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Investigation of the *in vitro* and *ex vivo* acetylcholinesterase and antioxidant activities of traditionally used *Lycopodium* species from South America on alkaloid extracts

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A B S T R A C T

Ethnopharmacological relevance: The study was aimed at evaluating medicinal and therapeutic potentials of two Lycopodiaceae species, *Lycopodium clavatum* (L.) and *Lycopodium thyoides* (Humb. & Bonpl. ex Willd), both used in South American folk medicine for central nervous system conditions. Alkaloid extracts were evaluated for chemical characterization, acetylcholinesterase and antioxidant activities.
Materials and methods: The alkaloid extracts obtained by alkaline extraction were determined for each species by GC/MS examination. The evaluation of the anticholinesterase and the antioxidant activities of the extracts were tested by determining *in vitro* and *ex vivo* models. Effects on acetylcholinesterase (AChE) were tested *in vitro* using rat brain homogenates and *ex vivo* after a single administration (25, 10 and 1 mg/kg i.p.) of the alkaloid extracts in mice. The *in vitro* antioxidant effects were tested for the 2-deoxyribose degradation, nitric oxide (NO) interaction, 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity and total reactive antioxidant potential (TRAP). After an acute administration (25 and 10 mg/kg i.p.) of the extracts in middle-aged (12 months) mice, the antioxidant effects were estimated through the thiobarbituric acid reactive substances test (TBARS), and the antioxidant enzymes activities for catalase (CAT) and superoxide dismutase (SOD) were measured.
Results: AChE activity was inhibited *in vitro* by the alkaloid-enriched extracts of both *Lycopodium* species in a dose and time-dependent manner in rat cortex, striatum and hippocampus. A significant inhibition was also observed in areas of the brain after acute administration of extracts, as well as decreased lipid peroxidation and increased CAT activity in the cortex, hippocampus and cerebellum. A moderate antioxidant activity was observed *in vitro* for the extracts. Chemically, the main alkaloids found for the two species were lycopodine and acetyldihidrolycopodine.
Conclusion: This study showed that the biological properties of the folk medicinal plants *Lycopodium clavatum* and *Lycopodium thyoides* include AChE inhibitory activity and antioxidant effects, two possible mechanisms of action in Alzheimer's related processes.
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1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease of this century and the most prevalent cause of dementia among the elderly. About 4.6 million new cases of dementia are reported every year worldwide, and it is estimated that 71% of dementia cases will occur in the developing countries by 2040 (Ferri et al., 2005; Kalaria et al., 2008). This irreversible neurological disorder is characterized by memory impairment, cognitive dysfunction, behavioral disturbances and deficits in activities in daily living (Cummings et al., 1988).

In the past decade, treatment for AD has largely involved replacement of neurotransmitters that are known to be lacking, mostly based on the inhibition of acetylcholinesterase (AChE), an important approach that is founded on the cholinergic hypothesis for the disease (Francis et al., 1999). As one of the most remarkable

Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase; AE, alkaloid extract; i.p., intraperitoneal; LC, *Lycopodium clavatum*; LT, *Lycopodium thyoides*.

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biochemical changes in AD patients is a reduction of acetylcholine levels in the hippocampus and cortex of the brain, the use of cholinesterase inhibitors is the most commonly used treatment for the symptoms of mild AD, improving the cholinergic deficit (Lahiri et al., 2002). However, the drugs that are currently used for the treatment of AChE inhibitory activity (e.g. rivastigmine, galanthamine and donepezil) are extremely limited and possess some considerable side effects, related to cholinergic stimulation in brain and peripheral tissues (Thompson et al., 2004; Fang et al., 2008). Recently, multiple lines of evidence indicate that reactive oxygen species are associated with the pathogenesis of AD, given that some cellular characteristics of this disease are either causes or effects of oxidative stress (Zhu et al., 2004; Sultana et al., 2006). Antioxidants may therefore be applied as neuroprotective agents (Moreira et al., 2005), since oxidative damage can be observed before the formation of specific pathological β-amyloid plaques. Considering the complex pathology of AD, the current strategies to develop new agents focus on compounds with multiple potencies, and plants are an important source of these compounds.

A number of plants have been used in traditional medicine remedies to treat memory and cognitive disorders, such as *Huperzia serrata* (Qian Ceng Ta), which has been used for centuries in China for the treatment of contusions, strains, swelling and schizophrenia (Skolnick, 1997). Huperzine A and B, two alkaloids found in this species, have proved to be potent, reversible and selective inhibitors for AChE activity (Liu et al., 1986; Ayer, 1991). Moreover, huperzine A displayed a remarkable activity on memory and learning (Zhang et al., 1991), as well as *in vivo* antioxidant activity against free radicals and β-amyloid-induced cell toxicity (Wang et al., 2001; Xiao et al., 2002). For this reason, this compound has been marketed in China as a new drug for AD, and some synthetic derivatives are being developed as drug candidates in China and Europe (Ma et al., 2007). Since then, *Lycopodium* alkaloids, a group of metabolites occurring in the family Lycopodiaceae, have become a new potential source of pharmacologically interesting natural products that have led to the identification of over 200 alkaloids from 54 *Lycopodium* species so far (Ma and Gang, 2004).

Lycopodium clavatum (L.) and *Lycopodium thyoides* (Humb. & Bonpl. ex Willd) (Lycopodiaceae), commonly known as “club mosses”, are creeping perennial species found in almost all parts of the world, although they are considered to be montane plants in the Neotropics (Øllgaard, 1992). Both species are well known for their rich alkaloid content, which has been extensively reviewed (Ayer and Dikko, 1974; MacLean, 1985; Ma and Gang, 2004) and characterized by the absence of huperzines, more commonly found in the *Huperzia* genus. Aerial parts of *Lycopodium clavatum* are popularly used as carminatives, laxatives and diuretics (Schauenberg and París, 1972; Nuñez and Cantero, 2000); the entire plant is used in South America as an aphrodisiac (Hurrel and de la Sota, 1996) and in China for the treatment of mental disorders (Duke and Ayensu, 1985). The Quechuas, an Andean ethnic group from Ecuador, use infusions of *Lycopodium thyoides* as a traditional medicine to treat parturition disorders, and the whole plant is believed to act as a central nervous system stimulant for young children with motor problems, in order to improve their resistance to the labor (Ellemann, 1990; Navarrete et al., 2006). Therefore, in this study, the hypothesis that the alkaloid extracts from *Lycopodium* species can inhibit the activity of the enzyme AChE, and also display antioxidant activities, was tested.

2. Materials and methods

2.1. Plant material

Lycopodium thyoides and *Lycopodium clavatum* were collected in the city of Bom Jesus, Rio Grande do Sul, Brazil in November 2007, and identified by Prof. Dr. Sérgio L. Bordignon, Fundação La Salle. The voucher specimens (collection numbers HAS 45875 and HAS 45878, respectively) were deposited in the Herbarium of the Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, Brazil.

2.2. Preparation of the total alkaloid extracts (AE)

The dried and ground aerial parts from *Lycopodium thyoides* (1.69 kg) and *Lycopodium clavatum* (1.35 kg) were separately extracted with *n*-hexane using a Soxhlet extractor. Afterwards, defatted plant material was exhaustively macerated with methanol and the resulting crude extracts were dried under reduced pressure. Each extract was then suspended in 5% HCl and partitioned with CH_2Cl_2. The aqueous layers were alkalinized with 0.1 N NaOH until pH 12, and then extracted again with CH_2Cl_2; the organic layers were dried over Na_2SO_4 and evaporated to give the crude alkaloid extracts AELT (*Lycopodium thyoides*) and AELC (*Lycopodium clavatum*).

