The anxiolytic-like effects of *Aloysia polystachya* (Griseb.) Moldenke (Verbenaceae) in mice

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Abstract

The aim of the present work is to demonstrate the putative sedative and anxiolytic-like effects of a hydro-ethanolic extract obtained from the aerial parts of *Aloysia polystachya* (Verbenaceae) in male mice using several behavioural assays. Groups of male mice orally treated with doses of 1.0, 10.0 and 100.0 mg/kg of the extract did not show any significant alteration of their locomotor activity, body temperature or motor coordination. The same treatment increased the duration of the sleeping time induced by 30.0 mg/kg i.p. of sodium pentobarbital. However, the sleeping time induced by ethyl ether was not modified by the oral administration of the extract, not confirming the putative sedative effect of the plant. The ethanolic extract also significantly increased the percentage of both entries (1.0 and 100.0 mg/kg) and the time spent (10.0 and 100.0 mg/kg) into the open arms of the elevated plus maze (EPM). Nevertheless, the binding of ^{3}H-flunitrazepam (^{3}H-FNZ) to the benzodiazepine binding site (BDZ-bs), in washed crude synaptosomal membranes from rat cerebral cortex, was not affected by the semi-purified components from *Aloysia polystachya*. These results indicate an anxiolytic-like profile of action for the extract of *Aloysia polystachya* without sedative side effect, being this activity probably mediated by other mechanism than BDZ-bs modulation at the $GABA_A$ receptors.

Keywords: *Aloysia polystachya*; Verbenaceae; Anxiolytic; Elevated plus maze; Hole-board; Open-field; Benzodiazepine binding assay

1. Introduction

The family *Verbenaceae* comprises about 175 genus and 2300 species, distributed in the tropics and subtropics, mainly in the temperate zone of Southern Hemisphere (Oliveira de Figueiredo et al., 2002). Mental ailments are heterogeneous diseases and will probably require a selected arsenal of drugs with different modes of action for successful treatment of their various manifestations (Baldessarini, 1990). Anxiety disorders are among the most prevalent of all psychological problems worldwide (Roselind Lieba et al., 2005). Benzodiazepines (BDZs) are considered safe drugs and are widely prescribed for their anxiolytic, muscle relaxant, sedative-hypnotic and anticonvulsant actions (Woods et al., 1992). However, they may produce side effects, such as sedation and myorelaxation that are considered as unwanted effects in an anxiolytic drug (Thiebot, 1985; Nutt, 1986; Griffiths and Sannerud, 1987; Kales et al., 1987; Miller et al., 1989; Izquierdo et al., 1990; Rickels et al., 1990; Pratt, 1991; Gallagher and Primus, 1992). On the other hand, the existence of natural flavonoids that possess anxiolytic effect not associated with myorelaxant, amnestic or sedative actions has been demonstrated (Medina and Marder, 1996; Marder and Paladini, 2002). Although alternative treatments are increasingly being used to alleviate affective disorders, strong evidence to recommend the use of herbal medicines for several illnesses is still scarce (Ernst, 2000).

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Aloysia polystachya (Griseb.) Moldenke (Verbenaceae), popularly named "burrito" in Paraguayan folk medicine, is a well-known medicinal plant which has been used for a wide variety of indications, including digestive and respiratory tract disorders (González Torres, 1996). Besides that, in a personal survey among users performed in Luque District, Paraguay, its use as sedative or to treat "nervous diseases" has been mentioned, similarly as reported by Del Vitto et al. (1997) in Argentina. Leaves of *Aloysia polystachya* are used, in Argentina, for respiratory diseases (colds and cough), gastrointestinal pain (Filipoy, 1994), as antiemetic (Martinez Crovetto, 1981) and sedative remedy (Del Vitto et al., 1997). Therapeutic actions of other species of *Aloysia* (i.e., *Aloysia triphilla*) include febrifuge, sedative, stomachic, diuretic and antispasmodic activities (Oliveira de Figueiredo et al., 2002). However, we found no scientific references or experimental evaluation regarding its central nervous system activity, as well as informations about the toxicity of *Aloysia polystachya*. Concerning its chemical composition, the presence of monoterpenes (carvacrol, carvone, eucarvone, limonene, (−)limonene, α-pinene, sabinene, (+)sabinene, (−)thujone, α-thujone, α-(−)thujone, β-thujone, isothujone, (+)isothujone), in leaf essential oil, was revealed by several studies (Fester et al., 1956; Huergo and Retamar, 1973; Gatto et al., 1981).

The present study was undertaken to determine the acute toxicity (LD_{50}), the general behavioural activity and the influence of the crude hydro-ethanolic extract of *Aloysia polystachya* (CEAp) on a variety of both in vivo and in vitro tests associated to anxiolytic effects. Our results encourage us to pursue the identification of the molecules associated to the anxiolytic effect observed in CEAp.

