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Artigo

Hypnotic effect of ecdysterone isolated from *Pfaffia glomerata* (Spreng.) Pedersen

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RESUMO: "Efeito hipinótico de ecdisterona isolada de *Pfaffia glomerata* (Spreng.) Pedersen". Neste trabalho foi avaliado, em roedores, o efeito depressor das frações clorofórmio ($CHCl_3$), acetato de etila (EtOAc) e n-butanol, obtidas das partes subterrâneas de *Pfaffia glomerata*, empregando-se o teste de tempo de sono barbitúrico como referência. Somente a fração lipofílica ($CHCl_3$:EtOAc, 1:1, m/m) (i.p. 500 mg/kg; v.o. 1000 mg/kg) potenciou o tempo de sono induzido por pentobarbital. A ecdisterona foi isolada e identificada como constituinte majoritário (1,4% m/m) desta fração, através de cromatografia líquida de alta eficiência e métodos espectroscópicos, respectivamente. Este composto potenciou o tempo de sono barbitúrico (100 mg/kg, i.p.; 400 mg/kg, v.o), sem causar hipotermia. Nestas mesmas doses, a ecdisterona não alterou a performance dos animais no rota-rod, esquiva inibitória e labirinto em cruz-elevado, além de não alterar o padrão de convulsões induzidas por pentilenotetrazol. Este perfil indica que esta substância, nestas doses, não apresenta perfil ansiolítico ou neurotóxico. Estes resultados indicam que a ecdisterona é o componente responsável pela ação hipnótica apresentada pela fração lipofílica obtida das partes subterrâneas de *P. glomerata*.

Unitermos: *Pfaffia glomerata*, Amaranthaceae, ecdisterona, efeito depressor, sistema nervoso central, tempo de sono induzido por pentobarbital.

ABSTRACT: In this study the depressant effect of fractions from *P. glomerata* was initially evaluated using the mice barbiturate sleeping time test as reference. The fractions tested were the $CHCl_3$, the EtOAc, the n-BuOH and the aqueous fraction obtained from *P. glomerata* subterraneous parts. Only the pretreatment with the lipophilic fraction ($CHCl_3$: EtOAc, 1:1, w/w) increased the barbiturate sleeping time (i.p 500 mg/kg; v.o. 1000 mg/kg). Ecdysterone, the main substance isolated from this lipophilic fraction, was identified by spectroscopic methods and its content in the ethanol extract was determined as 1.4% (w/w) by HPLC. In order to investigate the hypothesis of ecdysterone displaying a depressant effect on nervous central system, an evaluation toward the hypnotic-sedative and anxiolytic effects of this drug was carried out. Ecdysterone 100 mg/kg, i.p, increased the barbiturate sleeping time without provoking hypothermia; when administered by oral route its minimal effective dose was 400 mg/kg. On the other hand, ecdysterone (100 mg/kg, i.p; 400 mg/kg, p.o) did not impair motor coordination and was ineffective on pentylenetetrazole-induced convulsion, elevated plus-maze and step-down inhibitory avoidance tests, indicating that at these doses the drug does not present an anxiolytic profile and does not cause manifest neurotoxic effects as well. In conclusion, the lipophilic fraction from *P. glomerata* presents a hypnotic effect being ecdysterone one of the compounds responsible for this CNS activity.

Keywords: *Pfaffia glomerata*, Amaranthaceae, ecdysterone, central depressant effect, pentobarbital-induced sleeping time.