2.3. Chemical composition

The alkaloid extracts were submitted to GC–MS in order to separate and identify the present components, by means of an Agilent 7890A directly coupled to a quadrupole mass spectrometer Agilent 5975C and equipped with a capillary column HP 30, 30 m in length. The analysis was performed using an injection volume of 0.5 μL with He as a carrier, with a flow rate of 1 mL/min. Temperature program: 230 °C (2 min), 230–250 °C at 20 °C/min, 250 °C (2 min), 250–280 °C at 5 °C/min and 280 °C (5 min). The temperatures of the injector, interface and ion source were 300, 280 and 280 °C, respectively. The ionization energy was 70 eV and individual identification of the samples was made by matching scores from our own MS library of *Lycopodium* alkaloids, comparison with the literature data and standards co-injections. Retention indexes of the alkaloids were recorded by co-injecting 1 μL of 1 mg/mL of the standard calibration *n*-hydrocarbon mixture (C9–C36) and 1 μL of 1 mg/mL AELC and AELT solutions, using the temperature program: 60–300 °C at 3 °C/min. The nuclear magnetic resonance (NMR) spectra in $CDCl_3$ of the main alkaloids were obtained using a Bruker DPX400 spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C.

2.4. Animals

Experiments were performed using adult male Wistar rats (60 days old, weighing 240–260 g) and Swiss albino male mice (CF1 strain, 3 and 12 months old, weighing 35–50 g); all obtained from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS). Animals were maintained under controlled temperature (23 ± 1 °C) and light-dark cycles of 12 h, with free access to food and water. The research protocols with animals followed throughout our study were designed to minimize suffering and limit the number of animals sacrificed. The project was approved (#2007942, 2008) by the University Ethics Committee.

2.5. Drugs and reagents

Acetylthiocholine iodate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), AAPH (2,2-azobis[2-methylpropionamidine]dihydrochloride), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 2-deoxyribose, glycine, Griess reagent, sodium nitroprusside, huperzine A and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and the buffer salts were purchased from Merck (Darmstadt, Germany). All other chemicals and organic solvents were of analytical grade. Stock solutions of AELC and AELT

were initially dissolved in DMSO and further diluted to various concentrations in 20 mM phosphate buffer immediately before use, in order to achieve final concentrations of DMSO of not greater than 1%. For the intraperitoneal (i.p.) administrations, extracts were dissolved in DMSO and saline solution 0.9% g (w/v) NaCl to obtain the final concentrations in a 20% (v/v) DMSO solution.

2.6. Anticholinesterasic activity assays

AChE activity was measured by the colorimetric method of Ellman et al. (1961) with some minor modifications, adapted for determining the enzyme activity in supernatants of brain homogenates.

2.6.1. In vitro analysis for AChE activity

Rats were separately decapitated, the brains quickly removed and placed on an ice-cold plate. Cortices, striata and hippocampi were dissected, weighed and homogenized in cold 10 mM Tris–HCl buffer, pH 7.2, containing 160 mM sucrose. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C, and the resulting clear supernatants were used as enzyme sources that were divided into aliquots and stored at −20 °C. Briefly, enzyme samples in 20 mM phosphate buffer (pH 7.4) were incubated in the presence of 10 mM DTNB solution with different concentrations of each AE in buffer (final concentrations from 50 to 1000 μg/mL). The enzyme reaction was initiated by the addition of 0.8 mM acetylthiocholine iodide after the pre incubation times of 0, 15 and 30 min. Substrate hydrolysis was monitored by the formation of a yellow anion of 5-thio-2-nitrobenzoic acid at 415 nm, every 30 s during 2.5 min, by a SpectraMax 190, 96-well plate reader. Enzyme activity was estimated through differences in ΔA/min, and the degrees of inhibition in each brain area and incubation time were calculated by comparison with its control (1% DMSO, results expressed as percentages of control); every experiment was performed in triplicate.

The kinetic parameters K_m (Michaelis–Menten constant) and V_{max} (maximal velocity) were determined by means of a Lineweaver–Burk plot over a substrate range of 0.01–0.08 mM after 30 min of incubation of striatum samples with three different concentrations of AELC and AELT (50, 100 and 250 μg/mL).

2.6.2. Ex vivo analysis for AChE activity

Three-months old mice were randomly divided into 9 groups ($n = 6$) and each received a single i.p. injection (0.1 mL/10 g) of saline solution, vehicle (20% DMSO), AELC (25, 10 and 1 mg/kg), AELT (25, 10 and 1 mg/kg) or huperzine A (0.5 mg/kg). Animals were killed by decapitation 60 min after each administration. Subsequently, brains were removed; cortices and hippocampi were dissected separately and processed as described above. Total AChE activity was measured as triplicates in aliquots of brain homogenates, the enzymatic inhibition is expressed as the percentage of the control (DMSO-treated mice).

2.7. Antioxidant activity assays

2.7.1. Assay of 2-deoxyribose degradation

The formation of OH (hydroxyl radicals) was indirectly estimated by the spectrophotometric measurement of the 2-deoxyribose oxidative degradation, through the production of malondialdehyde, which condensates with 2-thiobarbituric acid (TBA) (Hermes-Lima et al., 1994). The reaction was carried out in a 20 mM phosphate buffer (pH 7.2) medium containing AELC and AELT at final concentrations ranging between 50 and 2500 μg/mL with 5 mM 2-deoxyribose and 100 mM H_2O_2. The reaction was started by the addition of Fe^{2+} (6 mM final concentration) to the solutions and was carried out for 15 min at room temperature. The reaction was stopped by the addition of 4% phosphoric acid followed by 1% TBA (in 50 mM NaOH). Solutions were boiled for 15 min at 100 °C and then cooled at room temperature. The absorbance was measured at 532 nm and percent values were determined by comparing the absorbance of control (Fenton reagents) and test samples.

2.7.2. Scavenging activity of the nitric oxide (NO) radical

The interaction of the alkaloid extracts (in final concentrations of between 50 and 2500 μg/mL) with nitric oxide was assessed by the nitrite detection method (Green et al., 1982). NO was generated from spontaneous decomposition of 200 nM sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Nitric oxide interacts with oxygen to produce stable products, leading to the formation of nitrites, which were measured by the Griess reaction. The reaction mixture containing 10 mM sodium nitroprusside in phosphate buffer and the extracts were incubated at 37 °C for 1 h. An aliquot was taken and homogenized with Griess reagent (0.1% α-naphthylethylenediamine in water and 1% sulfanilic acid in 5% H_3PO_4). The concentration of nitrite was assayed at 540 nm and calculated with reference to the absorbance of the standard nitrite solutions. The inhibition results were expressed as percentages in relation to the non-treated control.

2.7.3. DPPH radical scavenging activity

The free radical scavenging effect of the extracts was assessed by the discoloration of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), as previously reported (Mensor et al., 2001). The extracts were assayed in a range of 10–500 μg/mL. The quenching of free radicals by extracts was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical. A freshly prepared DPPH solution (0.1 mM) was used for the assays, samples were dissolved in methanol and the methanolic solution of DPPH served as a control. The degree of discoloration indicates the free radical scavenging efficiency of the substances. The percentage of DPPH discoloration was calculated as previously described.

2.7.4. Total reactive antioxidant potential (TRAP) assay

An adapted method of TRAP to measure luminol-enhanced chemiluminescence (Lissi et al., 1992) was used to determine the capacity of the alkaloid extracts to trap a flow of water-soluble peroxyl radicals produced through thermal decomposition of ABAP (2,2′-azobis (2-amidinopropane) dihydrochloride). Briefly, the reaction mixture contained the free radical source (ABAP, 10 mM) and 4 mM luminol in the 0.1 M glycine buffer (pH 8.6). The incubation of this mixture at 20 °C generates an almost constant light intensity that was measured in a scintillation counter (Beckman), working in the out of coincidence mode. For the determination of the free radical scavenging potential, increasing concentrations of AELC and AELT in methanol were added to the system (from 50 to 2500 μg/mL). The results of the chemiluminescence emission were expressed as area under the curve (AUC).

2.8. Ex vivo antioxidant assay

Mice (twelve-months old) were divided into 6 groups ($n = 6$). Saline solution, vehicle (20% DMSO), AELC (25 and 10 mg/kg) or AELT (25 and 10 mg/kg) were injected i.p. (0.1 mL/10 g). Animals were decapitated at 60 min after drug administration and each brain was quickly excised. Hippocampi, cerebral cortices and cerebella were dissected out, brain tissues were homogenized in 10 vol. of ice-cold 20 mM phosphate buffer (pH 7.4) containing EDTA (1 mM) and phenylmethylsulfonyl fluoride (1 mM). The homogenates were centrifuged at 10,000 × *g* for 10 min; the supernatants were employed for the assays.