2. Materials and methods

2.1. Plant material

Aerial parts of cultivated *Aloysia polystachya*, in blossom, were collected in the Botanical Garden for Medicinal Plants of the Facultad de Ciencias Quìmicas, San Lorenzo, Paraguay, December 2001. A voucher herbarium specimen has been deposited at the Department of Botany under the number Ortiz 1498. Fresh samples were air-dried in the shadow and grounded, yielding 1474 g of powder which was extracted with a mixture of ethanol:water (60:40) by a conventional reflux method for 1 h at 50 °C in a bathing apparatus. The extraction was repeated two times and the filtered hydro-ethanolic extracts were mixed and evaporated under reduced pressure. The concentrated extract (CEAp) was frozen and finally freeze-dried to yield 208.7 g (14.48%) of dry extract which was used in all biological tests.

2.2. Animals

Swiss albino male mice, weighing between 20 and 30 g, kept under controlled conditions (12-h dark/12-h light cycle, 23–25 °C temperature and 50–60% humidity), were used. All experiments were conducted in accordance with international standards of animal welfare and the experimental protocols were approved by the local Animal Care and Use Committee (#23080.001 156/2001-50/UFSC). The minimum number of animals and duration of observation required to obtain consistent data were used. Behavioural experiments were conducted from 10:00 a.m. to 2:00 p.m. The animals received a standard food pellet and before the experiments they were fasted overnight with water ad libitum.

2.3. Drugs

Sodium chloride and Tris–HCl were obtained from Sigma Chemical Company (St. Louis, MO, USA), diazepam (Valium) from Roche Pharmaceutical Co., Ltd. (Argentina), pentobarbital (Nembutal) from Abbott (Japan), sodium thiopental from Fada, Biochemie Gesellschaft m.b.H., Kundl/Tirol (Austria), ^{3}H-flunitrazepam (^{3}H-FNZ) (81.8 Ci/mmol; New England Nuclear, NEN), dimethylsulfoxide, ethanol, ethyl ether, Tween 80 and propylenoglycol for pharmaceutical use were locally purchased.

2.4. Acute toxicity (LD_{50}) and effects on gross behaviour

CEAp was dissolved in distilled water and administered intraperitoneally (30.0, 100.0, 300.0, 600.0 and 1000.0 mg/kg) and orally (100.0, 300.0, 600.0, 1000.0, 2000.0 and 3000.0 mg/kg) to groups of 10 mice each. Animals were kept under observation for the consecutive 48 h. In a different experimental set of assays, the effect on spontaneous behaviour of the mice was performed using the Hippocratic procedure (Irwin, 1968). The behavioural profile of the animals under the influence of CEAp was studied by placing the animals individually into their home cages. Changes in behaviour and autonomic activity were evaluated by direct and simple observation and scored (grades from 0 to 4+). Groups of 10 adult albino mice were administered with: (A) vehicle or CEAp (1.0, 10.0, 100.0 and 1000.0 mg/kg) i.p. or (B) vehicle or CEAp (100.0, 300.0, 1000.0 and 3000.0 mg/kg) p.o. Mice were kept under observation for 2 weeks, being checked once a day a the same time period.

2.5. Effect on normal body temperature

The rectal temperature of each mouse was measured prior to the experiment and 1 h after oral administration of each dose of CEAp (1.0, 10.0 and 100.0 mg/kg, p.o.) using a thermistor thermometer model MGA-III type 219 (San-ei Instruments). Male mice with a basal rectal temperature between 36 and 38 °C prior to the experiment were selected for the assay. Temperature changes in body temperature values before and after drug administration were recorded.

2.6. Barbiturate-induced hypnosis

Animals (20–30 g) were distributed into groups of twenty animals *per* dose and CEAp (1.0, 10.0, 100.0 and 1000.0 mg/kg) and saline (0.1 mL/10 g body weight) were orally administered to each group, and after 60 min, each animal was injected with sodium pentobarbital (30.0 mg/kg, i.p.). Diazepam

(0.5 mg/kg i.p.) was used as positive control (standard anxiolytic/hypnosedative drug) in this assay. The latency to the loss of righting reflex (induction time in seconds) and the time required to recover righting reflex or awakening (sleeping time in minutes) were registered for each animal (Carlini, 1973). In the second set of experiments different groups of animals were treated with: (a) 1.0, 10.0, 50.0, 150.0 and 300.0 mg/kg of CEAp and (b) vehicle (DMSO, 5%; Tween 80, 0.25%, 20%; and saline), respectively, by i.p. route. After 20 min, each animal was injected with a sub-hypnotic dose of sodium thiopental (35 mg/kg i.p.), and the sleeping time was determined (Ferrini et al., 1974). For the i.p. administration, CEAp was dissolved by the sequential addition of dimethylsulfoxide up to a final concentration of 5%, to ensure complete dissolution, a solution of 0.25% Tween 80 up to a final concentration of 20% and saline to complete 100% of final volume. The i.p. route was used to improve the bioavailability of the chemical components of the extract. Sodium thiopental was dissolved only in saline. The volume of i.p. injections was 0.15 mL/30 g of body weight. In each session, a control group receiving only vehicle was tested in parallel to those animals receiving drug treatments.

