatum (30—1000 μg/mL, $F_{(6,28)} = 82.3$; Fig. 3B) ($P < 0.05$). POEE appears to preferentially affect G1 and G4 in hippocampus (up to 75% and 60%), while G4 appears to be more markedly affected in frontal cortex and striatum (up to 58% and 75%, respectively). In the same concentration range POEE did not inhibit butyrylcholinesterase activity (hippocampus, $F_{(6,28)} = 10.9$; frontal cortex, $F_{(6,28)} = 14.9$; striatum, $F_{(6,28)} = 13.7$, data not shown) ($P > 0.05$).

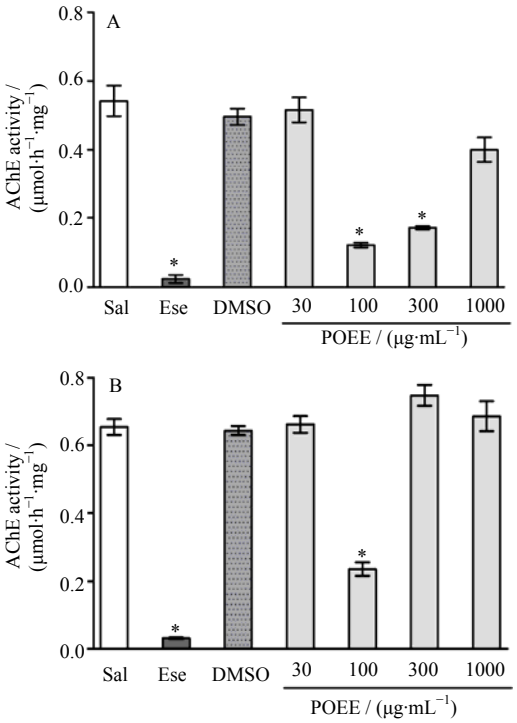

Fig. 1 Effects of POEE and Ese on G1 (A) and G4 (B) AChE isoforms in hippocampus of mice ($\bar{X} \pm s$)

All assays were performed in triplicate for five separate experiments. $^*P < 0.05$ *vs* control (Sal and DMSO). The figures were constructed using the software package Prism Graph Pad 5.0, same as below

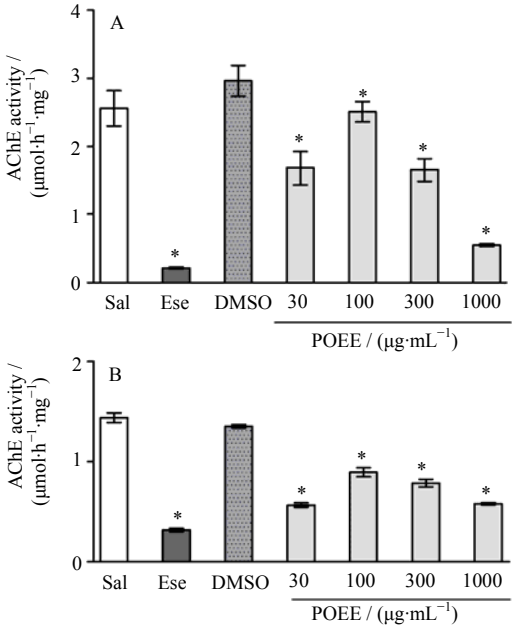

Fig. 2 Effects of POEE and Ese on G1 (A) and G4 (B) AChE isoforms in frontal cortex of mice

Kinetic analysis

The kinetic analysis performed in hippocampus by Lineawever-Burk plot revealed that POEE inhibited G1 in a competitive manner (Fig. 4A), whereas inhibition of G4 was uncompetitive (Fig. 4B). K_m and V_{max} values are shown in Table 1.

