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Geissospermum vellosii stembark
Anticholinesterase activity and improvement of scopolamine-induced memory deficits

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ABSTRACT

This study evaluated the cholinesterase inhibitory activity of an alkaloid-rich fraction of stembark from *Geissospermum vellosii* (PP), and its effect on memory tests in mice. PP inhibited rat brain and electric eel acetylcholinesterase, as well as horse serum butyrylcholinesterase in a concentration-dependent manner with mean IC_{50} values of 39.3 μg/mL, 2.9 μg/mL, and 1.6 μg/mL, respectively. The main alkaloid with anticholinesterase activity in PP was isolated and identified as geissospermine. PP significantly reduced scopolamine-induced amnesia in the passive avoidance and Morris water maze tests, at 30 mg/kg i.p. (given 45 min before the test sessions). At the highest effective dose (60 mg/kg), administration of PP did not result in noticeable peripheral or central cholinergic side effects. Only after administration of 200 mg/kg, mice showed convulsions affecting the whole body followed by death. These results show that compounds present in *G. vellosii* stembark have anticholinesterase activity, and that they can revert cognitive deficits in a model of cholinergic hypofunction.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, with important memory deficits as common early symptoms. Pharmacological and neurochemical evidences have correlated the severity of memory impairment with the degree of cholinergic hypofunction (Terry and Buccafusco, 2003). Therefore, centrally-acting cholinesterase inhibitors, which can effectively increase brain acetylcholine levels, alleviate symptoms and delay hospitalization, are the most prescribed pharmacological agents in the treatment of AD (Lleo et al., 2006). However, the occurrence of peripheral cholinergic side effects and hepatotoxicity with the use of some of these agents has limited their prescription, and there is still a need for the development of more potent agents, either natural products or synthetic analogues, with minimum side effects for the treatment of AD.

The growing involvement of alkaloids in the treatment of AD had its start with the indole alkaloid physostigmine, the prototype acetylcholinesterase (AChE) inhibitor, whose use has stimulated the synthesis of compounds with an improved clinical profile, like rivastigmine (Houghton et al., 2006). There are plants rich in alkaloids whose extracts or isolated compounds show inhibitory activity for AChE, butyrylcholinesterase (BChE) or both enzymes, including *Huperzia serrata* (Skolnick, 1997; Badia et al., 1998), *Stephania tetraanda* (Ogino et al., 1997; van Beek et al., 1984), *Stephania venosa* (Ingkaninan et al., 2001), *Narcissus confusus* and *N. perez-chiscanol* (López et al., 2002), *Stephania suberosa* and *Tabernaemontana divaricata* (Ingkaninan et al., 2003; Chattipakorn et al., 2006; Ingkaninan et al., 2006), *Chimarrhis turbinata* (Cardoso et al., 2004), *Peschiera australis* (Andrade et al., 2005), *Evodia rutaecarpa* (Park et al., 1996), and *Senna spectabilis* (sin. *Cassia spectabilis*, Leguminosae) (Viegas et al., 2005).

Geissospermum vellosii Fr. All [syn: *Geissospermum laeve* (Vell) Miers; *Geissospermum laeve* (Vell) Baillon; *Tabernaemontana laevis* (Vell)]; (Family: Apocynaceae) is a native tree of Brazil found primarily in the Northeast, South and Southeast regions (Pio Correa, 1984). Commonly this tree is named Pao pereira, Pao-de-pente, Pao-forquilha, Pereiroá, Ubá-açú, Triguaaba, Camará-de-bilro, Camará-do-

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mato, Canudo amargoso, or Pinguaciba (Pio Correa, 1984). Pao pereira stembarks are popularly used to treat malaria, poor digestion, constipation, dizziness, and as a febrifuge and "tonic" or "stimulant" (dos Santos et al., 1998). This tree, rich in indolic alkaloids, has been included by Gustavo Peckolt among the ten most useful medicinal Brazilian trees (Peckolt, 1942; dos Santos et al., 1998). Because of its properties, this plant was an object of extensive research interest at least since 1838, from both the chemical and pharmacological standpoints. The alkaloids of Pao pereira present various actions. For example, geissospermine, the most abundant alkaloid, has actions at the autonomic nervous system (Aurousseau, 1961a,b), whereas flavopereirine and its analogues have been proposed as useful drugs to treat HIV infection (Beljanski, 1994). In the last five decades, the activity of Pao pereira extracts has been linked to its observed curare-like activity (Rapoport et al., 1958).

The aim of this study was to evaluate the anticholinesterase activity of Pao pereira alkaloids in vitro and their memory-enhancing properties in vivo. We have initiated the prospection of stembarks to obtain fractions containing alkaloids and evaluated them for possible inhibitory activity of AChE and BChE, as well as for behavioral effects in mice against amnesia induced by scopolamine in two models, the Morris water maze and passive avoidance.

