Available online at www.sciencedirect.com
SCIENCE DIRECT®
Pharmacology, Biochemistry and Behavior 75 (2003) 645–650
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Ptychopetalum olacoides, a traditional Amazonian "nerve tonic", possesses anticholinesterase activity

Ionara Rodrigues Siqueira[a,b], Cíntia Fochesatto[a], Adriana Lourenço da Silva[c], Domingos Sávio Nunes[d], Ana Maria Battastini[a], Carlos Alexandre Netto[a,b], Elaine Elisabetsky[b,c,*]

[a]*Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 90035-003, Brazil*
[b]*Programa de Pós Graduação em Ciências Biológicas-Fisiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 90035-003, Brazil*
[c]*Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 90035-003, Brazil*
[d]*Departamento de Química, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, 84030-310, Brazil*

Received 11 November 2002; received in revised form 1 March 2003; accepted 10 March 2003

Abstract

The cholinergic hypothesis of Alzheimer disease (AD) has provided the rationale for the current pharmacotherapy of this disease, in an attempt to downgrade the cognitive decline caused by cholinergic deficits. Nevertheless, the search for potent and long-acting acetylcholinesterase (AChE) inhibitors that exert minimal side effects to AD patients is still an ongoing effort. Amazonian communities use traditional remedies prepared with *Ptychopetalum olacoides* (PO, Olacaceae) roots for treating various central nervous system conditions, including those associated with aging. The fact that PO ethanol extract (POEE) has been found to facilitate memory retrieval in the step down procedure in young and aged mice prompt us to evaluate its effects on AChE activity in memory relevant brain areas. POEE significantly inhibited AChE activity in vitro in a dose- and time-dependent manner in rat frontal cortex, hippocampus and striatum; a significant inhibition was also found in these same brain areas of aged (14 months) mice after acute administration of POEE (100 mg/kg ip). We propose that such AChE inhibitory activity is a neurochemical correlate of a number of therapeutic properties traditionally claimed for *P. olacoides*, particularly those associated with cognition.

Keywords: *Ptychopetalum olacoides*; Marapuama; Acetylcholinesterase; Striatum; Hippocampus; Frontal cortex; Cognitive deficits

1. Introduction

Cognitive impairment during normal aging as well as in the Alzheimer's disease (AD) is thought to be associated to the degeneration of the basal forebrain cholinergic neurons. The aging rat is a well-established model of spontaneous brain cholinergic hypoactivity, useful to study the effects of drugs on the cholinergic transmission, as well as on age-associated cognitive impairment (Quirion et al., 1995). Accordingly, transgenic mice overexpressing human acetylcholinesterase (AChE) in brain neurons have cognitive deterioration, corroborating the role of this system in the modulation of spatial memory (Andres et al., 1996; Beeri et al., 1995). In addition, brain cholinergic deficits, especially in the neocortex, amygdala and hippocampus are important neuropathologic findings in AD patients (Cummings and Back, 1998), usually accompanying abnormal components of the neuronal cytoskeleton, such as tangles and β-amyloid senile plaques (Vickers et al., 2000). The cholinergic hypothesis of Alzheimer disease (AD) has provided the rationale for the current pharmacotherapy of this disease, in an attempt to downgrade the cognitive decline caused by cholinergic deficits. Among the possible strategies for enhancing brain cholinergic activity, acetylcholinesterase inhibitors (AChEIs) have been the most extensively used for the symptomatic treatment of AD. Physostigmine and tacrine are the only AChEIs reasonably evaluated in AD patients, even though their use is limited by the short half-life and peripheral cholinergic side effects of physostigmine, and the dose-dependent hepatotoxicity of tacrine (Nordberg and Svensson, 1998; Watkins et al., 1994; Yoshida and

* Corresponding Author: Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, CP 5072, Porto Alegre, RS, 90041-970, Brazil. Tel.: +55-51-3316-3569; fax: +55-51-3316-3121.
E-mail address: elisasky@ufrgs.br (E. Elisabetsky).