2.7. Ether-induced hypnosis

To further evaluate the potentiation of hypnosis, 1 h after oral CEAp treatment, animals were placed in an ethyl ether (5 mL) saturated glass cage for 10 min (Vieira, 2001). The latency to the loss of righting reflex and the duration of sleep were recorded for up to 1 h using a stopwatch. Sleeping time was measured by the loss of the righting reflex, being the recovery of this reflex considered the hypnosis endpoint as previously described (Carlini et al., 1986). Diazepam was used as standard drug (positive control).

2.8. Open-field test

The open-field activity was measured in a Plexiglas cage (height: 17.0 cm; length: 30.0 cm; width: 30.0 cm) with black floor marked with white lines in 10 cm^2 areas. One hour after p.o. CEAp administration, each mouse was placed in the centre of the arena and its ambulation (peripheral and central area), rearing, grooming and defecation were recorded for 5 min (De Lima, 2002). The number of grid lines crossed by both hind feet in a 5-min period was counted as an index of ambulation. After each trial, the open-field apparatus was wiped clean with ethanol (10%) solution.

2.9. Hole-board test

The hole-board activity was measured in a Plexiglas cage (height: 15.0 cm; length: 40.0 cm; width: 40.0 cm) with black floor marked with white lines limiting areas of 10 cm^2. A total of 16 holes (diameter: 2.0 cm) in equidistant position were arranged in the arena. One hour after the p.o. CEAp administration each mouse was placed in the centre of the arena and its defecation, ambulation (peripheral and central area), rearing, grooming and head-dipping in the holes were recorded for 5 min (De Lima, 2002). After each trial, the hole-board apparatus was wiped clean with ethanol (10%) solution.

2.10. Rota-rod test

Mice were placed on a rotating rod (2.5 cm diameter divided in six equal compartments, rotating at 12 rpm). Animals remaining on the rod for 2 min in two successive trials (24 h before experiment) were selected for testing. Groups often mice were treated with: (A) vehicle (0.1 mL/10 g body weight p.o.), (B) doses of 1.0, 10.0, 100.0 and 1000.0 mg/kg of CEAp p.o. and (C) dose of 0.5 mg/kg i.p. of diazepam. After 60 min of the CEAp treatment, they were placed on the spinning bar of the rota-rod apparatus for 1 min. The time spent (in s) on the rotating rod was recorded (De Lima, 2002).

2.11. Elevated plus-maze test

The elevated plus-maze test has been widely validated to measure anxiety in rodents (Lister, 1987, 1990). The apparatus was made of transparent Plexiglas and consists of a plus-shaped maze formed by two opposite open arms (arm length: 30.0 cm; arm width: 5.0 cm); crossed with two arms enclosed by walls (height: 15.0 cm). The open and enclosed arms converge into a central platform (5.0 cm × 5.0 cm). The maze is elevated at 40.0 cm from ground level by wood bearing and it is placed in a room illuminated with red light (15 W). Each animal was placed on the centre of the apparatus, facing an enclosed arm. The total time spent on open arms, the number of open arms entries and the total entries into the enclosed arms were recorded scored for a period of 5 min. After each trial, the elevated plus-maze apparatus was wiped clean with ethanol (10%) solution.

2.12. Biochemical experiments

Analytical HPLC of fractions of 2 mg of CEAp were performed using a C18 reversed phase column (218TP54; The Separation Group, Hesperia, CA, USA). Elution was carried out with a lineal gradient of 10–40% acetonitrile in water, in 20 min and followed by a lineal gradient of 40–80% acetonitrile in water, in 10 min, at a flow rate of 1 mL/min. The detection was done at $\lambda = 280$ nm. The activity in the benzodiazepine binding assay of 2 mL fractions collected between the retention times of 5 min up to the end of the chromatogram was analyzed.

The binding of ^{3}H-FNZ (81.8 Ci/mmol; New England Nuclear, NEN) to BDZ-bs in washed crude synaptosomal membranes from rat cerebral cortex was carried out as described by Medina et al. (1990). For each assay of the inhibition experiments, triplicate samples of the membranes, containing 0.2–0.4 mg protein were suspended in a final volume of 1 mL of 25 mM Tris–HCl buffer, pH 7.4, in the presence of a solution of the sample assayed. The incubation was carried out at 4 °C for 60 min with 0.4 nM ^{3}H-FNZ. Non-specific binding was determined in parallel incubations in the presence of 10 mM FNZ, and represented 5–15% of the total. The assays were terminated by filtration under vacuum through Whatman GF/A glass-fibre filters, and three washes with 3 mL each of incubation medium.

Filters were counted after addition of Optiphase 'Hisafe' 3 (Wallac Company, Turku, Finland) liquid scintillation cocktail.

2.13. Statistical analysis

The results are expressed as mean ± S.D, and the statistical analysis of the data was performed by the Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA. Probability level less of 0.05 was considered as statistically significant.