2. Methods

2.1. Extraction and isolation

Stembarks of *G. vellosii* were dried at room temperature (30–35 °C). The dried material was ground, macerated with 95% ethanol six times (2 days each, during 12 days) and dried by evaporating the ethanol extracts (2 days) under reduced pressure. The yield of the ethanolic extracts from stembarks was 3.6% of dried materials. Water was added to the dried material and the mixture was deffated with hexane (1:5). The deffated aqueous extract was extracted with chloroform ($CHCl_3$) at different pH values (3.0; 5.0; 7.0; 9.0 and 12.0). Fractions were eluted in thin layer chromatography (TLC) and revealed with Dragendorff's reagent. Fractions at pH values of 7.0, 9.0 and 12.0 presented orange colored points, characteristic of alkaloids. A TLC procedure for cholinesterase inhibitors was used for a preliminary bioguided selection of these fractions (see below).

2.2. Cholinesterase inhibition assays

2.2.1. TLC bioautographic assay

Cholinesterase inhibitory activity was detected by a TLC bioautographic assay (Rhee et al., 2001). Pao pereira fractions obtained at pH 7.0, 9.0 and 12.0 were dissolved in methanol (MeOH) to a concentration of 5 mg/mL. Then 2 µL of each fraction was spotted on the silica gel TLC plate and developed in the solvent $CHCl_3$–MeOH 75:25. Two microliters of physostigmine 24 µM in MeOH were also spotted as a reference compound, used as a positive control. After developing, the TLC plate was sprayed with DTNB/acetylthiocholine or DTNB/butyrylthiocholine reagent (1:1 solutions of 2 mM in Tris–HCl buffer) until saturation of the silica. The plate was allowed to dry for 5 min and then AChE or BChE solution (3 U/mL in Tris–HCl buffer) was sprayed. After a while, a yellow background appeared, with white spots showing the inhibitory compounds. The fractions deemed active did not interfere with the thiocholine–DTNB reaction, as determined in control assays for false positive AChE and BChE inhibition (Rhee et al., 2003).

2.2.2. Microplate assay

The assay for measuring cholinesterase activity was modified from that described by Ellman et al. (1961). Briefly, brain tissue from adult Wistar rats was homogenized at 8% w/v in 0.1 M sodium phosphate buffer (pH 7.4), with added NaCl 0.9% (w/v) and Triton X-100 0.05% v/v. Cholinesterase activity was examined in 96-well microplates by adding 5 µL of rat brain homogenate 80 mg/mL (or AChE 2.5 U/mL, or BChE 2.5 U/mL in phosphate buffer), 5 µL of DTNB 0.01 M, and 185 µL of phosphate buffer (or dimethyl sulfoxide [DMSO] 1% or tacrine 10 µM, in phosphate buffer) or 185 µL of sample dissolved in buffer containing no more than 1% DMSO. The mixture was incubated for 10 min at 21–23 °C. Following incubation, 5 µL of substrate (acetylthiocholine 0.02 M or butyrylthiocholine 0.04 M, in deionized water) was added. The microplate was then immediately read at 412 nm for 5 min by a SpectraMax 250 microplate reader (Molecular Devices). In every experiment, cholinesterase-independent (nonspecific) substrate hydrolysis was determined by including one experimental group treated with tacrine 10 µM. The maximal velocities of reaction (V_{max}) were determined in two or three replicates per condition; these were averaged and expressed as percent activity relative to control (solvent) after subtracting the rate of nonspecific hydrolysis. The selected sample fraction was tested in five to six concentrations (0.0003–1.0 mg/mL). Only the concentration of 1.0 mg/mL was prepared in DMSO 1%, being diluted to lower concentrations with phosphate buffer. Acetylcholinesterase (AChE) type III, from electric eel 1.0 mg protein/mL, aqueous solution, 1.070 U/mg protein, butyrylcholinesterase (BChE) from horse serum 13 U/mg solid, 20 U/mg protein, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), Tris (hydroxy methyl amino methane), acetylthiocholine iodide, butyrylthiocholine iodide, physostigmine (eserine), and DMSO were all obtained from Sigma.

2.3. Behavioral experiments

2.3.1. Animals

Male Swiss albino mice (weighting 25–35 g) were kept in the experimental room for one week prior to testing, and had free access to food and water. Animals were housed 3–4 per cage in acclimatized conditions (21 ± 2 °C) and maintained on a 12/12 h light/dark cycle with lights on at 6:00 am. The behavioral tests were performed in the same room and all experiments were conducted during the daylight period. Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (U. S. National Institutes of Health).

2.3.2. Passive avoidance

Methods were a modification of those outlined by Cumin et al. (1982). The apparatus consisted of a box (20 × 20 cm) with a wooden platform (4 × 4 × 4 cm) and a grid floor connected to a stimulator. Mice were submitted to an acclimation session where they were individually transferred from plastic cages to the passive avoidance apparatus, which they could freely explore during 10 s, being immediately removed to the cage afterwards. After 24 h (training trial), animals were injected i.p. with vehicle (DMSO 1% in saline; 0.01 mL/g), or PP (10, 30 or 60 mg/kg), and after 15 min with saline or scopolamine (1 mg/kg; Sigma). Thirty minutes later, mice were individually placed on the wooden platform, and as soon as they touched the grid floor with the four paws, received a punishing electrical shock (0.6 mA/3 s). Animals showing a step-down latency above 15 s in the training session were excluded from the experiment. The step-down latency was measured in the training trial and after 24 h (retention trial), and no shock was applied in the latter session (maximum of 120 s).