0091-3057/03/$ – see front matter © 2003 Published by Elsevier Science Inc.
doi:10.1016/S0091-3057(03)00113-8

Suzuki, 1993). While newer AChE inhibitors, such as donepezil and huperazine, have been recently introduced in the European market (Rogers and Friedhoff, 1996; Sugimoto et al., 1992), the identification of a potent long-acting AChE inhibitor with fewer side effects in AD patients would be highly advantageous.

Brazil houses an enormous biological and cultural diversity, with several medicinal plants identified by local peoples as being useful for treating CNS disorders. Within the context of drug development, the interest in traditional medical systems has been mostly focused on its plant-based remedies, with the underlying understanding that some species may contain therapeutically useful compounds (Abelson, 1990; Cragg and Newman, 2001). *Ptychopetalum olacoides* Bentham (PO, Olacaceae), known as "Marapuama" (and/or Muirapuama and Mirantã among others) is widely consumed throughout the Amazon region. Local communities use PO-based alcoholic infusions for the treatment of CNS-related ailments and/or during highly stressful periods (Elisabetsky, 1987; Grenand et al., 1987; Siqueira et al., 1998; Steinmetz, 1962). The frequencies of elders amongst users, and by patients recovering from conditions associated with damage to the central nervous system (such as stroke), are of particular interest (Elisabetsky and Siqueira, 1998). It is noteworthy that PO has nowadays been included in dozens of herbal drugs or multivitamin dietary supplements at the international market, based on claims of enhanced physical and cognitive performance.

Recognizing the value of traditional knowledge, and the various CNS activities identified with an ethanol extract obtained from PO roots (Siqueira et al., 1998; da Silva et al., 2002a), including the enhancement of memory retrieval in young and aged mice (da Silva et al., 2002b), the purpose of this study was to evaluate the effects of POEE on AChE activity.

2. Material and methods

2.1. Plant material

Roots of *P. olacoides* Bentham (PO, Olacaceae) were collected at the State of Pará (Brazil), and identified by Mr. Nelson Rosa (voucher deposited at the Goeldi Museum herbarium, MG 108036).

The plant species was further authenticated through quantitative analysis for lupeol in the roots using the method described by Auterhoff and Momberger (1971); we found an average lupeol yield of 0.28% in the roots, in accordance with the data published by these authors for this species.

2.2. Ethanol extract

The extractive procedure has been detailed elsewhere (Siqueira et al., 1998). Briefly, milled root barks were extracted (Sohxlet) with ethanol and dried under reduced pressure, resulting in the POEE (brown syrup, yield 6%).

2.3. AChE activity

AChE activity was determined by slight modifications of the colorimetric method described by Ellman et al. (1961), using acetylthiocholine iodide as a substrate.

2.3.1. In vitro

Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout the study. Five male Wistar rats (housed with food and water ad libitum, light–dark cycles of 12 h) with 3 months of age were used. Each animal was decapitated, the brain quickly