3. Results

3.1. Acute toxicity, effects on general behaviour and body temperature of CEAp

Doses up to 3000.0 mg/kg p.o. and 1000.0 mg/kg i.p. of CEAp did not produce any evident toxic symptoms in mice for 2 weeks (Table 1). The dose of 100.0 mg/kg i.p. provoked abdominal writhing and piloerection, whereas the dose of 1000.0 mg/kg i.p. induced a slight decrease in locomotor activity and an increase of the breath frequency in the general behavioural studies. Body temperature of the animals was not modified by the CEAp administration (1.0, 10.0 and 100.0 mg/kg, p.o.).

3.2. Effect of CEAp on pentobarbital and ether-induced sleep

The effect on pentobarbital-induced sleep of oral administrations of CEAp is shown in Fig. 1. The latency values, up to the dose of 1000.0 mg/kg of CEAp, were not significantly different from vehicle-treated animals (Fig. 1A). CEAp, at doses of 1.0, 100.0 and 1000.0 mg/kg p.o., significantly augmented the sleeping time induced by pentobarbital ($P < 0.05$, Fig. 1B). The effect of CEAp on thiopental induced sleep is shown in Fig. 2. Doses of 150.0 or 300.0 mg/kg i.p. produced a significant increase in

Table 1
Effect of acute administration of increasing doses of *Aloysia polystachya* for assessing acute toxicity

Dose (mg/kg of CEAp[a])	Locomotion	Respiration	Autonomic symptom	Death
Oral (p.o.)				
100.0	0	0	0	0
300.0	0	0	0	0
600.0	+1	0	0	0
1000.0	+1	+1	0	0
2000.0	0	+1	0	0
3000.0	−1	+2	0	0
Intraperitoneal (i.p.)				
30.0	0	0	0	0
100.0	0	0	P	0
300.0	0	0	P	0
600.0	−1	+1	P	0
1000.0	−2	+3	P	0

No effect: 0; *increase effect*: slight +1, moderated +2, strong +3, severe +4; *decrease effect*: slight −1, moderated −2, strong −3, severe −4; P: piloerection.
[a] $N = 10$ animals/group.

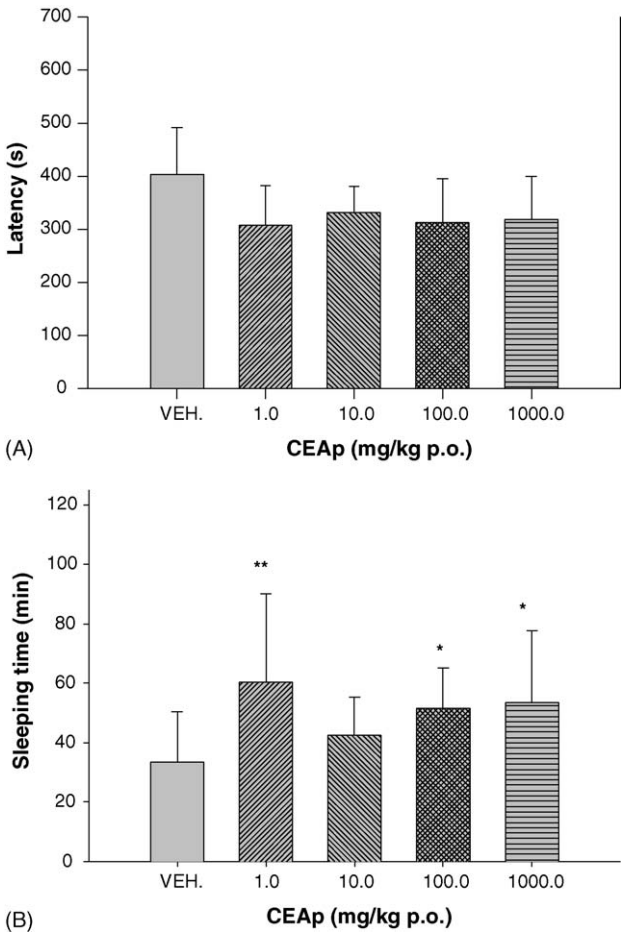

Fig. 1. Effect of the vehicle (0.1 mL/10 g body weight) (VEH), and increasing doses of CEAp (1.0, 10.0, 100.0 and 1000.0 mg/kg p.o.) of *Aloysia polystachya* on (A) latency and (B) the sleeping time induced by pentobarbital (30 mg/kg i.p.) in mice. Each bar represents the mean ± S.D. of 20 animals. $^{**}P < 0.01$; $^{*}P < 0.05$, significantly different from vehicle, Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA.