2.8.1. Lipid peroxidation assay

Lipoperoxidation was evaluated by thiobarbituric acid reactive substances (TBARS) tests during an acid-heating reaction, as previously described (Draper and Hadley, 1990). Aliquots of samples were incubated with 10% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was heated (25 min) in a boiling water bath. TBARS was determined by reading the absorbance of the pink-colored complex formed in a spectrophotometer at 532 nm. 1,1,3,3-Tetramethoxypropane, which is converted to malondialdehyde, was used as standard.

2.8.2. Determination of antioxidant enzyme activity

Catalase (CAT) activity was assayed, as described by Aebi (1984). Briefly, homogenates were incubated with 10% ethanol and 10% triton and the activity was assayed at 37 °C by determining the rate of degradation of H_2O_2 at 240 nm in 20 mM potassium phosphate buffer (pH 7.0). One unit is defined as 1 pmol of H_2O_2 consumed per minute and the specific activity is reported as units per mg protein. Superoxide dismutase (SOD) activity was accessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm (Boveris, 1984) and the results are expressed as units of SOD/mg of protein.

2.9. Protein assay

All results were normalized by the protein concentrations, which were determined by Peterson's modification of the procedure of Lowry et al. (1951) using bovine serum albumin as standard.

2.10. Statistical analysis

Results are expressed as mean ± standard error mean (S.E.M.). Differences between the treatments and controls were evaluated by ANOVA followed by Duncan's multiple range test whenever necessary; linear regression was further used to verify the dose–response relationship. A $p < 0.05$ was considered to be significant. Data related to K_m and V_{max} were analyzed by one-way ANOVA.

3. Results

3.1. Chemical composition

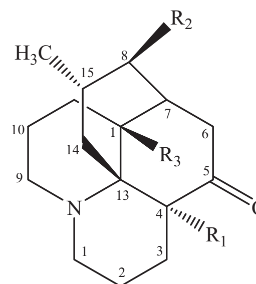
The yield of the extracts was 0.34% and 0.38% of dried materials for *Lycopodium clavatum* and *Lycopodium thyoides*, respectively. GC–MS analysis performed for the extracts of each species revealed the presence of a number of known *Lycopodium* alkaloids (Fig. 1) belonging to the lycopodane and flabellidane groups in different percentages. Both species had lycopodine and acetyldihydrolycopodine as their main alkaloids, as well as lycodoline, anhydrolycodoline, lycodine and α-obscurine, as shown in Figs. 1A and 2A. Additionally, *N*-methyllycodine and traces of clavolonine were detected in *Lycopodium clavatum*, whereas for *Lycopodium thyoides* the alkaloid flabellidine was found. The ^1H and ^{13}C NMR spectra of the main compounds found in extracts, lycopodine and acetyldihydrolycopodine is shown in Table 2.

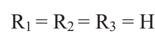
3.2. Acetylcholinesterase inhibition

No significant differences in AChE activity (ΔA/min) were found with phosphate buffer or DMSO over time. Both alkaloid extracts of *Lycopodium* significantly inhibited AChE activity *in vitro* in a dose and time-dependent manner in all brain structures studied (Figs. 2 and 3). At 30 min of incubation with AELC, there were dose/effect associations with Pearson's coefficients as follows: $r^2 = 0.9227$ ($p < 0.001$) for cortex (Fig. 2A), $r^2 = 0.8731$ ($p < 0.001$) for striatum (Fig. 2B) and $r^2 = 0.9139$ ($p < 0.001$) for hippocampus (Fig. 2C), while for AELT $r^2 = 0.8752$ ($p < 0.001$) was found for cortex (Fig. 3A), $r^2 = 0.8904$ ($p < 0.001$) for striatum (Fig. 3B) and $r^2 = 0.8868$ ($p < 0.001$) for hippocampus (Fig. 3C). Whereas the AChE inhibition seems to be stabilized after 15 min of incubation in the presence of AELC in the cortex, with AELT it continues to drop thereafter. As for striatum, treatment with AELC also promotes a stabilization of enzymatic inhibition after 15 min, the same effect was found for higher concentrations of AELT (500 and 1000 μg/mL), while for 50 and 250 μg/mL, a continuous drop in inhibition was observed. In the hippocampus, treatment with both alkaloid extracts seemed not to affect AChE inhibition over time. In cortex, striatum and hippocampus, the enzymatic activities reached a maximal inhibition of 73.3%, 62% and 59% in the presence of AELC, whereas for AELT this effect was 81.6%, 75% and 78.5%, respectively, both at 1000 μg/mL after 30 min of incubation.

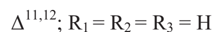
The double-reciprocal Lineweaver–Burk plot for rat striatal acetylcholinesterase assays with 30 min of incubation (Fig. 4) indicated that, in the presence of increasing concentrations of AELC (50, 100 and 250 μg/mL), V_{max} values are reduced (from 0.32 to 0.23, 0.16 and 0.12 μM of acetylthiocholine hydrolyzed/mg

Lycopodane group

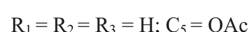




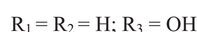
Lycopodine



Anhydrolycodoline



Acetyldihidrolycopodine



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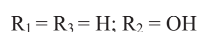
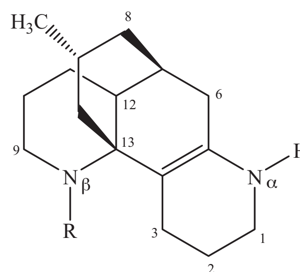
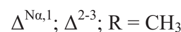


Clavolonine

Flabellidane group

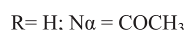


Lycodine



N-methyllycodine

α-obscurine



Flabellidine

Fig. 1. Structures of the alkaloids detected in the alkaloid extracts of *Lycopodium clavatum* (AELC) and *Lycopodium thyoides* (AELT).