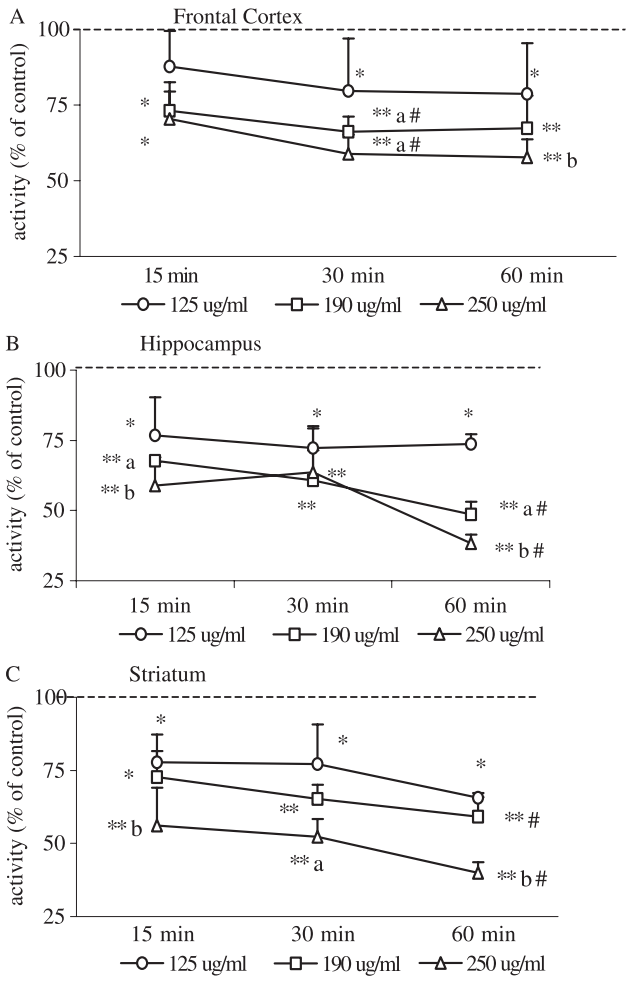

Fig. 1. Effect of POEE (125, 190 and 250 μg/ml) on AChE activity in rat frontal cortex, hippocampus and striatum. AChE activity (as percentage of the DMSO control group) at 15, 30 and 60 min of incubation. Each value represents mean ± S.E.M. ($n = 5$). * $P < .05$, * * $P < .01$ vs. control, paired Student's t test; a = $P < .05$ vs. 125 μg/ml and b = $P < .05$ vs. 125 and 190 μg/ml, ANOVA/Duncan; # = $P < .05$ vs. previous incubation time, ANOVA/Duncan.

removed, placed on ice-cold plate and washed with iced buffer (0.5 M sodium phosphate, pH 7.5); the frontal cortex, hippocampi and striata were rapidly removed, homogenized in 40, 40 and 200 volumes of buffer, respectively, and centrifuged at $900 \times g$ for 10 min. The resulting supernatants were used as the enzyme source. All steps were carried out at 4 °C.

Since DMSO was proven to be inactive in this assay (Park et al., 1996), the extract POEE was thoroughly macerated with DMSO, and further diluted to 2.5 mg/ml in buffer immediately before use; 2% was the maximum final concentration of DMSO in the assays. At this point POEE was centrifuged to eliminate colored substances that, although negligent in terms of total weight, could interfere with the experiment. Aliquots of the 2.5 mg/ml POEE solution were further diluted with buffer (so as to attain 125, 190 and 250 μg/ml as final concentrations in the assays), and incubated at 25 °C for 15, 30 or 60 min with the enzyme source; the total volume of reaction mixtures was 1 ml (100 μl of POEE, 10 μl of enzyme, 600 μl distilled water and 100 μl of Ellman's reagent [10 mM 5-5′-dithio-bis(2-nitrobenzoic acid)]; each sample of enzyme source was worked out in triplicates. The blank reading was obtained for each reaction mixture after 10 min of incubation, before the addition of acetylthiocholine iodide (ASCh, 75 mM). Absorbance (412 nm) readings were obtained for 5 min at 30-s intervals thereafter. AChE activity was estimated through differences in dA/min, and the degrees of inhibition in each brain area and incubation time were calculated by comparison with its control (DMSO 100%, results expressed as percentage of control).

Michaelis–Menten constant (Km) and V_{max} were calculated by means of a Lineweaver–Burk plot with initial velocities obtained over a substrate range of 50 to 250 μM, using striatum samples after 60 min of incubation.

2.3.2. Ex vivo

Swiss albino male mice of 14 months of age (CF1 strain, housed with food and water ad libitum, light–dark cycles of 12 h) were used. DMSO 20% ($N=5$) or POEE 100 mg/kg ($N=5$) were administered intraperitoneally (0.1 ml/10 g). The mice were killed 120 min after injection and brain regions dissected and processed as described above. Total AChE activity was measured (triplicates) in aliquots of brain homogenates; AChE inhibition is expressed as percentage of control (DMSO-treated mice).

2.4. Protein assay

Total protein concentrations were determined as described by Bradford (1976), using bovine serum albumin as standard.

2.5. Statistical analysis

Differences in AChE activity from the control baseline (DMSO) were assessed by using paired t tests ($P<.05$). ANOVA/DUNCAN was used to identify differences among doses and periods of incubation; linear regression was further used to verify dose–response relationship.