2.3.3. Water maze

Maze was a white circular pool (180 cm in diameter, 50 cm height) filled with a mixture of water and white ink to a depth of approximately 25 cm. Eight cue cards with different colors and shapes were placed on the internal wall. The pool was divided into four equal quadrants and a platform (8 cm in diameter) was submerged 0.5 below the opaque surface. Mice were injected i.p. with vehicle (DMSO 1% in saline; 0.01 mL/g), tacrine (10 mg/kg) or PP

(10 or 30 mg/kg in DMSO 1%) and after 15 min, saline or scopolamine i.p. (1 mg/kg). Thirty minutes after the second i.p. injection, mice were placed by hand into the water facing the wall of the pool, and were allowed 120 s to find the hidden platform. When successful, the mouse was given 15 s on the platform to watch the spatial cues. If the animal failed to find the platform in this time, it was placed on the platform for 15 s. Animals had 5 trials per day (separated by 10 min), during five consecutive days. In each trial the animal was released from a different start point in the pool, but the escape platform was kept on the same position (at the center of the 4th quadrant).

2.3.4. Locomotor activity, cholinergic side effects and toxicity

Motor impairment was evaluated in a protocol based on Fujimori and Cobb (1965), with an apparatus that consisted of a base platform on a rotating rod of 3-cm diameter with a non-skid surface. The rod was placed at a height of 15 cm from the base (Rota-Rod instrument; AVS, Brazil). In each trial the mouse was placed on the rotating axis, which then accelerated up to 10 rpm in 3 min. Mice were trained during 7 days, 6 trials per day at 30-min intervals, and those that could stay on the rod for at least 3 min were selected for the experiment. In the test session, PP or vehicle was administered i.p. and the time to fall was recorded in 6 trials at 30-min intervals. After 5 min on the rotating rod, the animals returned to the cage. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 180 s (10 rpm). During the intervals of the test session in the rota-rod, exploratory activity was evaluated in the open-field test, which consisted of recording the number of line crossings for 3 min, in a $15 \times 30 \times 38$ cm wooden box with a 5-cm square grid marked on the floor. The concentration of PP administered was 30 mg/kg, based on its ameliorating effect on the amnesia induced by scopolamine.

Acute toxicity of the PP extract was evaluated during 24 h after a single injection (1–300 mg/kg i.p.), in dose groups of 3 or 4 mice, including a vehicle control (DMSO 1% in saline). Mice were observed for lacrimation, salivation, diarrhea, tremor, locomotion and mortality.

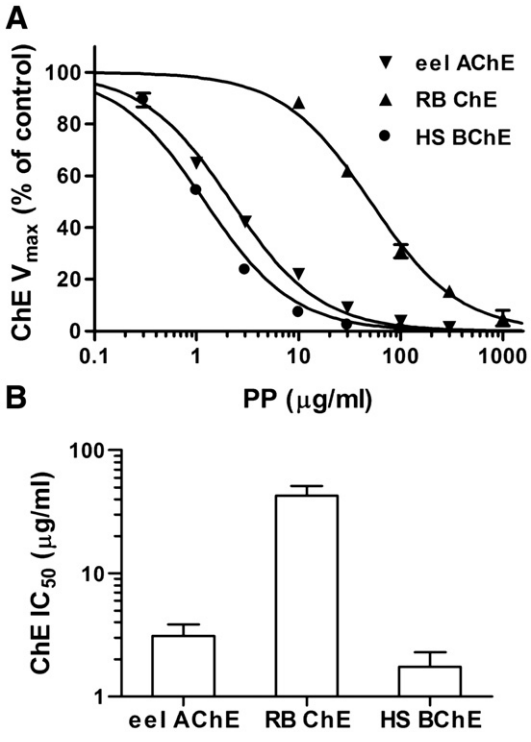

Fig. 1. Cholinesterase inhibition by PP. Concentration–response curves (A) were obtained in three enzyme preparations: *E. electricus* (eel) AChE, rat brain (RB) ChE, and horse serum (HS) BChE. Each curve represents means ± S.D. from a representative experiment with assays in triplicate, together with the regression line. The IC_{50} obtained from independent experiments is summarized in graph B (mean ± S.E.M., $n = 3$–4 complete curves).