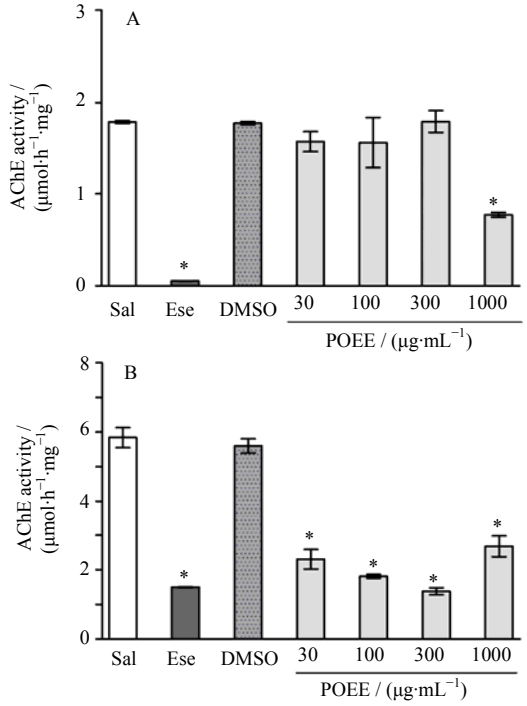

Fig. 3 Effects of POEE and Ese on G1 (A) and G4 (B) AChE isoforms in striatum of mice

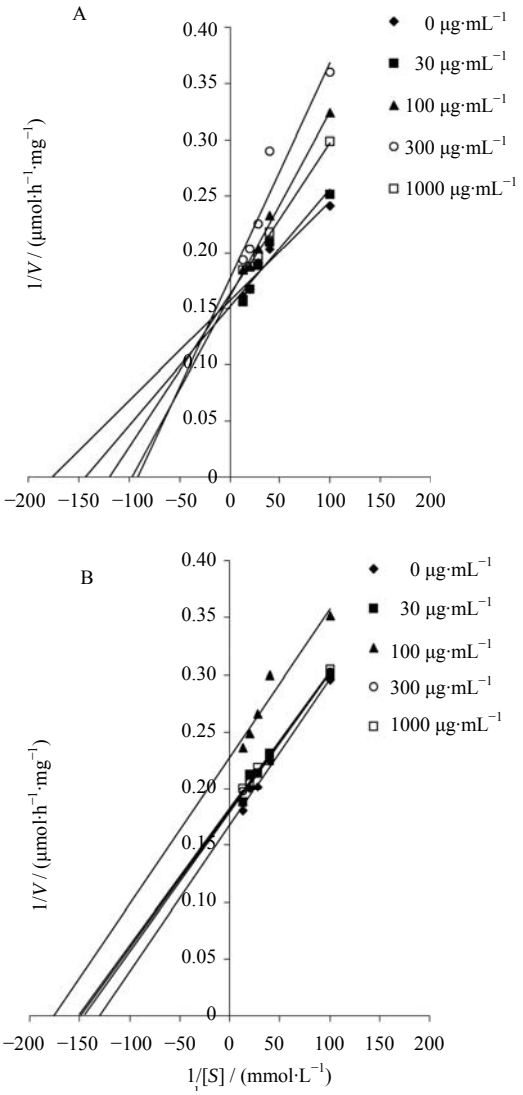

Fig. 4 Lineweaver-Burk representation of G1 AChE (A) and G4 AChE (B) inhibition by POEE in hippocampus with ATC as substrate (n = 5)

Double reciprocal plot was constructed in Microsoft Excel 2003 by plotting 1/*V* against 1/*S* analyzed over a range of substrate concentration (0.01－0.075 mmol/L) with or without POEE (30, 100, 300, and 1000 μg/mL)

Discussion

Adding to earlier analysis on the effects of POEE on AChE activity, this study shows that POEE markedly inhibits G1 and G4 AChE isoforms in brain areas relevant for cognitive processes, such as hippocampus, frontal cortex, and striatum. In frontal cortex, the concentration-effect curve appears to be bell shaped, whereas for hippocampus and striatum a U shaped curve is apparent. Differential effects of drugs in different brain areas are apparently more the rule than the exception (Sanacora, Treccani, and Popoli, 2012; Ellenbroek, 2012); This may in fact be beneficial to the overall drug effect considering the region specific expression of central nervous system disease (Correll, 2011).