3. Results

No significant differences in AChE activity (dA/min) were found with phosphate buffer (data not shown) or DMSO over time; the absolute mean(±S.D.) dA/min for DMSO control groups were 0.0050(0.0011), 0.0054(0.0003) and 0.0056(0.0006) for frontal cortex, 0.0040(0.0005), 0.0039(0.0006) and 0.0041(0.0003) for hippocampus, and 0.0047(0.0004), 0.0049(0.0004) and 0.0051(0.0005) for striatum at 15, 30 and 60 min, respectively.

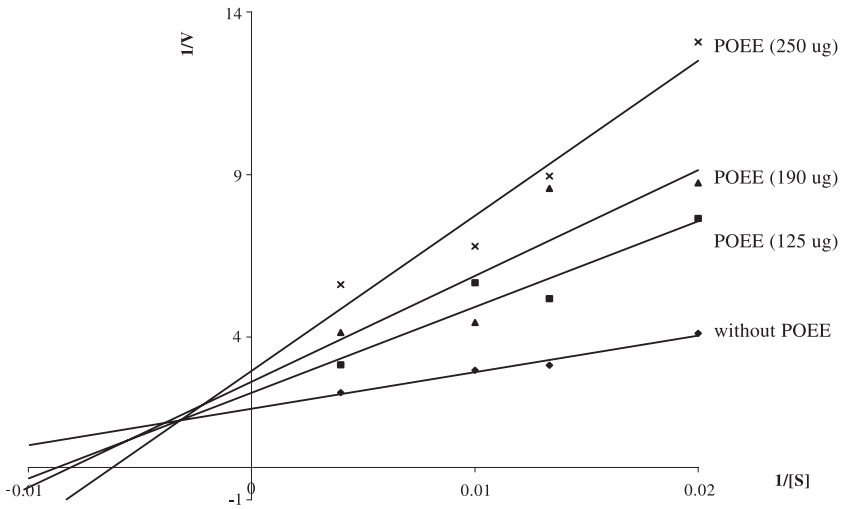

Fig. 2. Lineweaver–Burk plots of AChE activity over a range of substrate concentrations (50 to 250 μM) from striatum samples, in the absence or presence of POEE (125, 190 and 250 μg/ml).

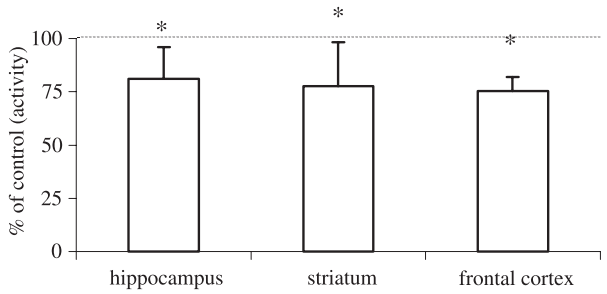

Fig. 3. Effect of acute administration of POEE (100 mg/Kg ip) on AChE activity in mice frontal cortex, hippocampus and striatum. Enzyme activity is expressed as percentage of control (DMSO). Each value represents mean ± S.E.M. ($n = 5$). * $P < .05$, Student's t test for paired (control/test) samples.

POEE significantly inhibited in vitro AChE activity, in a dose- and time-dependent manner in all brain structures studied (Fig. 1). At 60 min of incubation there were clear dose/effect associations, with Pearson coefficients as follows: r^2=.9979 ($P < .0001$) for frontal cortex (Fig. 1A), r^2=.9447 ($P < .001$) for hippocampus (Fig. 1B), and r^2=.9251 ($P < .001$) for striatum (Fig. 1C). Whereas the AChE inhibition seems to be stabilized after 30 min in the cortex, it continues to drop thereafter in both hippocampus and striatum.

Double-reciprocal Lineweaver–Burk plot for rat striatal AChE assays with 60 min of incubation (Fig. 2) indicates that in the presence of increasing POEE concentrations (125, 190 and 250 μg/ml) V_{max} values are reduced (from 0.55 to 0.43, 0.38 and 0.34 μM of acetylthiocholine hydrolyzed/mg/min, respectively) and Km values are increased (from 61.2 to 114.6, 124.5 and 150.8 μM, respectively), a pattern common to competitive/noncompetitive type inhibitors.