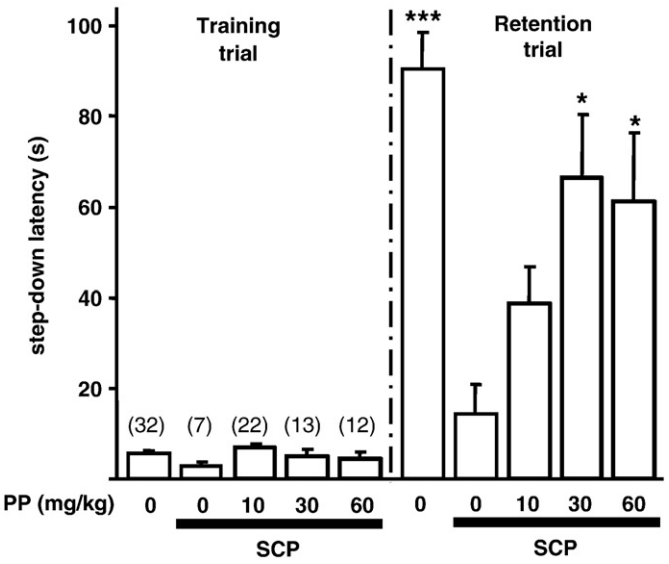

Fig. 2. Effects of PP on scopolamine-induced amnesia in the step-down passive avoidance test in mice. In order to induce amnesia, mice were treated with scopolamine (SCP; 1 mg/kg, i.p.) 30 min prior to the training (left panel) and retention trial (right panel). Vehicle or PP (10, 30 or 60 mg/kg) was administered i.p. 15 min before scopolamine. Data are expressed as means ± S.E.M. The number of mice in each group is indicated in parentheses. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, as compared with scopolamine-treated control group (C + SCP), Mann–Whitney *U* one-tailed test.

2.4. Statistical analysis

For the concentration–inhibition curves of ChE activity, the IC_{50} was determined by nonlinear regression based on a single-site model described by the expression $V_{max}\ \% = 100 / (1 + [PP] / IC_{50})$. Results are reported as geometric means and 95% confidence intervals of the IC_{50} obtained independently from different animals or different preparations of purified enzyme. The behavioral tests were analyzed with ANOVA on ranks, followed by Mann–Whitney rank sum test for particular inter-group comparisons.

3. Results

3.1. Cholinesterase inhibitory activity of G. vellosii stembark

Pao pereira fractions at pH 7 and 9 showed one spot of inhibition of AChE activity in Ellman's TLC enzyme assay. The spots were similar in retention factor (Rf = 0.67) and were revealed with Dragendorff's reagent, indicating that the substances with inhibitory activity in the two fractions could be the same and were alkaloids (data not shown). Although Dragendorff-positive spots with similar Rf were seen in fractions of pH 5 and 12, these fractions, in the concentrations administered, were not effective in inhibiting AChE. The fraction obtained at pH 7, which we named "PP", was available in larger mass and was chosen for the pharmacological studies. A sample of PP was further separated by chromatography in neutral alumina and eluted with a hexane–ethyl acetate–methanol system. Among the 14 subfractions collected, the one in 100% ethyl acetate yielded colorless crystals. This crystalline sample showed the same Rf as the active constituent of PP and inhibited both AChE and BChE in the bioautographic TLC assay. The crystals were then analyzed by ^{13}C, ^{1}H and bidimensional NMR spectroscopy, allowing the unequivocal identification as the previously described indole alkaloid geissospermine (Goutarel et al., 1978).

Concentration–inhibition curves of AChE were obtained for the PP in rat brain homogenates and in enzyme purified from electric eel. To establish enzyme selectivity, the inhibitory activity on horse serum BChE was also assayed by the same method. Fig. 1A shows the data from representative experiments for electric eel and rat brain AChE as well as horse serum BChE. In Fig. 1B, the inhibitory potency of PP in the different enzyme preparations can be compared. The IC_{50} of PP for inhibiting AChEs was 39. 3 μg/mL (17.7–86.9 μg/mL; $n=4$) and 2.9 μg/mL (1.1–7.8 μg/mL; $n=3$) in rat brain and electric eel, respectively. The IC_{50} of PP for inhibiting horse serum BChE was 1.6 μg/mL (0.5–5. 5 μg/mL; $n=3$).

3.1.1. Effect of PP on passive avoidance

After 30 min of a single i.p. injection of scopolamine (1 mg/kg), mice reduced the step-down latency from 90.4 ± 7.9 s to 16.7 ± 6.4 s. This deficit in passive avoidance learning and behavior was significantly reduced by pretreatment with PP. Mice injected with PP 30 and 60 mg/kg 45 min before the training trial showed step-down latencies of 66.4 ± 14.0 and 61.2 ± 15.1 s, respectively ($P<0.05$; Fig. 2).

3.1.2. Effect of PP on spatial memory

Fig. 3 illustrates swim paths of mice in the fourth trial of the second and fifth days of tests in the water maze. Mice tended to explore all four quadrants of the pool and the area close to the wall in the first and second days. Thereafter, they changed this search strategy, and on the fifth day vehicle-treated control mice swam in the direction of the hidden platform. However, scopolamine-treated mice took longer swimming paths, spending nearly the same time in different quadrants of the pool. Vehicle-treated mice showed a marked reduction in escape latencies from 86.2 ± 5.4 s to 35.4 ± 6.4 s, in the first and fifth days, respectively. Escape latencies remained essentially unchanged throughout the 5-day testing period in mice given scopolamine (1 mg/kg). Thus, at day 5, scopolamine-treated mice spent 103.5 ± 8.3 s to reach the hidden platform, indicating impairment of spatial memory. Mice pretreated with PP 30 mg/kg had an escape latency of 66.4 ± 11.2 s on the fifth day, showing a significant effect against the amnesia induced by scopolamine ($P<0.05$; Fig. 3B). The effect of PP (30 mg/kg) was similar to that of tacrine (10 mg/kg), which led to an escape latency of 59.4 ± 13.7 s on day 5.