A limitation of this study is the lack of clear concentration-response relationships. Siqueira *et al* (2003) reported a concentration-dependent inhibitory effect of the same extract used in this study on total AChE in cortex, hippocampus, and striatum. Though unexpected, this result may be related to a less than perfect separation of G1 and G4, the fact that POEE is in fact a complex mixture likely composed of active and inactive compounds, and/or the presence of more than one structurally related compounds with anticholinesterase properties but differential G1 and G4 affinity. Inasmuch as this is a caveat that would require additional experiments for further clarification, the inhibition of AChE here reported is in agreement with the *ex vivo* histochemical demonstration of AChE inhibition in mice brain treated orally with POEE (Figueiró *et al*, 2010), the reversal of amnesias (da Silva *et al*, 2004; 2009), and a refinement of initial *in vitro* studies for indicating G1 and G4 activities. It has been argued that an increased Gl/G4 ratio is found in AD patients due to the differential decrease in G1 and G4 expression, suggesting that an optimum AChE-Is for treating AD

Table 1 Effect of POEE on K_m and V_{max} of hippocampus G1 and G4 AChE

Isoforms	POEE / ($\mu g \cdot mL^{-1}$)	V_{max} / ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)	K_m / ($\mu g \cdot mL^{-1}$)
G1 AChE	0	5.86	7.91
	30	6.27	9.14
	100	6.11	10.63
	300	5.68	11.06
	1000	6.45	12.84
G4 AChE	0	6.05	8.29
	30	5.74	7.61
	100	4.11	6.0
	300	4.36	6.05
	1000	4.43	6.37

V_{max} and K_m were measured on Lineweaver-Burk double reciprocal plots varying the concentration of the substrate ATC from 0.01 to 0.075 mmol/L, and using increasing POEE concentration (0, 30, 100, 300, and 1000 μg/mL) in hippocampus

would preferentially inhibit G1 (Siek *et al*, 1990; Schegg *et al*, 1992). Oddly, the highly promnesic and neuroprotective alkaloid huperzine A is a G4-selective inhibitor in multiple brain areas but effective in the models of β-amyloid-induced dementia of mice (Zhao and Tang 2002; Wang, Zhang, and Tang, 2001). As mentioned above, this is also the case with Donepezil (Rakonczay, 2003; Lane, Kivipelto, and Greig, 2004; Lane, Potkin, and Enz, 2006).

Although a definite analysis requires isolated compounds and their various combinations, in this study POEE shows a clearly selective pattern of inhibition for different AChE isoforms. The data indicate that while G1 is preferentially inhibited by POEE in hippocampus, G4 is more strongly affected in frontal cortex and striatum. We argue that this could be a desirable profile in the context of cognitive deficits, considering the high density of cholinergic projections to the cortex and striatum, and the usefulness of conserving cytosolic acetylcholine (pools) to replenish synaptic vesicles in a highly active cognition site such as hippocampus. Though the clinical relevance of such neuropharmacological differences in AChE inhibition profiles remains to be determined, isoform- and region-specific relative potencies of AChE-inhibition are factors that are bound to influence efficacy as well as tolerability profiles.

The kinetic analysis indicates that, at least for hippocampus, POEE-induced AChE inhibition also differs regarding the two AChE isoforms. For G1 POEE acts as a competitive inhibitor, whereas for G4 the inhibition is of an uncompetitive nature. The nature of inhibition may be determined by the micro-environment surrounding the active site, which at the same time is a contributing factor in determining the selectivity for both substrate and AChE isoforms (Zhao and Tang, 2002). As for isoforms specificity, a detailed understanding of the interaction of G1 and G4 with POEE requires the structural elucidation of the active compound(s).