INTRODUCTION

The genus *Pfaffia* (Amaranthaceae) comprises about ninety species distributed through Central and South America, twenty-seven of them being described in Brazil (Taniguchi et al., 1997). *Pfaffia paniculata*, popularly known as "Brazilian ginseng" (Oliveira, 1986), is the most employed and commercialized species in Brazil as a surrogate for *Panax* spp. (ginseng - Araliaceae). Furthermore, the substitution of *P. paniculata* by *Pfaffia glomerata* is also common due to falsification or botanical misidentification (De-Paris et al., 2000). Recently some quality parameters to differentiate between *P. paniculata* and *P. glomerata* roots have been described considering their botanical and chemical characteristics (Gosmann et al., 2003). Ecdysterone (Figure 1) was only found in *P.glomerata*, as already described (Shiobara et al., 1993), so it seems that

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this compound could be a good marker for differentiation of both species. Allantoin, ecdysteroids, pfaffic acid and their glycosides (nortriterpene saponins), stigmasterol and sitosterol have been isolated from subterraneous parts of *Pfaffia* species (Nakai et al., 1984, Nishimoto et al., 1984, Takemoto et al., 1982).

Pharmacological studies with *P. glomerata* roots evidenced a gastroprotective effect probably mediated by histaminergic pathway and an enhanced production of nitric oxide in the stomach (Freitas et al., 2003, 2004). An ethanol extract of this species did not show antiviral, antiproliferative, antifungal or MAO inhibitory activities *in vitro* (Gosmann et al., 2003). A crude hydroalcoholic extract of *P. glomerata* roots presented analgesic and anti-inflammatory activities (Neto et al., 2005).

Regarding to the central nervous system action, the administration of a crude ethanol extract of *P. glomerata* by intraperitoneal route produces a depressor effect in the barbiturate sleeping time test and an amnesic effect in adult rodents (De-Paris et al., 2000; Vigo et al., 2003). On the other hand, Marques et al. (2004) reported a barbiturate sleeping time decrease and an improvement in learning and memory in old mice chronically treated.

The aim of this work was to evaluate the central nervous activity of *P. glomerata* fractions and its main isolated compound toward the hypnotic-sedative, anxiolytic and memory effects.

MATERIAL AND METHODS

Plant material

Pfaffia glomerata (Spreng.) Pedersen subterraneous parts were obtained from the cultivated area of the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA/UNICAMP, Campinas, SP, Brazil) and a voucher specimen is kept in the herbarium-UNICAMP (CPQBA 0238). Roots and rhizomes (subterraneous parts) from *P. glomerata* were reduced to small pieces, dried in a circulating air stove (40 ºC) and then triturated to powder.

Ethanol extract and fractions from *Pfaffia glomerata*

The ethanol extract from *P. glomerata* subterraneous parts was prepared as already described (De-Paris et al., 2000). The fractions from *P. glomerata* subterraneous parts (1000 g) were obtained using soxhlet during 12 h and, successively, solvents of increasing polarity to obtain the $CHCl_3$ (2 g, 0.2% w/w), the EtOAc (7 g, 0.7% w/w) and the n-BuOH (16 g, 1.6% w/w) fractions, which were evaporated, separately, to dryness. The remainder vegetal residue was submitted to decoction under stirring during 1 h, and then the resulting aqueous fraction (600 g, 60% w/w) was lyophilized. $CHCl_3$ and EtOAc fractions were pooled due to similar TLC profile. The fractions used in pharmacological experiments were named CAE ($CHCl_3$ and EtOAc, 1:1, w/w), BUT (n-BuOH) and AQU (aqueous).

Isolation of ecdysterone

The main constituent in the organic fraction was isolated from *P. glomerata* roots (1500 g) through soxhlet using EtOAc. EtOAc fraction was concentrated until half volume and cooled resulting in a precipitate with a major compound which was purified using $CHCl_3$ until obtaining a white powder (5 g, 0.3% yield, w/w, relating to the dried plant). The isolated product was identified as ecdysterone (ECD) (Figure 1) by spectroscopic and HPLC analysis. FAB-MS spectrum was performed on a MS50 spectrometer. ^1H and ^{13}C NMR spectra were recorded on Bruker AMX 500 spectrometer.

Ecdysterone: White powder. UV λ_{max} 242 (EtOH); FABMS *m/z* = 503.2 $[M + Na]^+$, 481.3 $[M + H]^+$; ^1H and ^{13}C NMR (500 MHz, C_5D_5N) the same as Nishimoto et al. (1987); HPLC: the same as under quantification. Chromatographic peak was identified at 242 nm by comparison of the retention time (3.40 min) and co-chromatography to ecdysterone Sigma®.