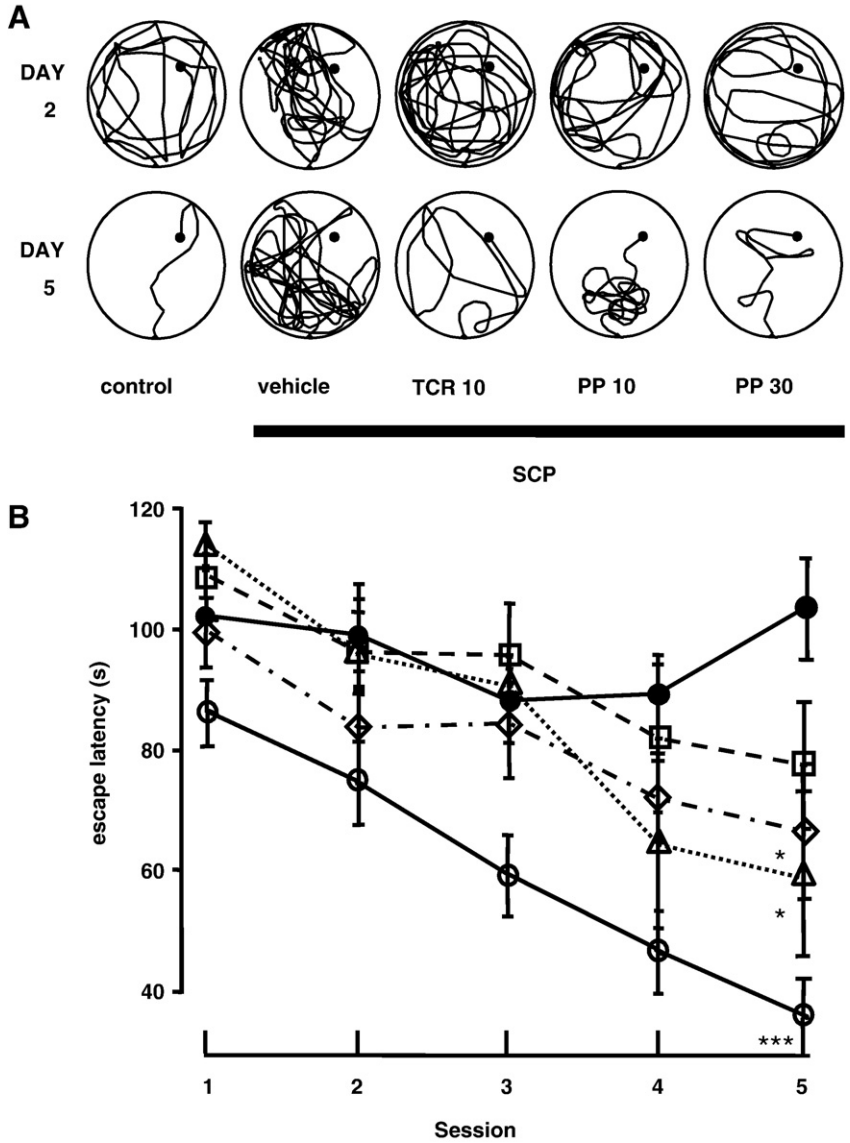

Fig. 3. A: Representative search strategy of mice in the Morris water maze in the fourth trial on the second and fifth days. Traces show the swim path of mice injected i.p. with vehicle ($n=21$), scopolamine (SCP 1 mg/kg) ($n=9$), tacrine (TCR, 10 mg/kg) + SCP ($n=6$), and PP (10 and 30 mg/kg) + SCP ($n=6$ and $n=7$, respectively). B: PP administered at 30 mg/kg i.p. reduced scopolamine-induced amnesia. Effects of vehicle (○) and scopolamine 1 mg/kg (●), tacrine (10 mg/kg; ◊) and PP (10 and 30 mg/kg; □ and Δ, respectively) on the escape latency (s) in the Morris water maze. Asterisks represent significant differences between groups and controls given vehicle and 1 mg/kg i.p. of scopolamine on the fifth day. The number of mice in each group is indicated in parentheses. Mann–Whitney *U* one-tailed test: $^{*}P<0.05$ and $^{***}P<0.001$.

Table 1
Effect of PP on central cholinergic side effects and lethality.

Group (treatment)	Tremor/micromovements[a]	Death (%)
Vehicle	0	0
Tacrine 50 mg/kg[b]	+++	100
PP 60 mg/kg	0	0
PP 100 mg/kg	+	0
PP 200 mg/kg	+++	100
PP 300 mg/kg	+++	100

[a]Data represent observations of the intensity of micromovements: 0, no tremor, +, tremors affecting the head; +++, generalized convulsion followed by death. Vehicle, tacrine and PP were administered intraperitoneally and mice ($n=4$/group) observed for 24 h.
[b]Data from Castro et al. (2008).