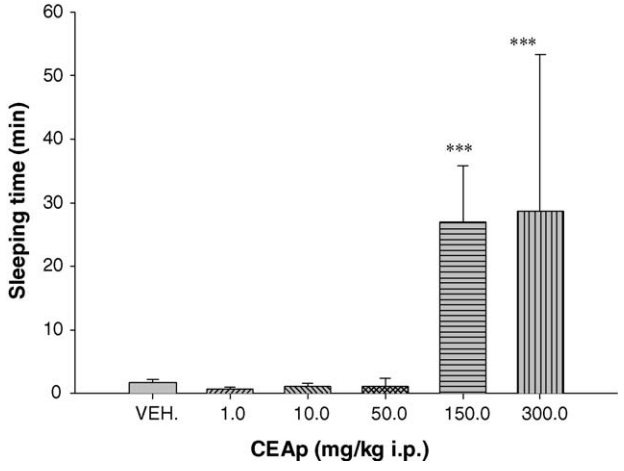

Fig. 2. Effect of the vehicle (0.15 mL/30 g body weight) (VEH), and increasing doses of CEAp (1.0, 10.0, 50.0, 150.0 and 300.0 mg/kg i.p.) of *Aloysia polystachya* on the sleeping time induced by thiopental (35 mg/kg i.p.) in mice. Each bar represents the mean ± S.D. of 6–10 animals. $^{***}P < 0.001$, significantly different from vehicle, Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA.

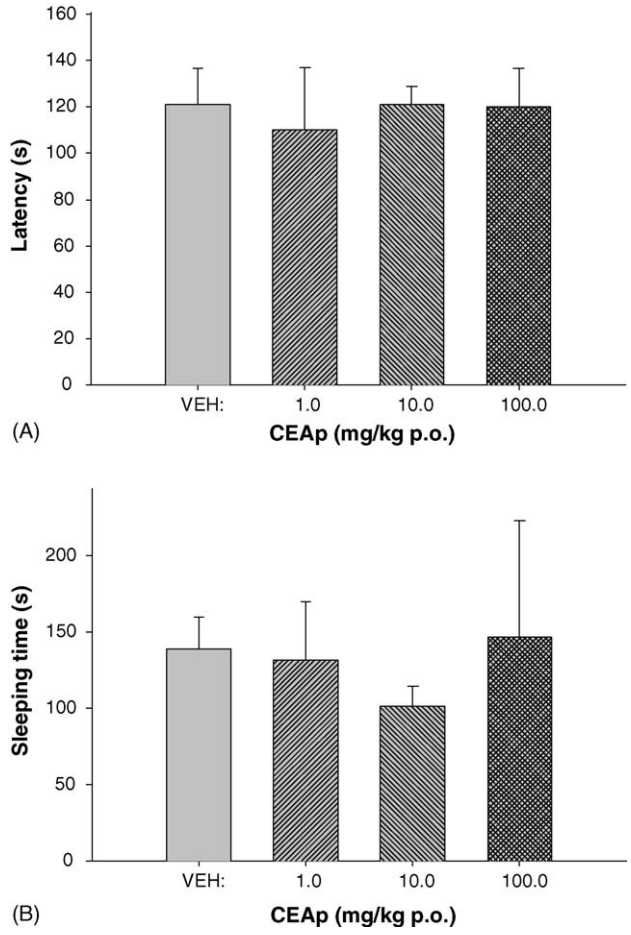

Fig. 3. Effect of the vehicle (0.1 mL/10 g body weight) (VEH), and increasing doses of CEAp (1.0, 10.0 and 100.0 mg/kg p.o.) of *Aloysia polystachya* on (A) latency and (B) the sleeping time induced by ethyl ether (5 mL/10 min) in mice. Each bar represents the mean ± S.D. of 20 animals. $^{*}P < 0.05$, significantly different from vehicle, Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA.

the sleeping time induced by thiopental ($P < 0.001$). On the other hand, CEAp produced no significant effect on the sleeping time induced by ethyl ether (Fig. 3).

3.3. Effect of CEAp on the open-field, hole-board and rota-rod tests

No differences in ambulatory (total, centre and peripheral) and emotional (rearing, grooming and defecation) behaviour of mice were observed in the open-field test (data not shown). Also, no effect on the motor coordination of mice was observed in the rota-rod study (data not shown). The effect of CEAp on the hole-board test is shown in Fig. 4. The dose of 10.0 mg/kg p.o. significantly increased the number of head-dipping behaviour ($P < 0.05$).

3.4. Effect of CEAp on the elevated plus-maze test

CEAp at doses of 1.0 and 100.0 mg/kg p.o. produced anxiolytic-like effects as determined by the increase in the percentage of open arm entries ($P < 0.05$ and <0.001, respectively;

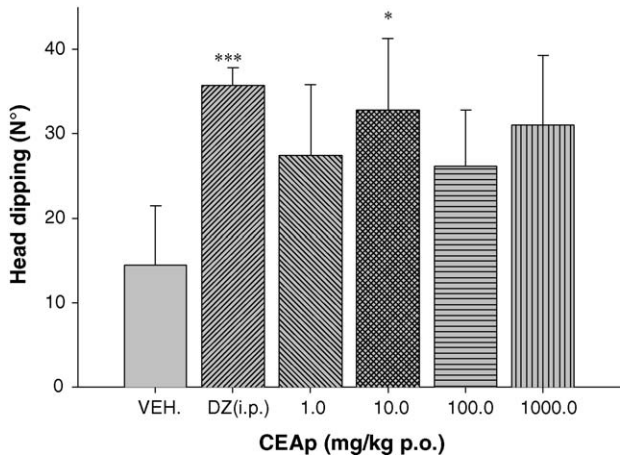

Fig. 4. Number of head dips registered in a 5 min session in the hole-board test performed 1 h after the administration of vehicle (VEH, p.o.), diazepam (DZ, 0.5 mg/kg, i.p.) or crude hydro-ethanolic extract of *Aloysia polystachya* (CEAp, 1.0, 10.0, 100.0 and 1000.0 mg/kg, p.o.). Each bar represents the mean ± S.D. of 10 animals. $^{*}P < 0.05$; $^{***}P < 0.001$, significantly different from vehicle, Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA.