HPLC quantification of ecdysterone

The quantification of ecdysterone (ECD) present in the ethanol extract of *P. glomerata* was carried out in a liquid chromatograph Shimadzu LC-10A as already described using an HPLC methodology previously validated (Zimmer et al., 2005).

Animals

Adult male Wistar rats (weight 200-300 g) and adult male CF1 mice (weight 25-30 g) from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS, Brazil) breeding colony were used. The animals were housed in plastic cages, five by cage, under a 12 h light/dark cycle (lights on at 7:00 a.m.) at constant temperature of 23 ± 1 °C with free access to standard certified rodent diet and tap water. All experiments were performed between 10:00 and 16:00 h.

All experiments were approved by the Research Ethical Committee of Universidade Federal do Rio Grande do Sul (# 2003236).

Drugs and treatments

Pentobarbital sodium salt (PTB, Abbot®, São Paulo, SP, Brazil), pentylenetetrazole (PTZ, Sigma®, St. Louis, MO) and diazepam (DZP, Valium®, Roche®) were used.

The aqueous fraction, pentobarbital, pentylenetetrazole were dissolved in physiological saline (NaCl 0.9%). Other fractions, ecdysterone and diazepam were suspended in saline with the addition of polysorbate 80 at 1% v/v. All administrations were made in a volume of 1 ml/100 g body weight (mice), except for the inhibitory avoidance task, where the rats were treated with a 2 ml/kg volume. When oral route was used, all the animals were fasted for 6 h before testing.

Barbiturate sleeping time test

Different groups of mice were treated with different *P. glomerata* fractions or ecdysterone, saline (SAL), saline + polysorbate 80 1% (TWE) and diazepam by intraperitoneal and oral routes. Thirty minutes after intraperitoneal injection and 60 min after gavage, all groups received pentobarbital (40 mg/kg, i.p.) and the time elapsed between the loss and voluntary recovery of the righting reflex was recorded as sleeping time. A ceiling of 240 min was imposed in this measure, i.e., animals whose sleeping time was over 240 min were counted as 240 min. Sleep latency was also recorded. The room temperature was kept at 23 ± 1 °C.

Rota-rod motor coordination test

The rota-rod consisted of a cylinder of 3 cm of diameter at a height of 21 cm from the base. One day before testing, mice were placed on the cylinder for training during 5 min. On the test day, the animals were placed on the bar and selected based on their ability to remain at least 90 s continuously on the rotating bar at the speed of 5 rpm. Immediately after, the selected mice were treated with the test substances and replaced on the cylinder 60 min later. In both sessions, the parameters registered were number of falls and the maximum time of permanence on the bar through 5 min. The following groups were tested: TWE (saline + polysorbate 80 1%, n = 9), ecdysterone 400 mg/kg (n = 9), ecdysterone 800 mg/kg (n = 8) and diazepam (5 mg/kg, n = 10). All groups were treated orally.

Effect on pentylenetetrazole-induced convulsions

Groups of mice were treated with ecdysterone 100 mg/kg (n = 13), TWE (saline + polysorbate 80 1%, n = 14) and diazepam (1 mg/kg, n = 10) by intraperitoneal route. pentylenetetrazole (80 mg/kg i.p.) was given 30 min after the intraperitoneal administration. The latency and duration of the first convulsion and number of death were taken into account.

Elevated plus-maze

The elevated plus-maze consists of two open arms (30 x 10 cm) and two enclosed arms (30 x 10 x 15 cm), arranged in such a way that the two arms of each type were opposite one another. The maze is 50 cm

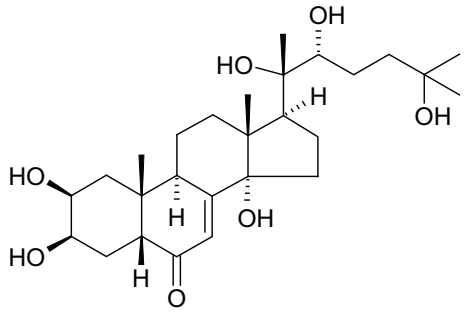

Figure 1. Ecdysterone.