3.1.3. Effect on locomotor activity, cholinergic side effects and toxicity of PP

When administered at the most active dose found in the memory tests (30 mg/kg), PP did not increase the number of falls from the rotating rod in comparison with vehicle-treated mice, showing an average of 2 falls in 180 s at 10 rpm. Moreover, PP did not reduce the exploratory activity of mice in the open-field test. Treated mice showed 54 ± 16 line crossings in 3 min, which is slightly greater than vehicle-treated animals, which showed 33 ± 7 crossings. However, further experiments are necessary to elucidate if PP induces hyperactivity in mice.

Administration of PP from 1 to 30 mg/kg did not result in detectable peripheral or central cholinergic side effects. At the dose of 60 mg/kg, it was possible to observe some degree of abdominal discomfort, but no central cholinergic side effect, such as tremor (Table 1). However, at the dose of 100 mg/kg, mice showed mild tremor restricted to the head which disappeared after a few minutes, but no animal died up to 24 h of observation. After the administration of 200 mg/kg, mice showed convulsions affecting the whole body followed by death after 30 min of the injection. At the dose of 300 mg/kg, mice died after a maximum of 10 min of the administration. Table 1 compares these results with those previously obtained for tacrine at 40 and 50 mg/kg, which induced tremor followed convulsions affecting the whole body, and death in 50% and 100% of animals, respectively.

4. Discussion

Pao pereira fraction at pH 7 (PP) showed inhibitory effects on both AChEs and in BChE in a concentration-dependent manner. The IC_{50} value for electric eel AChE was about 15 times lower than that of rat brain AChE. It is known that there is considerable interspecies variability in the sensitivity of AChE to anticholinesterase compounds (Silver, 1974; Vigny et al., 1978). For example, tacrine, the first drug used in the treatment of AD, and (−)-huperzine A, a selective inhibitor of AChE, are less active toward human than bovine AChE (Camps et al., 2000). Atack et al. (1989), comparing the sensitivity of human and electric eel AChE to physostigmine and its analogues also observed different inhibitory potencies between species. Another possible explanation for the lower potency in rat brain could be the partition into lipids and protein binding of PP, which would lead to a lower free fraction of the active compound(s) in the tissue homogenate suspension, compared to the purified eel AChE solution.

PP was approximately 25 times more potent against BChE than rat brain AChE, but only 1.8 times more potent against eel AChE. Notwithstanding the differences in species and preparations, these data indicate that PP inhibited both cholinesterases, with a slight selectivity towards BChE. It has recently been established that BChE has an important function in the constitutive hydrolysis of acetylcholine in the central nervous system, where it could play a more extensive role in normal cholinergic transmission (Mesulam et al., 2002). It has been shown that in patients with AD the level of AChE activity declines and the activity of BChE increases. The ratio between BChE and AChE can change from 0.6 in the normal brain to as high as 11 in cortical areas affected by the disease (Greig et al., 2002).

Numerous plants and their constituents generally reputed to enhance cognitive function in traditional practices of medicine have been evaluated on pharmacological targets relevant to AD. Some of them have shown significant inhibition of ChEs. López et al. (2002) have evaluated different extracts of *Narcissus* (Amaryllidaceae) bulbs for inhibition of electric eel AChE and the most effective was *N. confusus* ($IC_{50}=53.8$ μg/mL). Ingkaninan et al. (2003) demonstrated that methanolic extracts from roots of *T. divaricata* (Apocynaceae) and *S. suberosa* (Menispermaceae) inhibited electric eel AChE activity by 93.5 and 91. 9%, respectively, at 0.1 mg/mL. Recently the same group reported IC_{50} values for *T. divaricata* extract of 2.6 μg/mL in electric eel AChE and 76.9 μg/mL in horse serum BChE, and also identified bisindole alkaloids that may be responsible for this activity (Chattipakorn et al., 2006; Ingkaninan et al., 2006). Adsersen et al. (2006) found that *Corydalis* (Papaveraceae) methanolic extracts of tubers (0.1 mg/mL) showed marked inhibitory effect on electric eel AChE (92–97%) and were positive in the Dragendorff test for alkaloids after TLC. Likewise, other reports of the screening of plant extracts indicate that those containing alkaloids are more likely to be active in cholinesterase assays (Trevisan and Macedo, 2003; Orhan et al., 2004; Houghton et al., 2006). Based on the TLC assay, at least one alkaloid, geissospermine, may account for the observed anticholinesterase activity of PP. Indeed, anticholinesterase activity of isolated alkaloids from *G. vellosii* has been reported in abstract form (Tanae et al., 2006). Considering the relatively high potency of PP compared to other extracts tested in similar assays (eel AChE), the active constituents must be potent inhibitors of the enzyme, possibly with IC_{50} in the submicromolar range.

The Pao pereira fraction at pH 7 (PP) showed a dose-dependent reversal of cognitive deficits induced by scopolamine in mice, but its effects were reduced at higher doses. This reduced effect was not due to motor impairments because PP had no effect on the number of line crossings in the open-field test and in motor coordination assessed in the rota-rod test. Moreover, this inverted U-shape curve has been observed elsewhere with anticholinesterase agents such as physostigmine, tacrine and metriphonate (Braida et al., 1996). It is possible that PP at higher doses leads to excessive stimulation of pre-synaptic muscarinic M_2 receptors, resulting in reduced release of acetylcholine. Indeed, it has been shown that tacrine reduces the release of ACh, and this effect is antagonized by a selective antagonist of M_2 receptors, AF-DX 116 (Svensson et al., 1996).