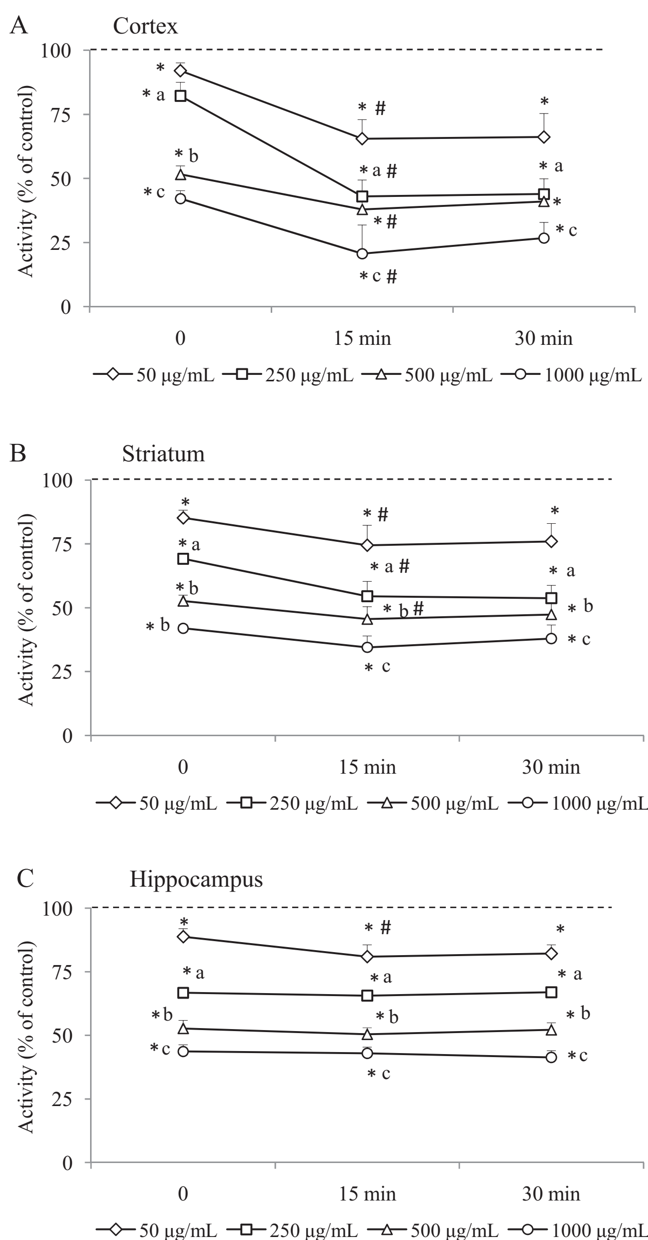


Fig. 2. Effect of *Lycopodium clavatum* AE (50, 250, 500 and 1000 µg/mL) on AChE activity in rat cortex, striatum and hippocampus. Results are expressed as AChE activity (percentage of the DMSO control group) at 0, 15 and 30 min of incubation. Each value represents mean ± S.E.M. ($n = 5$). *$p < 0.05$ vs. control, paired Student's *t* test; (a) $p < 0.05$ vs. 50 µg/mL, (b) $p < 0.05$ vs. 250, (c) $p < 0.05$ vs. 500, ANOVA/Duncan; (#) $p < 0.05$ vs. previous incubation time, ANOVA/Duncan.

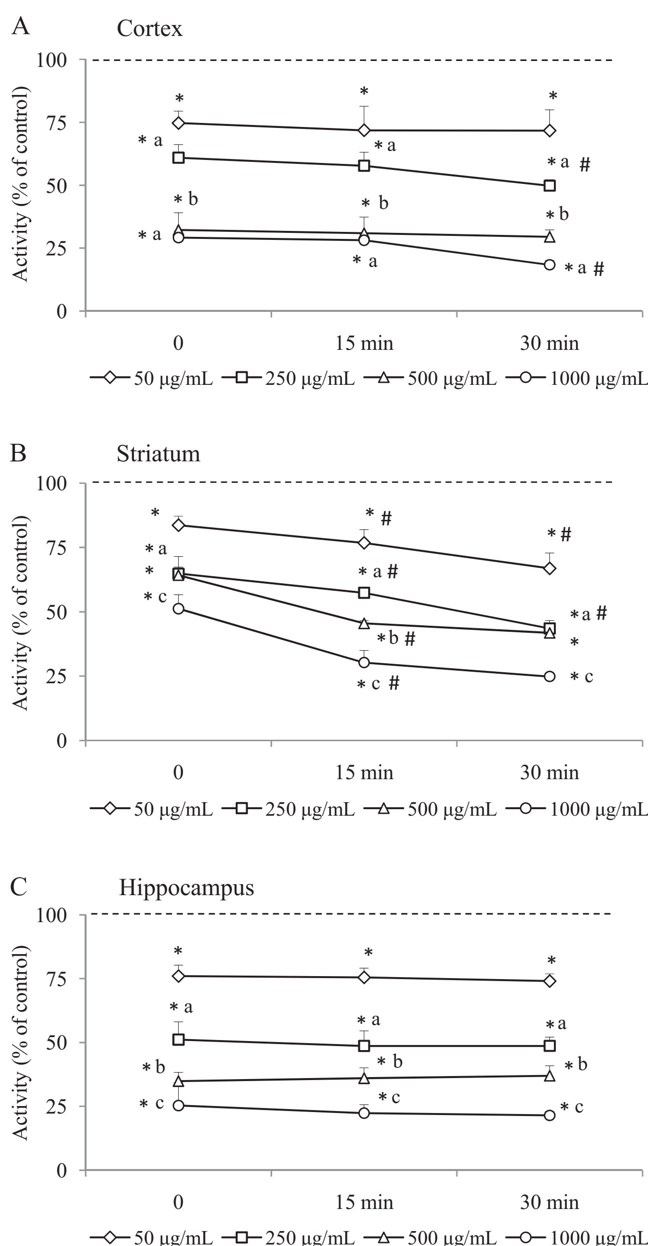


Fig. 3. Effect of *Lycopodium thyoides* AE (50, 250, 500 and 1000 µg/mL) on AChE activity in rat cortex, striatum and hippocampus. Results are expressed as AChE activity (percentage of the DMSO control group) at 0, 15 and 30 min of incubation. Each value represents mean ± S.E.M. ($n = 5$). *$p < 0.05$ vs. control, paired Student's *t* test; (a) $p < 0.05$ vs. 50 µg/mL, (b) $p < 0.05$ vs. 250, (c) $p < 0.05$ vs. 500, ANOVA/Duncan; (#) $p < 0.05$ vs. previous incubation time, ANOVA/Duncan.

of protein/min, respectively) and K_m values are increased (from 0.05 to 0.07, 0.09 and 0.12 µM, respectively). In the case of AELT, the same concentrations also induced a decrease in V_{max} (from 0.26 to 0.17, 0.13 and 0.11 µM of acetylthiocholine hydrolyzed/mg of protein/min, respectively), and an augment in K_m (from 0.05 to 0.07, 0.09 and 0.11 µM), a pattern common to competitive/noncompetitive type inhibitors.

Table 1 shows the effects of a single administration of the alkaloid extracts (25, 10 and 1 mg/kg, i.p.) on AChE activity in the cortex and hippocampus of mice. As observed, treatment with the extracts correlated with enzymatic inhibition in all of the brain structures studied; when treated with AELC, decreases in AChE activity were in the range of 46.5, 37.2 and 20% (cortex) and 43, 18.7 and 6.2% (hippocampus), respectively for each dose, while for AELT, decreases in enzymatic activity were in the order of 43.5, 40.7 and 28.3% (cortex) and 54.7, 33.6 and 10% (hippocampus). At 0.5 mg/kg, huperzine A showed an inhibitory effect of 64.3% for cortex and 47.5% for hippocampus.

3.3. Antioxidant activities

Table 2 summarizes the antioxidant effects of each AE and the reference free radical scavenger boldine against 2-deoxyribose degradation, nitric oxide production, DPPH discoloration and total reactive antioxidant potential. At the final concentration of 2500 µg/mL, the inhibitory power against 2 deoxyribose degradation comprised 25–32%, and for nitric oxide production 50–60% for AELC and AELT. Both extracts were also able to reduce the DPPH

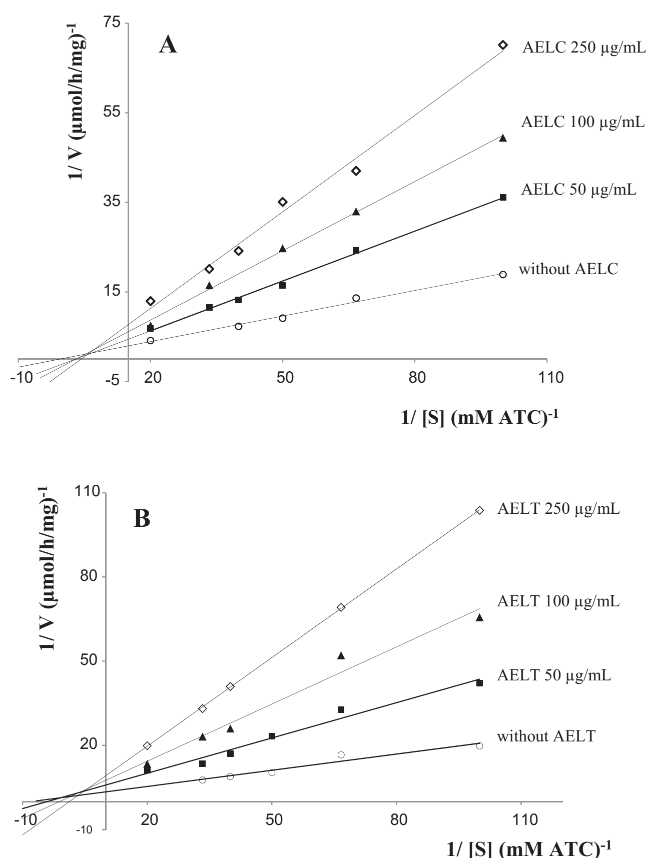


Fig. 4. Lineweaver–Burk plots of AChE activity over a range of substrate concentrations (0.01–0.08 mM) from striatum samples in the absence or presence of AE (50, 100 and 250 µg/mL) of *Lycopodium clavatum* (A) and *Lycopodium thyoides* (B).