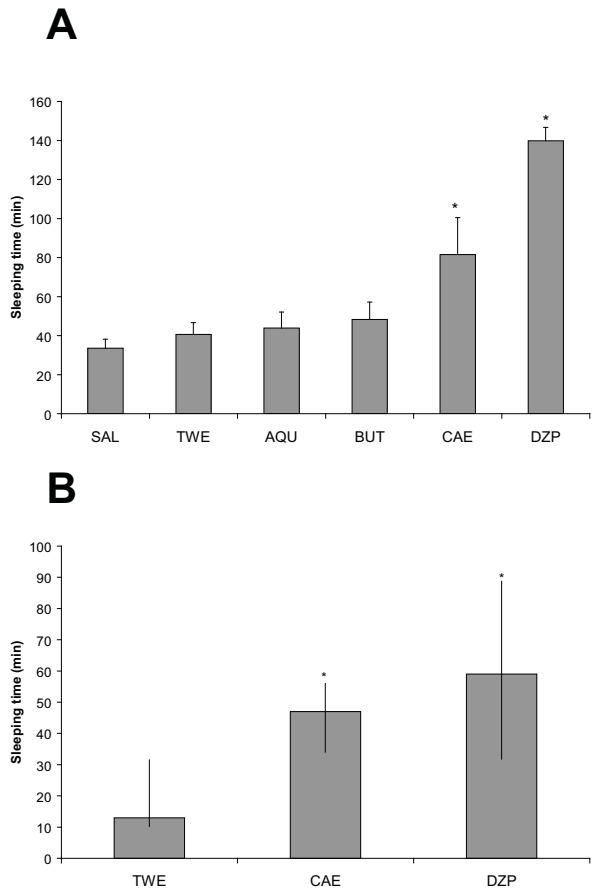

Figure 2. Effects of pretreatment with *P. glomerata* fractions (AQU, BUT and CAE) and diazepam (DZP) on sleeping time induced by pentobarbital (40 mg/kg i.p.) in mice.
A) Treatments: SAL - saline i.p. (n = 18); TWE - saline + polysorbate 80 1% i.p. (n =.13); AQU 500 mg/kg i.p. (n = 9); BUT 500 mg/kg i.p. (n = 9); CAE 500 mg/kg i.p. (n = 10); DZP 1 mg/kg i.p. (n = 16). The data are reported as mean ± standard error (*different from SAL and TWE groups. ANOVA; $F_{5,74}$=25.71; $p < 0.001$).
B) Treatments: TWE - saline + polysorbate 80 1% p.o. (n = 11); CAE 1000 mg/kg p.o. (n = 10); DZP 2 mg/kg p.o. (n = 13). The data are reported as median and interquartile intervals (*different from TWE group. Kruskal-Wallis; H = 12.65; p = 0.002).