PP significantly decreased scopolamine-induced amnesia in the passive avoidance and water maze test at 30 mg/kg, which is five and three times, respectively, higher than the effective dose of tacrine in these tests. Similarly, while tacrine in the concentration of 50 mg/kg resulted in convulsion followed by death in 100% of mice, it was necessary 4 times this concentration (200 mg/kg) to produce the same level of lethality. However, tacrine was more effective in inducing diarrhea, lacrimation and salivation. These results suggest that compounds present in the stembark from *G. vellosii* are more selective than tacrine regarding central versus peripheral cholinergic effects.

In conclusion, PP showed a potent anticholinesterase activity in vitro, with slight selectivity for BChE over AChE, and showed memory-enhancing effects in vivo, in a model of cholinergic deficit that has been validated for the development of drugs for the symptomatic treatment of AD. Geissospermine was identified as the main cholinesterase inhibitor in PP, although the presence of other active compounds cannot be excluded. Future studies with isolated constituents from *G. vellosii* are necessary to test whether the anticholinesterase activity underlies the positive cognitive effects of the extract.

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References

Adsersen A, Gauguin B, Gudiksen L, Jäger AK. Screening of plants used in Danish folk medicine to treat memory dysfunction for acetylcholinesterase inhibitory activity. J Ethnopharmacol 2006;104:418–22.

Andrade MT, Lima JA, Pinto AC, Rezende CM, Carvalho MP, Epifanio RA. Indole alkaloids from *Tabernaemontana australis* (Müell. Arg) Miers that inhibit acetylcholinesterase enzyme. Bioorg Med Chem 2005;13:4092–5.

Atack JR, Yu QS, Soncrant TT, Brossi A, Rapoport SI. Comparative inhibitory effects of various physostigmine analogs against acetyl- and butyrylcholinesterases. J Pharmacol Exp Ther 1989;249:194–202.

Aurousseau MM. Etude pharmacodynamique de la geissospermine. Alcaloïde principal du *Geissospermum laeve* (Vellozo) Baillon (Apocynacées). Ann Pharm Fr 1961a;19: 175–89.

Aurousseau MM. Etude comparée de quelques propriétés pharmacodynamiques de la geissospermine et de ses produits d'hydrolyse ou de reduction. Ann Pharm Fr 1961b;19:515–9.

Badia A, Banos JE, Camps P, Contreras J, Gorbig DM, Munoz-Torrero D, Simon M, Vivas NM. Synthesis and evaluation of tacrine–huperzine A hybrids as acetylcholinesterase inhibitors of potential interest for the treatment of Alzheimer's disease. Bioorg Med Chem 1998;6:427–40.

Beljanski M. Flavopereirine-based pharmaceutical composition for treatment of HIV infection. Patent family written in French. PCT Int. Appl.; 1994. 33pp.

Braida D, Paladini E, Griffini P, Lamperti M, Maggi A, Sala M. An inverted U-shaped curve for heptylphysostigmine on radial maze performance in rats: comparison with other cholinesterase inhibitors. Eur J Pharmacol 1996;302:13–20.

Camps P, Achab RE, Morral J, Munöz-Torreno D, Badia A, Banos JE, Vivas NM, Barril X, Orozco M, Luque FJ. New tacrine–huperzine A hybrids (huprines): highly potent tight-binding acetylcholinesterase inhibitors of interest for the treatment of Alzheimer's disease. J Med Chem 2000;43:4657–66.

Cardoso CL, Castro-Gamboa I, Silva DHS, Furlan M, Epifanio RA, Pinto AC, Rezende CM, Lima JA, Bolzani VS. Indole glucoalkaloids from *Chimarrhis turbinata* and their evaluation as antioxidant agents and acetylcholinesterase inhibitors. J Nat Prod 2004;67:1882–5.

Castro NG, Costa RS, Pimentel LS, Danuello A, Romeiro NC, Viegas Jr C, et al. CNS-selective noncompetitive cholinesterase inhibitors derived from the natural piperidine alkaloid (−)-spectaline. Eur J Pharmacol 2008;580:339–49.

Chattipakorn S, Pongpanparadorn A, Pratchayasakul W, Pongchaidacha A, Ingkaninan K, Chattipakorn N. *Tabernaemontana divaricata* extract inhibits neuronal acetylcholinesterase activity in rats. J Ethnopharmacol 2006;110:61–8.

Cumin R, Bandle EF, Gamzu E, Haefely WE. Effects of the novel compound aniracetam (Ro 13-5057) upon impaired learning and memory in rodents. Psychopharmacol (Berl) 1982;78:104–11.

dos Santos NP, Pinto AC, Alencastro RB. Theodoro Peckolt, farmacêutico do Brasil Imperial. Quim Nova 1998;21:666–70.

Ellman GL, Courtney KD, Andres JRV, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase. Biochem Pharmacol 1961;07:88–95.