Fig. 3 shows the effects of a single POEE administration (100 mg/kg ip) on AChE activity in the frontal cortex, hippocampus and striatum of middle-aged (14 months old) mice. POEE treatment correlates with AChE inhibition in all of the three brain areas studied; decreases in AChE activity were in the range of 25% in the frontal cortex, 20% in the striatum and 15% in the hippocampus.

4. Discussion

It is now widely accepted that hypoactivity of the cholinergic system plays a central role in cognitive deficits (De Lacalle et al., 1996; Quirion et al., 1995; Taylor and Griffith, 1993). Accordingly, despite serious limitations in both efficacy and adverse effects, inhibition of AChE is the current basis for drug therapy in AD (Rogers and Friedhoff, 1996; Sugimoto et al., 1992; Watkins et al., 1994).

We have recently found that *P. olacoides* ethanol extract (POEE) improved long-term memory retrieval in adult mice in the step down inhibitory avoidance paradigm, in a dose-dependent way (significant improvement with 50 and 100 mg/kg), affecting neither memory consolidation nor task acquisition. Moreover, POEE 100 mg/kg acute ip was found to reverse the memory retrieval deficit of aged (14 months) mice in the same inhibitory avoidance paradigm (da Silva et al., 2002b). In the present study we report that POEE also has the ability to inhibit AChE as evaluated by in vitro and ex vivo assays, suggesting that improvement in cholinergic function is a neurochemical correlate of the behavioral results. Although a dose–effect analysis is desirable, a significant inhibition of AChE was observed ex vivo with mice treated with the same dose found to facilitate memory retrieval in adult and aged mice (da Silva et al., 2002b). It is noteworthy that the oral administration of huperzine A, a lycopodium alkaloid obtained from the Chinese medicinal herb *Huperzia serrata*, also results in various degrees of AChE activity inhibition in rat cortex, hypothalamus, hippocampus and striatum (Cheng and Tang, 1998; Cheng et al., 1996; Mckinney et al., 1991).

AChE activity was found to be inhibited in rat cortex, hippocampus and striatum homogenates incubated in vitro with POEE (125–250 μg/ml); a significant (15–25%) decrease in AChE activity was also observed in vivo in these memory relevant brain structures as evaluated 2 h after a single POEE (100 mg/kg ip) administration to middle-aged (14 months) mice. Although further experiments are needed for a definitive conclusion, POEE-induced AChE inhibition seems to be dose- and time-dependent, and more effective in hippocampus and striatum than frontal cortex. Differential effects may be related to the uneven existence of several molecular AChE forms on particular tissues or brain regions, favoring interactions with particular types of AChE (Bisso et al., 1991).

Neither the active compound(s) nor the mechanism(s) by which POEE inhibited AChE can be concluded at this point. Despite continuous reappearance of old data (Peckolt, 1901) at current literature (Duke, 1992), tertiary alkaloids could not be detected in the roots, even using specific extraction procedure for this class of compounds. Although Peckolt (1901) himself did not assert that the crystals (latter recognized as long chain esters of beta-sitosterol and lupeol) he isolated and named "muyrapuamin" were alkaloids, the description of his isolation methods may have been misinterpreted originating this confusion. In view of the in vitro assay here used, several inhibition mechanisms possibilities have to be considered (Cohen et al., 1991): active ingredient(s) from POEE could combine with free AChE, with AChE–ASCh complexes or with acylated AChE. The competitive/noncompetitive mode of inhibition, as suggested by the Lineweaver–Burk plot, indicates that active compound(s) is (are) able to interact with both the enzyme's active center and the enzyme–substrate complex. Interestingly enough, tacrine and donepezil currently used for the symptomatic treatment of AD are also of the mixed competitive/noncompetitive type (Snape et al., 1999).