radical at all tested concentrations; however, due to the turbidity in test conditions, concentrations of higher than 500 µg/mL could not be assayed. In the TRAP assay, the extracts acted as peroxyl scavengers, diminishing the chemiluminescence with induction times that were proportional to the concentrations added. In addition, a single i.p. administration of AELC and AELT (25 and 10 mg/kg) to 12-months old mice induced changes in all indexes of oxidative stress studied, with different patterns found in different brain regions. A significant decrease in lipoperoxidation (TBARS levels, Fig. 5) was observed in the cortex, hippocampus and cerebellum

Table 1
Effects of an acute administration of *Lycopodium clavatum* and *Lycopodium thyoides* AE (1, 10 and 25 mg/kg i.p.) and huperzine A (0.5 mg/kg i.p.) on AChE activity in mice cortex and hippocampus.

Treatment groups	Dose (mg/kg)	AChE inhibition (%)	
		Cortex	Hippocampus
AELC	25	46.8 ± 2.9*	44.2 ± 1.5*
	10	37.8 ± 2.1*	20.4 ± 1.6*
	1	21.4 ± 0.9*	6.7 ± 0.9
AELT	25	48.9 ± 1.7*	50.2 ± 1.3*
	10	41.7 ± 2.3*	31.5 ± 2.4*
	1	29.6 ± 2.2*	9.8 ± 1.1
Huperzine A	0.5	62.1 ± 2.5*	49.2 ± 0.9*

All assays were performed in triplicate. Enzyme inhibition is expressed as percentage of control (DMSO-treated mice). Each value represents mean ± S.E.M. ($n = 6$). ANOVA followed by Duncan's test.
* $p < 0.05$; significant from control.

for all extracts analyzed. In terms of antioxidant enzymes activity, SOD activity was significantly increased only in the cerebellum of AELT treated mice (Fig. 6A), while CAT activity was enhanced in all brain structures after treatment with both AE (Fig. 6B). Moreover, the SOD/CAT ratio was found to be significantly decreased in the cortex, hippocampus and cerebellum of AELC treated mice (Fig. 6C), while AELT promoted a decrease only at the higher dose, with the exception of the cortex, which showed a decrease in the enzymatic ratio at all doses.

4. Discussion and conclusions

The use of inhibitors of the acetylcholinesterase enzyme is currently widely accepted as the first line pharmacotherapy for symptomatic relief of mild to moderate AD, through an increase in acetylcholine levels at the synaptic site in the brain, improving the memory and cognitive deficits of the patients (Giacobini, 2003; Loizzo et al., 2008). Some potent inhibitors of AChE are derived from natural sources and most of them belong to the chemical class of alkaloids, including galanthamine, obtained from Amaryllidaceae species, and huperzine A, the most studied *Lycopodium* alkaloid. As such, the present study reports, for the first time, on the *in vitro* and *ex vivo* antioxidant and anticholinesterasic activities of the alkaloid extracts of two species belonging to the *Lycopodium* genus (Lycopodiaceae) and collected in Brazil: *Lycopodium clavatum* (L.) and *Lycopodium thyoides* (Humb. & Bonpl. ex Willd). These plants are well known for their alkaloid content, which has been extensively reviewed, and decoctions of the entire plants are used

Table 2
Inhibition profile of activities of the *Lycopodium clavatum* and *Lycopodium thyoides* AE against 2-deoxyribose degradation, nitric oxide production, DPPH radical scavenging activity and total reactive antioxidant potential (TRAP) assays as free radical scavengers.

Treatment groups	Dose (µg/mL)	2-Deoxyribose degradation	Nitric oxide production	DPPH decoloration	TRAP assay
AELC	2500	25.3 ± 2.7*	57.2 ± 1.6*	–	81.4 ± 1.8$^{*,+}$
	1000	8.6 ± 1.5	53.5 ± 1.1*	–	72.5 ± 1.1$^{*,+}$
	500	4.9 ± 0.7	49.2 ± 0.7$^{*,+}$	91.7 ± 0.6$^{*,+}$	45.3 ± 1.5$^{*,+}$
	250	3.2 ± 0.3	24.4 ± 1.3$^{*,+}$	85.6 ± 4.1$^{*,+}$	24.0 ± 0.8$^{*,+}$
	50	1.1 ± 0.02	2.9 ± 0.2	50.1 ± 3.2*	5.1 ± 0.2
AELT	2500	32.0 ± 2.1*	48.6 ± 2.1*	–	84.9 ± 2.1$^{*,+}$
	1000	25.4 ± 1.1*	43.3 ± 0.9$^{*,+}$	–	74.7 ± 1.9$^{*,+}$
	500	20.1 ± 1.3*	31.7 ± 1.9*	83.1 ± 1.3$^{*,+}$	33.5 ± 1.3$^{*,+}$
	250	12.8 ± 0.4*	23.5 ± 1.2*	68.7 ± 0.9$^{*,+}$	21.2 ± 1.2$^{*,+}$
	50	8.4 ± 0.4*	19.1 ± 0.9*	43.8 ± 0.2*	4.9 ± 0.5
Boldine	100	43.4 ± 2.7*	79.1 ± 3.8*	97.8 ± 2.9*	70.9 ± 3.5*
	50	18.5 ± 0.9*	59.4 ± 2.1*	70.4 ± 2.5*	55.9 ± 1.9*
	10	5.2 ± 0.4	22.7 ± 1.3*	53.8 ± 0.2*	18.8 ± 1.5*
	1	1.3 ± 0.4	12.0 ± 0.9*	15.1 ± 0.4*	3.1 ± 0.4

Results are expressed as % of activity relative to the corresponding control (mean ± S.E.M., 6–8 experiments run in triplicate). ANOVA followed by Duncan's test.
* $p < 0.05$ as compared to control group.
$^{+}$ $p < 0.05$ as compared to previous dose.

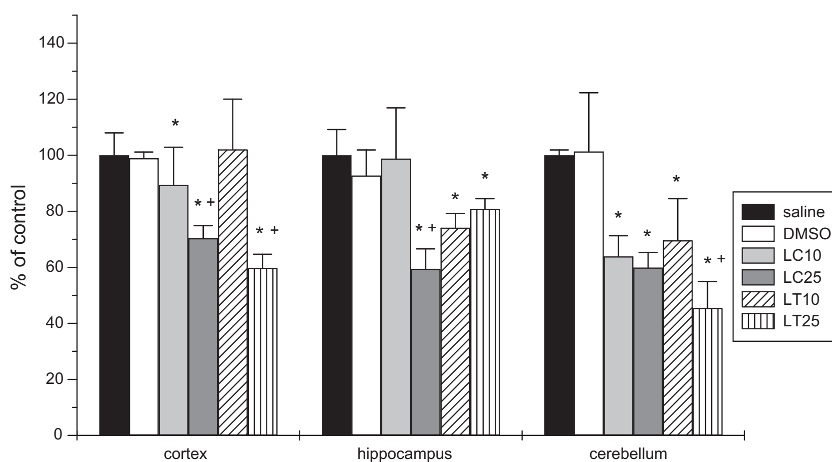


Fig. 5. Effects of AELC and AELT (10 and 25 mg/kg) on lipid damage (thiobarbituric acid reactive substance, TBARS) in brain regions from aging mice. Data is expressed as percentage of control DMSO (mean ± S.E.M.). The absolute mean TBARS values for saline group were 0.26 ± 0.04 (cortex), 0.19 ± 0.03 (hippocampus) and 0.27 ± 0.01 (cerebellum) (nmol MDA/mg protein). Data are referred to five separated experiments run in triplicate (ANOVA followed by Duncan's test). $^{*}p < 0.05$ as compared to saline group, $^{+}p < 0.05$ as compared to previous dose.