Fig. 5A). Doses of 10.0 and 100.0 mg/kg p.o. also produced an increase in time spent in the open arms of the plus maze ($P < 0.01$, Fig. 5B). No changes were observed in the total arm entries. Conversely, the number of entries and the time spent in the enclosed arms were reduced by the CEAp treatment (Fig. 6A and B).

3.5. Effect of CEAp on the binding of ^{3}H-FNZ to BDZ-bs

Semi-purified fraction of CEAp, obtained from HPLC chromatograms of 2 mg of plant extract, were not able to displace ^{3}H-FNZ binding to synaptosomal membranes of rat cerebral cortex (Fig. 7).

4. Discussion

This study was aimed to analyze the behavioural effects of the crude hydro-ethanolic extract of the aerial parts of *Aloysia polystachya* (CEAp), a plant popularly used to treat "nervous diseases" in Paraguayan traditional medicine. The results here presented show that CEAp exhibits low toxicity, no lethality, was well tolerated by mice, did not induce any significant changes in several behavioural and physiological parameters, showed a slight decrease in spontaneous locomotor activity and an increase in breath frequency (Table 1).

Treatment with CEAp reduced the latency of induction and increased the duration of the barbiturate-induced sleep (Figs. 1 and 2). CEAp given alone did not produce any sedation per se indicating no CNS depressant activity (Fig. 4). Since the sleeping time induced by sodium pentobarbital (intermediate-acting) and sodium thiopental (short-acting) is related to its central depressant properties, but can be modified by other factors such as pharmacokinetic interferences, we have investigated the CEAp activity in the sleeping time induced by ethyl ether, a hypnotic drug without liver bio-transformation. Our results

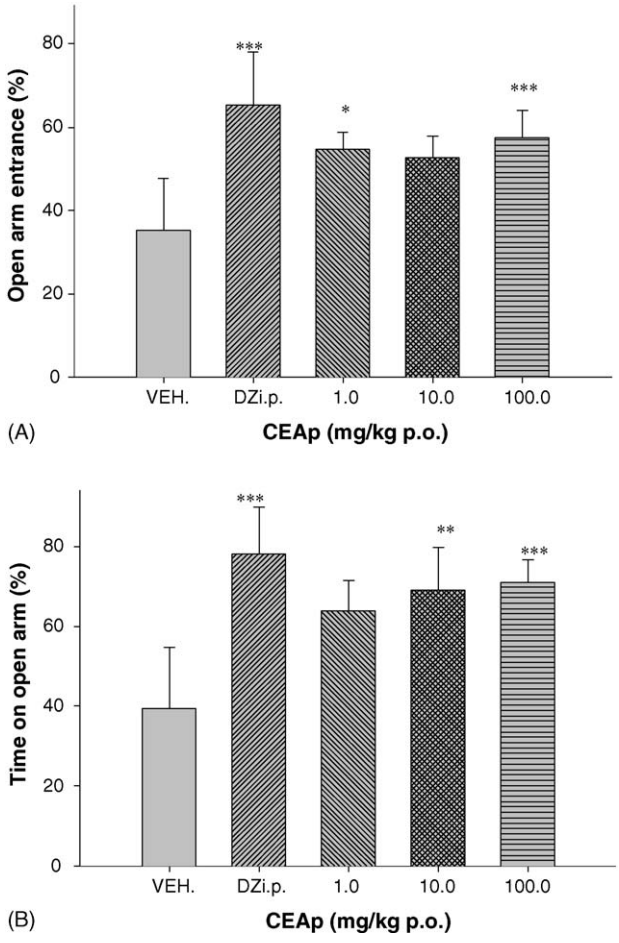

Fig. 5. Behavioural performance of mice registered in a 5 min session in the elevated plus maze performed 1 h after the injection of vehicle (VEH, p.o.), diazepam (DZ, 0.5 mg/kg, i.p.) or crude hydro-ethanolic extract of *Aloysia polystachya* (CEAp, 1.0, 10.0 and 100.0 mg/kg, p.o.). (A) Percentage of number of entries into the open arm and (B) percentage of time spent into the open arms. Each bar represents the mean ± S.D. of 10 animals. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA.