It has been reported that chronic treatment with *N-tert*-butyl-alpha-phenylnitrone (PBN) improves cognitive per-

formance of aged rats in the Morris water maze paradigm, an effect attributed to the neuroprotective action of PBN associated with its free radical scavenging activity (Carney et al., 1991). Interestingly enough, it was recently found that PBN also has anticholinesterase activity (Milatovic et al., 2000), a property unrelated to its free radicals scavenging action. Relevant to this discussion, previous studies showed that POEE has a marked scavenging activity against several free radicals (Siqueira et al., 2002). Moreover, a single administration of POEE reduced free radical generation as well as lipid peroxidation and protein-bound carbonyl levels in several brain regions of middle-aged mice (Siqueira et al., submitted for publication).

In conclusion, our study reveals a dose-dependent competitive/noncompetitive inhibitory effect of POEE on AChE activity of brain areas relevant to cognitive functions. Adding to previously reported data, this study further indicates that *P. olacoides* possesses multiple modes of action that are relevant for cognitive disorders and consistent with its traditional local use.

Acknowledgements

We gratefully acknowledge financial support received from FINEP/PRONEX, FAPERGS, CAPES, CNPq and PROPESQ-UFRGS. There is a patent request (PI0205432-9, INPI, Brazil) associated with this study.

References

Abelson PH. Medicine from plants. Science 1990;247:513.

Andres C, Beeri R, Huberman T, Shani M, Soreq H. Cholinergic drug resistance and impaired spatial learning in transgenic mice overexpressing human brain acetylcholinesterase. Prog Brain Res 1996;109: 265–72.

Auterhoff H, Momberger B. Der lipophile Inhaltsstoffe von Muira puama. Archiv Pharm Bericht 1971;304:223–8.

Beeri R, Andres C, Lev-Lehman E, Timberg R, Huberman T, Shani M, et al. Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. Curr Biol 1995;5:1063–71.

Bisso GM, Briancesco R, Michalek H. Size and charge isomers of acetylcholinesterase in the cerebral cortex of young and aged rats. Neurochem Res 1991;16:571–5.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

Carney JM, Starke-Reed PE, Oliver CN, Landum RW, Cheng MS, Wu JF, et al. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N-tert*-butyl-α-phenylnitrone. Proc Natl Acad Sci U S A 1991;88:3633–6.

Cheng DC, Tang XC. Comparative studies of huperzine A, E2020, and tacrine on behavior and cholinesterase activities. Pharmacol Biochem Behav 1998;60:377–86.

Cheng HC, Ren RG, Tang XC, Huperzine A. A novel promising acetylcholinesterase inhibitor. NeuroReport 1996;8:97–101.

Cohen SH, Chishti SB, Bell DA, Howard SI, Salih E, Cohen JB. General occurrence of binding to acetylcholinesterase-substrate complex in non-competitive inhibition and in inhibition by substrate. Biochim Biophys Acta 1991;1076:112–22.

Cragg GM, Newman DJ. Natural product drug discovery in the next millennium. Pharm Biol 2001;39:8–17.

Cummings JL, Back C. The cholinergic hypothesis of neuropsychiatric symptoms in Alzheimer's disease. Am J Geriatr Psychiatry 1998;6: S64–78.

da Silva AL, Bardini S, Nunes DS, Elisabetsky E. Anxiogenic properties of *Ptychopetalum olacoides* Benth.(Marapuama). PTR, Phytother Res 2002a;16:223–6.

da Silva AL, Bardini S, Netto CA, Nunes DS, Elisabetsky E. Facilitating effects of *Ptychopetalum olacoides* Bentham (Marapuama) on memory retrieval in young and old mice. Braz J Med Biol Res 2002b [submitted for publication].

De Lacalle S, Cooper JD, Svendsen CN, Dunnett SB, Sofroniew MV. Reduced retrograde labelling with fluorescent tracer accompanies neuronal atrophy of basal forebrain cholinergic neurons in aged rats. Neuroscience 1996;75:19–27.

Duke J. Handbook of phytochemical constituents of GRAS herbs and other economic plants. Boca Raton, FL: CRC Press; 1992. p. 497.

Elisabetsky E. From indigenous disease concepts to laboratory work hypothesis: the case of "nerve tonics" from the Brazilian Amazon. International Foundation for Science, Provisional Report Series, vol. 19. Stockholm, Sweden: IFS; 1987. p. S-11438.