in folk medicine for CNS-related conditions, as nerve tonics and for motor disorders (Duke and Ayensu, 1985; Navarrete et al., 2006). Some previous studies have found that a crude plant extract of *Lycopodium clavatum* growing in Turkey displayed a significant inhibitory effect on AChE *in vitro* (49.8%) at the concentration of 1000 μg/mL (Orhan et al., 2004); a bio-assay guided fractionation of the plant extract indicated triterpenoid α-onocerin as the main compound responsible for this activity (Orhan et al., 2003). Later, in contrast to these studies, no anti-AChE activity was found for this compound, and another triterpenoid, lyclavatol, was found to possess some inhibitory effect, although the presence of this compound does not fully explain the activity of the *Lycopodium clavatum* crude extract (Rollinger et al., 2005). Furthermore, an alkaloid extract for the same species was found to possess a significant *in vivo* anti-inflammatory activity, where the major alkaloid found for this extract was lycopodine, followed by dihydrolycopodine and lycodine, all of them belonging to lycopodane group (Orhan et al., 2007). On the other hand, to our knowledge no biological studies have been reported in the literature with the *Lycopodium thyoides* species so far.

In the present study, we report that the alkaloid extracts of both the *Lycopodium clavatum* and *Lycopodium thyoides* species have the ability to inhibit AChE and display antioxidant properties, as evaluated by *in vitro* and *ex vivo* assays. The AChE activity was found to be inhibited in the rat cortex, hippocampus and striatum homogenates incubated *in vitro* with AELC and AELT (50–1000 μg/mL); a significant (≈10–50%) decrease in AChE activity was also observed *ex vivo* in these memory relevant brain structures, as evaluated at 60 min after a single AELC and AELT (25, 10 and 1 mg/kg i.p.) administration to adult (3 months) mice. Although further experiments are needed for a definitive conclusion, the AChE inhibition induced by both alkaloid extracts seems to be dose and time-dependent, with AELT displaying a higher inhibitory activity when compared to AELC. Different effects on enzymatic inhibition may be related to varying concentrations of several molecular AChE forms in particular tissues or brain regions, favoring interactions with particular types of the enzyme in each (Bisso et al., 1991). Of note, i.p. administration of huperzine A, obtained from the Chinese medicinal herb *Huperzia serrata*, resulted in various degrees of AChE activity inhibition in the rat hippocampus, cortex, striatum and hypothalamus (Tang et al., 1994; Cheng and Tang, 1998), consistent with our findings for this alkaloid in the hippocampus and cortex (inhibition of 49.2 and 62.1%, respectively). Also, the observed levels of inhibition are comparable to those reported for donepezil and tacrine (30–46%) by using the same analytical method (Kosasa et al., 1999). The competitive/noncompetitive mode of inhibition, as suggested by the Lineweaver–Burk plots, indicates that active alkaloids in extracts are able to interact with both the enzyme active center and the enzyme–substrate complex. Interestingly, compounds with notorious efficacy for the symptomatic treatment of AD, such as tacrine galanthamine and donepezil, are also of the mixed type of inhibition (Snape et al., 1999; Khalid et al., 2005), as well as some plant extracts with reported neuroprotective effects (Siqueira et al., 2003).

Increasing evidence supports the role of oxidative damage to biomolecules in the pathogenesis of AD patients, suggesting that antioxidants may be recognized as a potential treatment for the disease (Lleó et al., 2006). In this scenario, the benefits of multifactorial medicines have been advocated, stimulating the search for new anti-AChE and antioxidant drugs, especially those derived from natural sources. Relevant to this discussion, our *in vitro* antioxidant tests revealed that extracts had poor activity against the formation of hydroxyl radicals a harmful radical formed *in vivo*, but were more potent in decreasing the concentration of nitrite after the spontaneous decomposition of sodium nitroprusside, indicating that the alkaloids of AELC and AELT may be able to scavenge nitric oxide. The scavenging effect on DPPH, which is related to lipid peroxidation (Rekka and Kourounakis, 1991), revealed a stronger antioxidant effect for the extracts, and may be attributed to their capacity of trapping free radicals by donating a hydrogen atom. TRAP assay is commonly associated with the ability of a given compound to scavenge peroxyl radicals, superoxide or luminol-derived radicals (Lissi et al., 1992), indicating the presence of alkaloids in the assayed extracts with dose-dependent antioxidant action, although less potent than the reference scavenger alkaloid boldine.

Additionally, acute treatments with AELC or AELT in middle-aged mice were found to reduce oxidative stress damage in specific brain areas. This is an important step to verify a physiological contribution to the cellular defenses against free radicals, by detecting the interaction of the extracts with the antioxidant systems of the brain samples. Our data showed that extracts diminished the formation of TBARS, an index of lipid peroxidation, in the cortex, hippocampus and striatum. Moreover, both AE enhanced CAT activity, therefore avoiding the accumulation of hydrogen peroxide. As for SOD, this enzyme's activity was only significantly enhanced in the cerebellum after the administration of AELT, indicating that extracts did not reach the brain areas studied, at least in significant amounts, or even that the extracts have a selective capacity