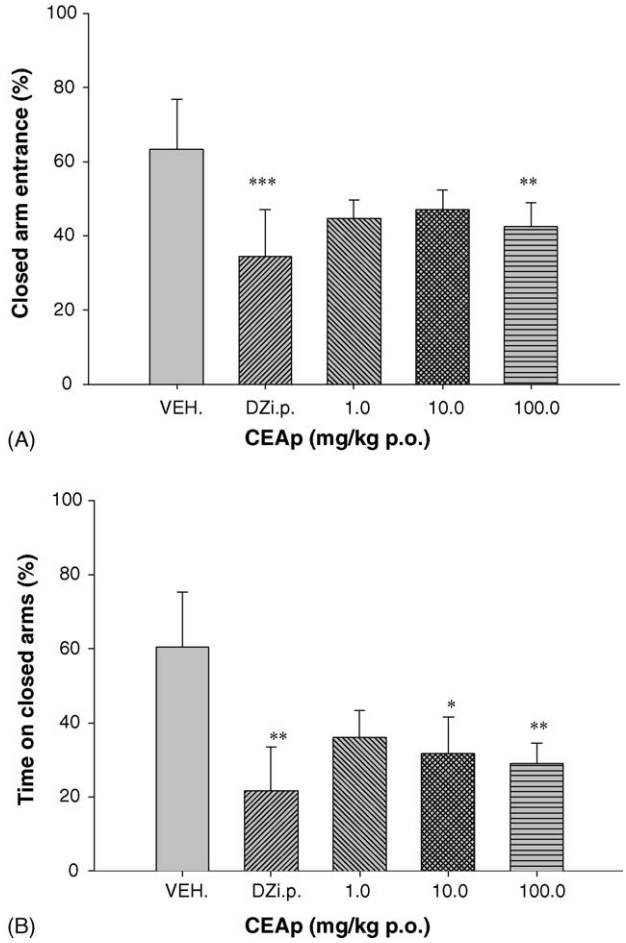

Fig. 6. Behavioural performance of mice registered in a 5 min session in the elevated plus maze performed 1 h after the injection of vehicle (VEH, p.o.), diazepam (DZ, 0.5 mg/kg, i.p.) or crude hydro-ethanolic extract of *Aloysia polystachya* (CEAp, 1.0, 10.0 and 100.0 mg/kg, p.o.). (A) Percentage of number of entries into the enclosed arm and (B) percentage of time spent into the enclosed arms. Each bar represents the mean ± S.D. of 10 animals. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, Dunn's Multiple Comparison test after Kruskal–Wallis non-parametric ANOVA.

showed no statistical difference between CEAp and vehicle-treated groups in the hypnosis induced by ethyl ether. These findings suggest that *Aloysia polystachya*, administered orally, is devoid of any hypnosedative activity and the potentiation of barbiturate-induced sleep observed here might probably be due to pharmacokinetic interactions between CEAp and pentobarbital. In this regard, there are few reports showing pharmacokinetic interaction of other plant species, such as *Smilax* sp., *Eucalyptus globulus*, *Blupeurum falcatum*, *Piper methysticum*, among others, with therapeutic drugs such as diazepam, alcohol, barbiturates and other psychopharmacological agents (Blumenthal et al., 1998; Cupp, 1999; WHO, 1999; Blumenthal, 2000). Both, pentobarbital and thiopental are metabolized in the liver by oxidative pathway that involves cytochrome P450, NADPH and molecular O_2 (Shah et al., 1996). A hypothesis to explain the enhanced barbiturate-induced sleep should be possibly an enzymatic inhibition of liver enzymatic system such as CYP 450 by CEAp which metabolizes intermediate and short-acting barbiturates. In this regard, administration of *Hypericum perforatum*, for example, affects the hepatic cytochrome P450 system, increasing the activity of its most abundant isoenzyme, CYP3A4, thereby probably lowering the activity of simultaneously administered drugs that are known substrates for this isoenzyme, including non-sedating antihistamines, oral contraceptives, certain anti-retrovirals, anti-epileptics, calcium channel blockers, cyclosporine, some chemotherapeutics, macrolide antibiotics and selected anti-fungals (Roby et al., 1999, 2000).

Results obtained in the open-field test showed that *Aloysia polystachya*, acutely administered, did not promote any ambulatory or emotional (rearing, grooming and defecation) changes of the mice, suggesting that this plant may not produce undesirable sedative side effects as shown by a reduction in locomotion, for example, which would agree to our previous observation with the enhanced barbiturate-induced hypnosis. Diazepam was applied as a positive control drug that, at the dose here used (anxiolytic), did not reduce locomotion in the open-field test when acutely