Elisabetsky E, Siqueira IR. Is there a psychopharmacological meaning for traditional tonics? In: Prendergast HD, Etkin N, Harris DR, Houghton PJ, editors. Plants for food and medicine. Kew, London: Royal Botanic Gardens; 1998. p. 373–85.

Ellman GL, Courtney D, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

Grenand P, Moretti C, Jacquemin H. Pharmacopées traditionnelles en Guyane. Editions de L'Orstom, Paris; 1987. p. 326–8.

Mckinney MA, Miller JH, Yamada F, Tuckmantal W, Kozikowski AP. Potencies and stereoselectivities of enantiomers of huperzine A for inhibition of rat cortical acetylcholinesterase. Eur J Pharmacol 1991; 203:303–5.

Milatovic D, Radic Z, Zivin M, Dettbam WD. Atypical effect of some spin trapping agents: reversible inhibition of acetylcholinesterase. Free Radic Biol Med 2000;28:597–603.

Nordberg A, Svensson AL. Cholinesterase inhibitors in the treatment of Alzheimer's disease: a comparison of tolerability and pharmacology. Drug Safety 1998;19:465–80.

Park CH, Kim SH, Choi W, Lee YJ, Kim JS, Kang SS, et al. Novel anticholinesterase and antiamnesic activities of dehydroevodiamine, a constituent of *Evodia rutaecarpa*. Planta Med 1996;62:405–9.

Peckolt T. Heil und Nutzpflanzen Brasiliens. Ber Dtsch Pharm Ges 1901; 11:40–7.

Quirion R, Wilson A, Rowe W, Aubert I, Richard J, Doods H, et al. Facilitation of acetylcholine release and cognitive performance by an M_2 muscarinic receptor antagonist in aged memory-impaired rats. J Neurosci 1995;15:1455–62.

Rogers SL, Friedhoff LT. The efficacy and safety of donepezil in patients with Alzheimer's disease: results of a US multicenter, randomized, double-blind, placebo-controlled trial. Dementia 1996;7:293–303.

Siqueira IR, Lara DR, Gaieski F, Silva FS, Nunes DS, Elisabetsky E. Psychopharmacological properties of *Ptychopetalum olacoides* (Olacaceae). Pharm Biol 1998;36:327–34.

Siqueira IR, Cordova CAS, Creczynski-Pasa TB, Elisabetsky E, Netto CA. Antioxidant action of an ethanol extract of *Ptychopetalum olacoides*. Pharmac Biol 2002;40:374–9.

Siqueira IR, Fochesatto C, Torres ILS, da Silva AL, Nunes DS, Elisabetsky E, Netto CA. Antioxidant activities of the ethanol extract from *Ptychopetalum olacoides* in mice brain. Pharmacol Biochem Behav 2003 [submitted for publication].

Snape MF, Misra A, Murray TK, de Souza RJ, Williams JL, Cross AJ, et al. A comparative study in rats of the in vitro and in vivo pharmacology of

the acetylcolinesterase inhibitors tacrine, donepezil and NXX-066. Neuropharmacology 1999;38:181–93.

Steinmetz EF. Muira puama (''potency wood''). Q J Crude Drug Res 1962;2:229–32.

Sugimoto H, Tsuchiya Y, Sugumi H, Higurashi K, Karibe N, Iimura Y, et al. Synthesis and structure–activity relationships of acetylcholinesterase inhibitors: 1-benzyl-4-(2-phthalimidoethyl)piperidine and related derivatives. J Med Chem 1992;35:4542–8.

Taylor L, Griffith WH. Age-related decline in cholinergic synaptic transmission in hippocampus. Neurobiol Aging 1993;14:509–15.

Vickers JC, Dickson TC, Adlard PA, Saunders HL, King CE, McCormack G. The cause of neuronal degeneration in Alzheimer's disease. Prog Neurobiol 2000;60:139–65.

Watkins PB, Zimmerman HJ, Knapp MJ, Gracon SI, Lewis KW. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. JAMA 1994;271:992–8.

Yoshida S, Suzuki N. Antiamnesic and cholinomimetic side-effects of the cholinesterase inhibitors, physostigmine, tacrine and NIK-247 in rats. Eur J Pharmacol 1993;250:117–24.