The results of this study are consistent with the traditional claims for *P. olacoides* based home remedies as well as with nootropic profile of POEE in mice. We reported that this standardized extract improved various phases of memory in different behavioral tasks (long-term memory retrieval and short-term memory acquisition, consolidation, and retrieval in step-down inhibitory avoidance and object recognition tasks) (da Silva *et al*, 2004; 2007). Moreover, POEE is able to reverse age-, scopolamine-, and MK-801-induced amnesias (da Silva *et al*, 2004; 2009). It is noteworthy that these effects are obtained with orally treated mice, while the signs expected from peripherally acting AChE with POEE given acutely or for 21 d are absent (da Silva *et al*, 2004; 2008).

Conclusion

This study refines the proposal that an improvement in cholinergic function in cognition relevant areas by POEE is likely to be a neurochemical correlation with its memory facilitating effects. The data also point to the relevance of giving specific attention to AChE molecular isoforms in developing AChE-Is effective for the treatment of cognitive disorders.

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References

Atack JR, Perry EK, Bonham JR, Perry RH, 1986. Molecular forms of acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease resemble embryonic development: A study of molecular forms. *Neurochem Int* 21: 381-396.

Barnes CA, Meltzer J, Houston F, Orr G, McGann K, Wenk GL, 2000. Chronic treatment of old rats with donepezil or galantamine: effects on memory, hippocampal plasticity and nicotinic receptors. *Neuroscience* 99(1): 17-23.

Brimijoin S, 1983. Molecular forms of acetylcholinesterase in brain, nerve and muscle: nature, localization and dynamics. *Prog Neurobiol* 21: 291-322.

Correll CU, 2011. What are we looking for in a new antipsychotic? *J Clin Psychiatry* 72(1): 9-13.

Cummings JL, Schneider E, Tariot PN, Graham SM, 2006. Behavioral effects of memantine in Alzheimer disease patients receiving donepezil treatment. Memantine MEM-MD-02 Study Group. *Neurology* 67(1): 57-63.

da Silva AL, da Silva Martins BD, Linck V, Herrmann AP, Mai N, Nunes DS, Elisabetsky E, 2009. MK801- and scopolamine-induced amnesias are reversed by an Amazonian herbal locally used as a brain tonic. *Psychopharmacology* 202: 165-172.

da Silva AL, Ferreira JG, da Silva Martins B, Oliveira S, Mai N, Nunes DS, Elisabetsky E, 2008. Serotonin receptors contribute to the promnesic effects of *P. olacoides* (Marapuama). *Physiol Behav* 95(1/2): 88-92.

da Silva AL, Piato AL, Bardini S, Netto CA, Nunes DS, Elisabetsky E, 2004. Memory retrieval improvement by *Ptychopetalum olacoides* in young and aging mice. *J Ethnopharmacol* 95: 199-203.

da Silva AL, Piato AL, Ferreira JG, Martins BS, Nunes DS, Elisabetsky E, 2007. Promnesic effects of *Ptychopetalum olacoides* in aversive and non-aversive learning paradigms. *J Ethnopharmacol* 109: 449-457.

Das A, Dikshit M, Nath C, 2001. Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. *Life Sci* 68: 1545-1555.

Ellenbroek BA, 2012. Psychopharmacological treatment of schizophrenia: What do we have, and what could we get? *Neuropharmacology* 62: 1371-1380.

Ellman GL, Courtney KD, Andre VJr, Featherstone RM, 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7: 27-30.

Figueiró M, Ilha J, Pochmann D, Porciúncula LO, Xavier LL, Achaval M, Nunes DS, Elisabetsky E, 2010. Acetylcholinesterase inhibition in cognition-relevant brain areas of mice treated with a nootropic Amazonian herbal (Marapuama). *Phytomedicine* 17(12): 956-962.

Francis PT, Palmer AM, Snape M, Wilcock GK, 1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry* 66(2): 137-147.

Giacobini E, 2003. Cholinesterases: new roles in brain function and in Alzheimer's disease. *Neurochem Res* 28: 515-522.

Hartmann S, Mobius HJ, 2003. Tolerability of memantine in combination with cholinesterase inhibitors in dementia therapy. *Int Clin Psychopharmacol* 18(2): 81-85.

Lane RM, Kivipelto M, Greig NH, 2004. Acetylcholinesterase and its inhibition in Alzheimer disease. *Clin Neuropharmacol* 27(3): 141-149.