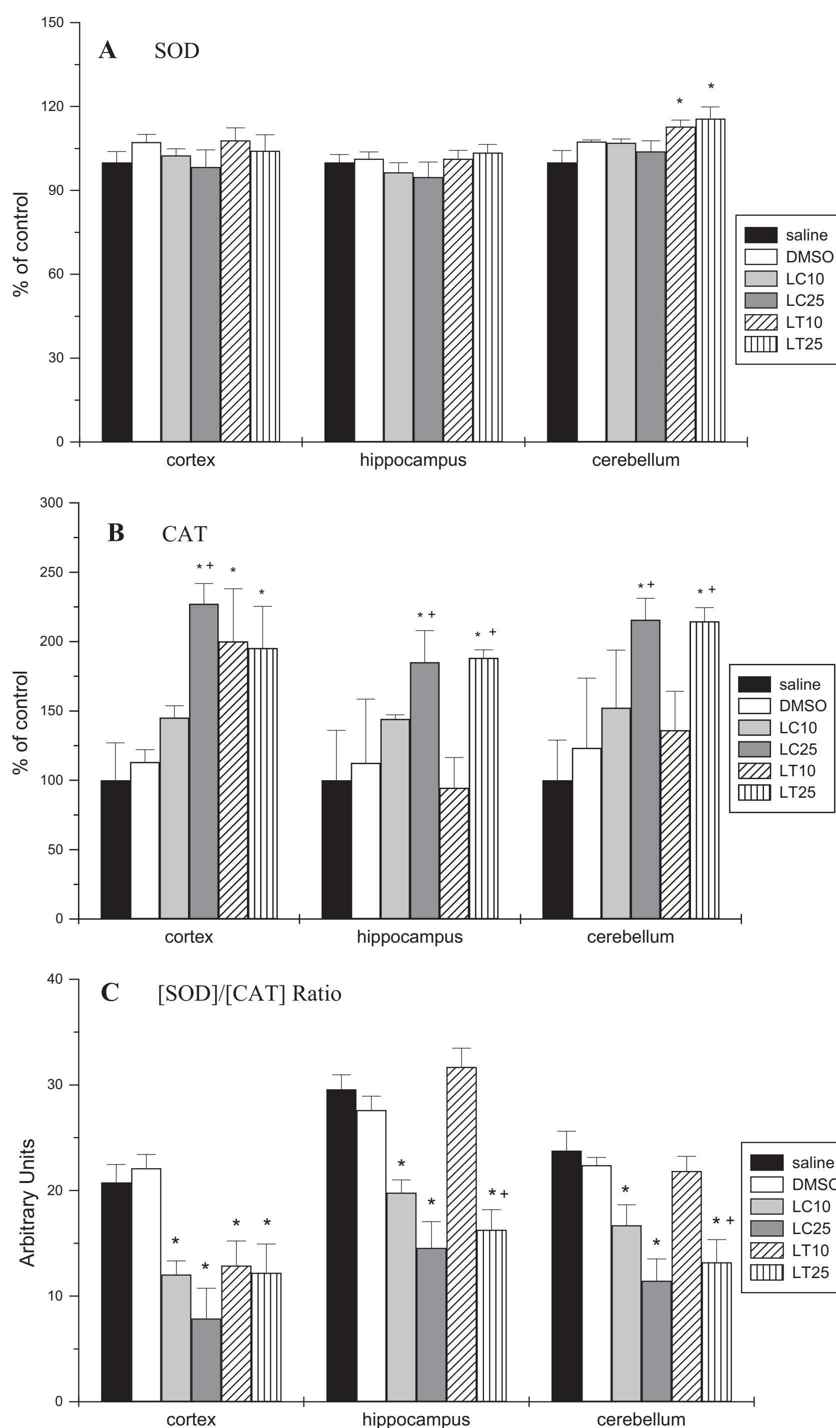


Fig. 6. Effects of AELC and AELT on antioxidant enzymes (SOD (A) and CAT (B)) activities and on [SOD]/[CAT] activity ratio (C) in brain regions from aging mice. Data is expressed as percentage of control DMSO (mean ± S.E.M.). The mean SOD values from saline groups were 19.5 ± 2.1 (cortex), 26.9 ± 1.2 (hippocampus) and 22.8 ± 2.4 U enzyme/mg protein (cerebellum). The mean CAT values from saline groups were 1.1 ± 0.08 (cortex), 1.0 ± 0.03 (hippocampus) and 1.2 ± 0.04 U enzyme/mg protein (cerebellum). Data are referred to five separated experiments run in triplicate (ANOVA followed by Duncan's test). $^{*}p < 0.05$ as compared to saline group, $^{+}p < 0.05$ as compared to previous dose.

for specific brain structures. The activity ratios between the antioxidant enzymes, such as SOD/CAT ratio, have been considered to be an index of oxidative status (Palomero et al., 2001). As such, the high activity of CAT combined with the decrease in SOD function in most brain regions might allow an imbalance in the physiologic generation of oxygen reactive species that in many cases act as cell signaling elements, compromising Ca^{2+} influx and the vesicular traffic through cell membranes (Halliwell, 2006; Forman, 2007).

Although some alkaloid extracts of Lycopodiaceae species, such as *Huperzia saururus* (Ortega et al., 2004) and *Lycopodium complanatum* (Orhan et al., 2009), have been already tested for *in vitro* AChE inhibitory activity, no study has been carried out on the anti-AChE effect for the constituents of the currently analyzed species, with the exception of anhydrolycodoline (IC_{50} = 191 μM) and lycodoline, which displayed poor effects against the enzyme (Halldorsdottir et al., 2010). Therefore, the inhibitory activities of the pure alkaloids are currently being studied in order to identify the active

compound(s) in the extracts. As expected, huperzine A and B were not detected in the AE, pointing out that the biological activity found may be due to other *Lycopodium* alkaloids. In addition, the constituents present in the alkaloid extracts, responsible for the anticholinesterase and antioxidant activities found after a single i.p. administration may have different activities and/or access different areas of brain, suggesting that pharmacokinetic studies to evaluate the distinct distribution of the compounds should be carried out.

The major finding of this study was that AELC and AELT demonstrate efficacy in crossing the blood–brain barrier, which may be an important property for medications for neurodegenerative diseases. In addition, alkaloid extracts had significant AChE inhibition and antioxidant effects (as demonstrated by *ex vivo* and *in vitro* animal models) in brain areas relevant to cognitive functions. Thus, this study provides further evidence that *Lycopodium clavatum* and *Lycopodium thyoides* possess multiple modes of action that are relevant to cognitive disorders, corroborating their traditional use by local communities in South America as medicines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2011.10.042.

References

Aebi, H., 1984. Catalase *in vitro*. Methods in Enzymology 105, 121–126.

Ayer, W.A., 1991. The *Lycopodium* alkaloids. Natural Product Reports, 455–463.

Ayer, W.A., Dikko, S., 1974. Alkaloids of *Lycopodium thyoides* and *L. contiguum*. Phytochemistry 13, 654–655.

Bisso, G.M., Briancesco, R., Michalek, H., 1991. Size and charge isomers of acetylcholinesterase in the cerebral cortex of young and aged rats. Neurochemical Research 16, 571–575.

Boveris, A., 1984. Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods in Enzymology 105, 429–435.

Cheng, D.C., Tang, X.C., 1998. Comparative studies of huperzine A, E2020, and tacrine on behavior and cholinesterase activities. Pharmacology, Biochemistry and Behavior 60, 377–386.

Cummings, J.L., Vinters, H.V., Cole, G.M., Khachaturian, Z.S., 1988. Alzheimer's disease: etiologies, pathophysiology, cognitive reserve and treatment opportunities. Neurology 51, S2–S17.

Draper, H.H., Hadley, M., 1990. Malondialdehyde determination as index of lipid peroxidation. Methods in Enzymology 186, 421–431.

Duke, J.A., Ayensu, E.S., 1985. Details of over 1200 medicinal plants of China and 579 brief details of their uses. Medicinal Plants of China, vol. II. Reference Publications Inc., Michigan, pp. 352–361.

Ellemann, L., 1990. Appendix to plants used by the Saraguros. In: Balslev, H., Øllgard, B. (Eds.), Plants Used by the Saraguros. Botanisk Institut Aarhus Universitet, Aarhus, pp. 1–46.

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

Fang, L., Kraus, B., Lehmann, J., Heilmann, J., Zhang, Y., Decker, M., 2008. Design and synthesis of tacrine–ferulic acid hybrids as multi-potent anti-Alzheimer drug candidates. Bioorganic and Medicinal Chemistry Letters 18, 2905–2909.

Ferri, C.P., Prince, M., Brayne, C., Brodati, H., Fratiglioni, L., Ganguli, M., Hall, K., Hasegawa, K., Hendrie, H., Huang, Y., Jorm, A., Mathers, C., Menezes, P.R., Rimmer, E., Scazufca, M., 2005. Global prevalence of dementia: a Delphi consensus study. Lancet 366, 2112–2117.

Forman, H.J., 2007. Use and abuse of exogenous H_2O_2 in studies of signal transduction. Free Radical Biology and Medicine 42, 926–932.

Francis, P.T., Palmer, A.M., Snape, M., Wilcock, G.K., 1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. Journal of Neurology Neurosurgery and Psychiatry 66, 137–147.

Giacobini, E., 2003. Cholinesterases: new roles in brain function and in Alzheimer's disease. Neurochemical Research 28, 512–522.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.K., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. Analytical Biochemistry 126, 131–138.

Halldorsdottir, E.S., Jaroszewski, J.W., Olafsdottir, E.S., 2010. Acetylcholinesterase inhibitory activity of lycopodane-type alkaloids from the Icelandic *Lycopodium annotinum* sp. *alpestre*. Phytochemistry 71, 149–157.

Halliwell, B., 2006. Oxidative stress and neurodegeneration: where are we now? Journal of Neurochemistry 97, 1634–1658.

Hermes-Lima, M., Wang, E.M., Shulman, H.M., Storey, K.B., Ponka, P., 1994. Deoxyribose degradation catalyzed by Fe(II) EDTA: kinetics aspects and potential usefulness for submicromolar iron measurements. Molecular and Cellular Biochemistry 137, 65–73.

Hurrel, J., de la Sota, E., 1996. Etnobotánica de las pteridófitas de los pastizales de altura de Santa Victoria (Salta, Argentina). Revista Museo La Plata XIV, Ser. Botánica, 343–364.

Kalaria, R.N., Maestre, G.E., Arizaga, R., Friedland, R.P., Galasko, D., Hall, K., Luchsinger, J.A., Ogunniyi, A., Perry, E.K., Potocnik, F., Prince, M., Stewart, R., Wimo, A., Zhang, Z., Antuono, P., 2008. Alzheimer's disease and vascular dementia in developing countries: prevalence, management, and risk factors. The Lancet Neurology 7, 812–826.