Fujimori H, Cobb D. Central nervous system depressant activity of MA 1337, 3-[3,4-m-chlorophenyl-1-piperazyl propil-2-4 (1H, 3H) quinozolinedone hydrocholride. J Pharmacol Exp Ther 1965;148:151–7.

Greig NH, Lahiri DK, Sambamurti K. Butyrylcholinesterase: an important new target in Alzheimer's disease therapy. Int Psychogeriatr 2002;14(Suppl 1):77–91.

Goutarel R, País M, Gottlieb HE, Wenkert E. ^{13}C NMR analysis of geissospermine and its indole alkaloid monomer fragments. Tetrahedron Lett 1978;19(14):1235–8.

Houghton PJ, Ren Y, Howes MJ. Acetylcholinesterase inhibitors from plants and fungi. Nat Prod Rep 2006;23:181–99.

Ingkaninan K, Changwijit K, Suwanborirux K. Vobasinyl-iboga bisindole alkaloids, potent acetylcholinesterase inhibitors from *Tabernaemontana divaricata* root. J Pharm Pharmacol 2006;58:847–52.

Ingkaninan K, Temkitthawon P, Chuenchom K, Yuyaem T, Thongnoi W. Screening for acetylcholinesterase inhibitory activity in plants used in Thai traditional rejuvenating and neurotonic remedies. J Ethnopharmacol 2003;89:261–4.

Ingkaninan K, Yuyaem T, Thongnoi W. Anticholinesterase activity in *Stephania venosa* Spreng. Naresuan Univ J 2001;9:35–40.

Lleo A, Greenberg SM, Growdon JH. Current pharmacotherapy for Alzheimer's disease. Annu Rev Med 2006;57:513–33.

López S, Bastida J, Viladomat F, Codina C. Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. Life Sci 2002;71:2521–9.

Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 2002;110:627–39.

Ogino T, Yamaguchi T, Sato T, Sasaki H, Sugama K, Okada M, Maruno M. Studies of inhibitory activity against acetylcholinesterase of new bisbenzylisoquinoline alkaloid and its related compounds. Heterocycles 1997;45:2253–60.

Orhan I, Sener B, Chouhary MI, Khalid A. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. J Ethnopharmacol 2004;91: 57–60.

Park CH, Kim S, Choi W, Lee Y, Kim J, Kang SS. Novel anticholinesterase and antiamnesic activities of dehydroevodiamine, a constituent of *Evodia rutaecarpa*. Planta Med 1996;62:405–9.

Pio Correa M. Diccionário de plantas úteis do Brasil e das exóticas cultivadas, vol. IV. Rio de Janeiro: Instituto Brasileiro de Desenvolvimento Florestal; 1984.

Rapoport H, Onak TP, Hughes NA, Reinecke MG. Alkaloids of *Geissospermum vellosii*. J Am Chem Soc 1958;80:1601–4.

Rhee IK, van de Meent M, Ingkaninan K, Verpoorte R. Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. J Chromatogr A 2001;915:217–23.

Rhee IK, van Rijn RM, Verpoorte R. Qualitative determination of false-positive effects in the acetylcholinesterase assay using thin layer chromatography. Phytochem Anal 2003;14:127–31.

Peckolt G. As dez árvores genuinamente brasileiras mais úteis na medicina. Rev Flora Med 1942;9:453–70.

Silver A. The biology of cholinesterases. North-Holland Research MonographsAmsterdam: Elselvier; 1974. p. 426–47.

Skolnick AA. Old Chinese herbal medicine used for fever yields possible new Alzheimer disease therapy. JAMA 1997;277:776.

Svensson AL, Zhang X, Nordberg A. Biphasic effect of tacrine on acetylcholine release in rat brain via M1 and M2 receptors. Brain Res 1996;726:207–12.

Tanae MM, Souccar C, Lapa AJ, Lima-Landman MTR. Molecular interaction of *Geissospermum*'s alkaloids with alpha 7 or muscle-type nicotinic receptors (nAChR) subtypes and with acetylcholinesterase (AChE). Acta Pharmacol Sin 2006;27(Suppl.1):347-347.

Terry Jr AV, Buccafusco JJ. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. J Pharmacol Exp Ther 2003;306:821–7.

Trevisan MT, Macedo FVV. Seleção de plantas com atividade anticolinesterásica para tratamento da doença de Alzheimer. Quím Nova 2003;26:301–4.

van Beek TA, Verpoorte R, Baerheim SA, Leeuwenberg AJM, Bisset NG, Tabernaemontana L. (Apocynaceae): a review of its taxonomy, phytochemistry, ethnobotany and pharmacology. J Ethnopharmacol 1984;10:1-156.

Viegas Jr C, Bolzani VS, Pimentel LS, Castro NG, Cabral RF, Costa RS, Floyd C, Rocha MS, Young MC, Barreiro EJ, Fraga CA. New selective acetylcholinesterase inhibitors designed from natural piperidine alkaloids. Bioorg Med Chem 2005;13:4184–90.

Vigny M, Bon S, Massoulié J, Leterrier F. Active-site catalytic efficiency of acetylcholinesterase molecular forms in *Electrophorus*, *Torpedo*, rat and chicken. Eur J Biochem 1978;85:317–23.