Lane RM, Potkin SG, Enz A, 2006. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. *Int J Neuropsychopharmacol* 9(1): 101-124.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265-275.

Massouliè J, 2002. The origin of molecular diversity and functional anchoring of cholinesterases. *Neurosignals* 11: 130-143.

Massouliè J, Pezzementi L, Bon S, Krejci E, Vallette FM, 1993. Molecular and cellular biology of cholinesterases. *Prog Neurobiol* 41: 31-91.

Mattson MP, 2004. Pathways towards and away from Alzheimer's disease. *Nature* 430: 631-639.

Panza F, D'Introno A, Colacicco AM, Capurso C, Pichichero G, Capurso SA, Capurso A, Solfrizzi V, 2006. Lipid metabolism in cognitive decline and dementia. *Brain Res Rev* 51: 275-292.

Pepeu G, Giovannini MG, 2009. Cholinesterase inhibitors and beyond. *Curr Alzheimer Res* 6: 1-11.

Piato AL, Rizon LP, Martins BS, Nunes DS, Elisabetsky E, 2009. Antidepressant profile of *Ptychopetalum olacoides* Benthan (Marapuama) in mice. *Phytother Res* 23: 519-524.

Rakonczay Z, 2003. Potencies, and selectivities of inhibitors of acetylcholinesterase and its molecular forms in normal and Alzheimer's disease brain. *Acta Biol Hung* 54: 183-189.

Rauk A, 2009. The chemistry of Alzheimer's disease. *Chem Soc Rev* 38: 2698-2715.

Sanacora G, Treccani G, Popoli, M, 2012. Towards a glutamate hypothesis of depression an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* 62: 63-77.

Schegg KM, Harrington LS, Neilsen S, Zweig RM, Peacock JH, 1992. Soluble and membrane-bound forms of brain acetylcholinesterase in Alzheimer's disease. *Neurobiol Aging* 13: 697-704.

Siek GC, Katz LS, Fishman EB, Korosi TS, Marquis JK, 1990. Molecular forms of acetylcholinesterase in subcortical areas of normal and Alzheimer disease brain. *Biol Psychiatry* 27: 573-580.

Siqueira IR, Cimarosti H, Fochesatto C, Nunes DS, Salbego C, Elisabetsky E, Netto CA, 2004. Neuroprotective effects of *Ptychopetalum olacoides* Bentham (Olacaceae) on oxygen and glucose deprivation induced damage in rat hippocampal slices. *Life Sci* 75(15): 1897-1906.

Siqueira IR, Fochesatto C, da Silva AL, Nunes DS, Battastini AM, Netto CA, Elisabetsky E, 2003. *Ptychopetalum olacoides*, a traditional Amazonian "nerve tonic", possesses anticholinesterase activity. *Pharmacol Biochem Behav* 75(3): 645-650.

Siqueira IR, Fochesatto C, Torres IL, da Silva AL, Nunes DS, Elisabetsky E, Netto CA, 2007. Antioxidant activities of *Ptychopetalum olacoides* ("muirapuama") in mice brain. *Phytomedicine* 14(11): 763-769.

Sonkusare SK, Kaul CL, Ramarao P, 2005. Dementia of Alzheimer's disease and other neurodegenerative disorders—memantine, a new hope. *Pharmacol Res* 51(1): 1-17.

Tariot PN, Farlow MR, Grossberg GT, Graham SM, McDonald S, Gergel I, 2004. Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: A randomized controlled trial. *JAMA* 291(3): 317-324.

Wang RH, Zhang Y, Tang XC, 2001. Huperzine A attenuates cognitive dysfunction and neuronal degeneration caused by β-amyloid protein (1－40) in rat. *Eur J Pharmacol* 21: 149-156.

Zhao Q, Tang XC, 2002. Effects of huperzine A on acetylcholinesterase isoforms *in vitro*: comparison with tacrine, donepezil, rivastigmine and physostigmine. *Eur J Pharmacol* 455: 101-107.