Khalid, A., Azim, M.K., Parveen, S., Rahman, A., Choudhary, M.I., 2005. Structural basis of acetylcholinesterase inhibition by triterpenoidal alkaloids. Biochemical and Biophysical Research Communications 331, 1528–1532.

Kosasa, T., Kuriya, Y., Matsui, K., Yamanishi, Y., 1999. Inhibitory effects of donepezil hydrochloride (E2020) on cholinesterase activity in brain and peripheral tissues of young and aged rats. European Journal of Pharmacology 386, 7–13.

Lahiri, D.K., Farlow, M.R., Greig, N.H., Sambamurti, K., 2002. Current drug targets for Alzheimer's disease treatment. Drug Development Research 56, 267–281.

Lissi, E., Pascual, C., Delcastillo, M.D., 1992. Luminol luminescence induced by 2,2′-azo-bis(2-amidinopropane) thermolysis. Free Radical Research Communications 17, 299–311.

Liu, J.S., Zhu, Y.L., Yu, C.M., Zhou, Y.Z., Han, Y.Y., Wu, F.W., Qi, B.F., 1986. The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. Canadian Journal of Chemistry 64, 837–839.

Lleó, A., Greenberg, S.M., Growdon, J.H., 2006. Current pharmacotherapy for Alzheimer's disease. Annual Review of Medicine 57, 513–533.

Loizzo, M.R., Tundis, R., Menichini, F., Menichini, F., 2008. Natural products and their derivatives as cholinesterase inhibitors in the treatment of neurodegenerative disorders: an update. Current Medicinal Chemistry 15, 1209–1228.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

Ma, X., Gang, D.R., 2004. The *Lycopodium* alkaloids. Natural Products Reports 21, 752–772.

Ma, X., Tan, C., Zhu, D., Gang, D.R., Xiao, P., 2007. Huperzine A from *Huperzia* species—an ethnopharmacological review. Journal of Ethnopharmacology 113, 15–34.

MacLean, D.B., 1985. *Lycopodium* alkaloids. In: Brossi, A. (Ed.), The Alkaloids: Chemistry and Pharmacology, vol. 26. Academic Press, Orlando, pp. 241–298.

Mensor, L.L., Menezes, F.S., Leitão, G.G., Reis, A.S., dos Santos, T.C., Coube, C.S., Leitão, S.G., 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytotherapy Research 15, 127–130.

Moreira, P.I., Siedlak, S.L., Aliev, G., Zhu, X., Cash, A.D., Smith, M.A., Perry, G., 2005. Oxidative stress mechanisms and potential therapeutics in Alzheimer's disease. Journal of Neural Transmission 112, 921–932.

Navarrete, H., León, B., Gonzales, J., Aviles, D.K., Lecaro, J.S., Mellado, F., Alban, J., Øllgaard, B., 2006. Helechos. In: Moraes, R., Øllgaard, B., Kvist, L.P., Borchsenius, F., Balslev, H. (Eds.), Botánica Económica de los Andes Centrales. Universidad Mayor de San Andrés, La Paz, pp. 385–411.

Nuñez, C., Cantero, J., 2000. Las plantas medicinales del sur de la província de Córdoba. Ed. Fundación Universidad Nacional Río Cuarto, Córdoba, pp. 1–144.

Øllgaard, B., 1992. Neotropical Lycopodiaceae—an overview. Annals of the Missouri Botanical Garden 79, 687–717.

Orhan, I., Terzioglu, S., Şener, B., 2003. α-onocerin: an acetylcholinesterase inhibitor from *Lycopodium clavatum*. Planta Medica 69, 265–267.

Orhan, I., Şener, B., Choudhary, M.I., Khalid, A., 2004. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. Journal of Ethnopharmacology 91, 57–60.

Orhan, I., Küpeli, E., Şener, B., Yesilada, E., 2007. Appraisal of anti-inflammatory of the clubmoss, *Lycopodium clavatum* L. Journal of Ethnopharmacology 109, 146–150.

Orhan, I., Ozcelik, B., Aslan, S., Kartal, M., Karaoglu, T., Şener, B., Terzioglu, S., Choudhary, M.I., 2009. *In vitro* biological activity screening of *Lycopodium complanatum* L. ssp. chamaecyparissus (A, Br.) Doll. Natural Product Research 23, 514–526.

Ortega, M.G., Agnese, A.M., Cabrera, J.L., 2004. Anticholinesterase activity in an alkaloid extract of *Huperzia saururus*. Phytomedicine 11, 539–543.

Palomero, J., Galán, A.I., Muñoz, M.E., Tuñon, M.J., González-Gallego, J., Jiménez, R., 2001. Effects of aging on the susceptibility to the toxic effects of cyclosporin A in rats. Changes in liver gluthatione and antioxidant enzymes. Free Radical Biology and Medicine 30, 386–845.

Rekka, E., Kourounakis, P.N., 1991. Effect of hydroxyethyl rutenosides and related compounds on lipid peroxidation and free radical scavenging activity-some structural aspects. Journal of Pharmacy and Pharmacology 43, 486–491.

Rollinger, J.M., Ewelt, J., Seger, C., Sturm, S., Ellmerer, E.P., Stuppner, H., 2005. New insights into the acetylcholinesterase inhibitory activity of *Lycopodium clavatum*. Planta Medica 71, 1040–1043.

Schauenberg, P., París, F., 1972. Guía de las plantas medicinales. Ed. Omega, Barcelona, pp. 1–364.

Siqueira, I.R., Fochesatto, C., da Silva, A.L., Nunes, D.S., Battastini, A.M., Netto, C.A., Elisabetsky, E., 2003. *Ptychopetalum olacoides*, a traditional Amazonian "nerve tonic", possesses anticholinesterase activity. Pharmacology, Biochemistry and Behavior 75, 645–650.

Skolnick, A.A., 1997. Old herbal Chinese medicine used for fever yields possible new Alzheimer disease therapy. Journal of the American Medical Association 277, 776.

Snape, M.F., Misra, A., Murray, T.K., de Souza, R.J., Williams, J.L., Cross, A.J., Green, A.R., 1999. A comparative study in rats of the *in vitro* and *in vivo* pharmacology of the acetylcholinesterase inhibitors tacrine, donepezil and NXX-066. Neuropharmacology 38, 181–193.

Sultana, R., Perluigi, M., Butterfield, D.A., 2006. Protein oxidation and lipid peroxidation in brain of subjects with Alzheimer's disease: insights into mechanism of neurodegeneration from redox proteomics. Antioxidants and Redox Signaling 8, 2021–2037.

Tang, X.C., Kindel, G.H., Kozikowski, A.P., Hanin, I., 1994. Comparison of the effects of natural and synthetic huperzine A on rat brain cholinergic function *in vitro* and *in vivo*. Journal of Ethnopharmacology 44, 147–155.

Thompson, S., Lanctot, K.L., Herrmann, N., 2004. The benefits and risks associated with cholinesterase inhibitor therapy in Alzheimer's disease. Expert Opinion on Drug Safety 3, 425–440.

Wang, R., Xiao, X.Q., Tang, X.C., 2001. Huperzine A attenuates hydrogen peroxide-induced apoptosis by regulation expression of apoptosis-related genes in rat PC12 cells. Neuroreport 12, 2629–2634.

Xiao, X.Q., Zhang, H.Y., Tang, X.C., 2002. Huperzine A attenuates amyloid β-peptide fragment 25-35-induced apoptosis in rat cortical neurons via inhibiting reactive oxygen species formation and caspase-3 activation. Journal of Neuroscience Research 67, 30–36.

Zhang, R.W., Tang, X.C., Han, Y.Y., Sang, G.W., Zhang, Y.D., Ma, Y.X., Zhang, C.L., Yang, R.M., 1991. Drug evaluation of huperzine A in the treatment of senile memory disorders. Acta Pharmacologica Sinica 12, 250–252.

Zhu, X., Raina, A.K., Lee, H.G., Casadesus, G., Smith, M.A., Perry, G., 2004. Oxidative stress signaling in Alzheimer's disease. Brain Research 1000, 32–39.