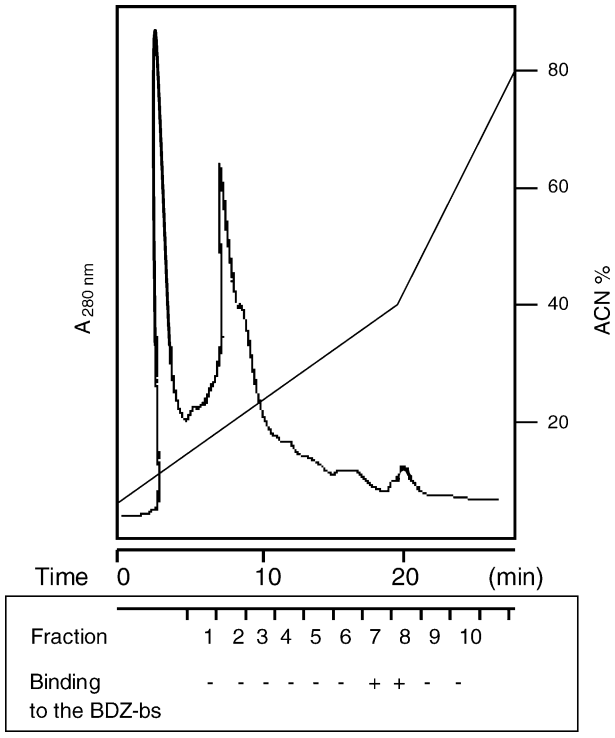

Fig. 7. Representative analytical HPLC chromatogram of the crude hydroethanolic extract of *Aloysia polystachya* (CEAp): HPLC fractionation was performed using 2 mg of CEAp in a LKB Pharmacia apparatus for analytical HPLC with a C18 reversed phase Vydac column. The extract was properly injected into the column and eluted using an aqueous acetonitrile (ACN) gradient, as indicated in the figure, at a flow rate of 1 mL/min. The detection was done at $\lambda = 280$ nm. The activity in the benzodiazepine binding assay of 2 mL fractions collected between the retention times of 5 min up to the end of the chromatogram was analyzed and are indicated as: inhibition = 10–30%, +, and inhibition <10%, −.

injected (Fisher and Hughes, 1996). A significant increase in the exploratory head-dipping behaviour was observed after the treatment with 10.0 mg/kg p.o. of the *Aloysia polystachya* extract, thus reinforcing the anxiolytic-like activity. No effect on motor coordination of mice was observed in the rota-rod studies confirming non-sedative or hynoptic-like activity, as seen in our results of ether-induced hypnosis.

On the other hand, several plants increase the exploration of open arms in the elevated plus-maze test and are used to diminish anxiety in folk medicine. Among them are *Panax ginseng* (Araliaceae; Bhattacharya and Mitra, 1991), *Passiflora coerulea* (Passifloraceae; Wolfman et al., 1994), *Matricaria recutita* (Compositae; Viola et al., 1995), *Azadirachta indica* (Meliaceae; Jaiswal et al., 1994), *Cassia siamea* (Caesalpiniaceae; Thongsaard et al., 1996), *Tilia tomentosa* (Tiliaceae; Viola et al., 1994), *Sesbania grandiflora* (Fabaceae; Kasture et al., 2000), *Zingiber officinale* (Zingiberaceae), *Ginkgo biloba* (Ginkgoaceae) (Hasenöhrl et al., 1996, 1998) and *Casimiroa edulis* (Molina-Hernández et al., 2004; Mora et al., 2005).

In this regard, in the elevated plus-maze test, the extract of *Aloysia polystachya* (1.0, 10.0 and 100.0 mg/kg, p.o.) increased the exploration and the time spent into the open arms (i.e., anxiolytic-like action) in a non-dose-related way. The number of entries and the time spent into the enclosed arms were reduced by the oral treatment with the CEAp (10.0 and 100.0 mg/kg) in comparison to the control values. The elevated plus-maze test is designed to detect the effect of anxiolytic drugs (Hogg, 1996). The apparatus has two narrow enclosed arms, which are bordered by high walls and two open arms that have essentially unprotected boards. Naive mice will normally prefer to spend much of their allotted time in the former. This preference appears to reflect an aversion towards open arms, generated by fear of open spaces (Rodgers and Dalvi, 1997). Drugs that increase the open arms exploration are considered anxiolytics and the reverse holds true for anxiogenic compounds (Handley and McBlane, 1993). In this study, diazepam was used as a positive control and, as expected, it increased the activity in the open arms of the elevated plus-maze apparatus, confirming its anxiolytic actions (Stock et al., 2000). Many benzodiazepines, and related compounds that bind to receptors in the central nervous system, have been already identified in certain plant extracts (Medina et al., 1990; Medina and Marder, 1996; Viola et al., 1994, 1995). In search of the underlying mechanism of anxiolytic-like effect of *Aloysia polystachya*, we have performed a complementary assay, the "in vitro" binding study in cerebral tissues. However, none of the fractions collected after analytical HPLC was able to displace [^{3}H]-FNZ to brain synaptosomal membranes, denoting that *Aloysia polystachya* acts probably by a different mechanism than the modulation of the benzodiazepine site of the $GABA_A$ receptors to induce its anxiolytic-like effect. Altogether, our results give support to the traditional use of *Aloysia polystachya* in Paraguayan folk medicine. This finding may provide important leads for the development of potent and selective anxiolytic agents which could also be devoid of any disadvantage arising from their oral uptake such as sedation. Studies are being carried out in our laboratories to isolate the active principle(s) of this plant, and to determine more specific activities of *A. polystachya* upon the central nervous system as well as to further investigate its underlying mechanism(s) of action.

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