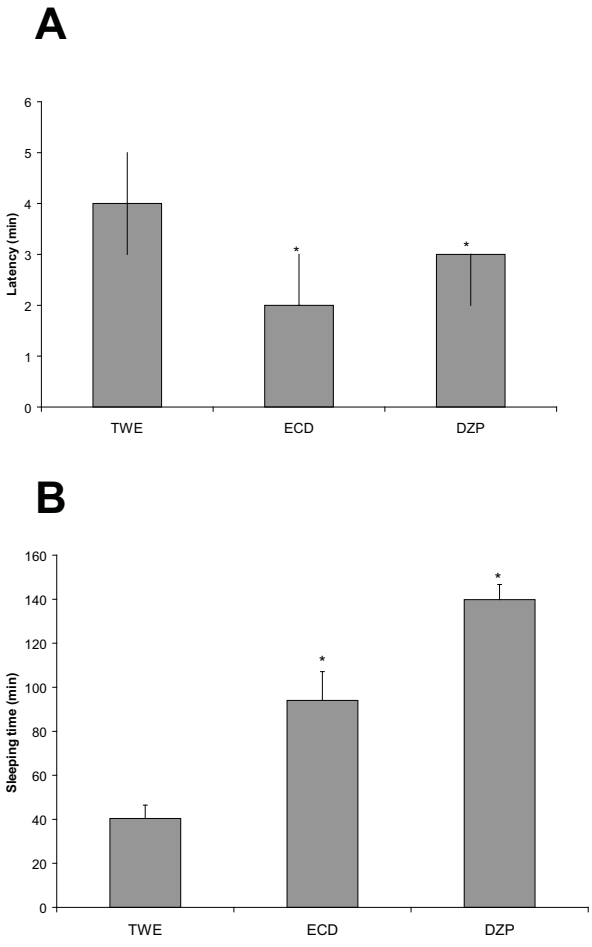

Figure 3. Effects of pretreatment with ECD (100 mg/kg i.p.; n = 12) and diazepam (DZP 1 mg/kg i.p.; n = 16) on latency (A) and sleeping time (B) induced by pentobarbital (40 mg/kg i.p.) in mice. Control group: TWE - saline + polysorbate 80 1% i.p. (n = 13).
A) The data are reported as median and interquartile intervals (*different from TWE group. Kruskal-Wallis; H = 17.16; $p < 0.001$).
B) The data are reported as mean ± standard error (*different from TWE group. ANOVA; $F_{2,40} = 33.63$; $p < 0.001$).

high and the tests were conducted under shadow. During a 5 min test period, the following measurements were recorded by two observers: the number of entries and the time spent in open and enclosed arms, the exploratory behavior (total number of arm entries) and the number of rearing and risk assessments of mice. The groups evaluated were: TWE (saline + polysorbate 80 1%, n = 8), ecdysterone 400 mg/kg (n = 8) and diazepam (2 mg/kg, n = 13). All groups were treated orally 60 min before the test.

Step-down inhibitory avoidance task

A 50 x 30 x 25 cm plastic box with a frontal glass wall and whose floor was made of parallel 10 mm caliber bronze bars was used. The left end of the grid

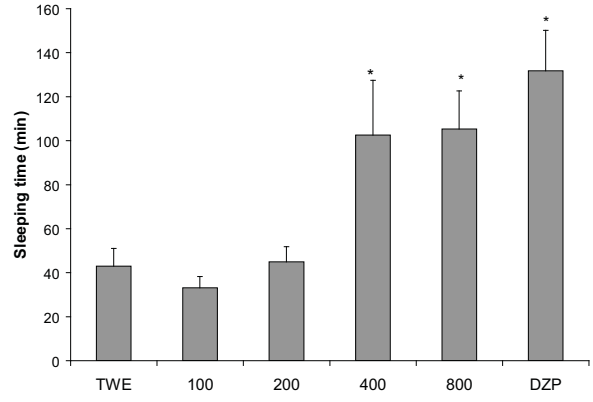

Figure 4. Effects of pretreatment with ECD (100, 200, 400 e 800 mg/kg p.o.; n = 12) and diazepam (DZP 2 mg/kg p.o.; n = 13) on sleeping time induced by pentobarbital (40 mg/kg i.p.) in mice. Control group: TWE - saline + polysorbate 80 1% p.o. (n = 13). The data are reported as mean ± standard error (*different from TWE group. ANOVA; $F_{5,69} = 8.29$; $p < 0.001$).

was occupied by a 10 cm wide, 4.5 cm high platform. The rats were gently placed on the platform facing the rear wall and their latency to step-down, placing their four paws on the grid was measured. After stepping-down the animals received a 0.4 mA, 2 s scrambled foot shock and were immediately withdrawn from the cage. Twenty-four hours later, the procedure was repeated but the foot shock was not given. Then, the step-down latency was taken a measure of memory retention. A ceiling of 180 s was imposed in this measure, i.e., animals whose test latency was over than 180 s were counted as 180 s. The following groups were tested: TWE (saline + polysorbate 80 1%, n = 8); ecdysterone 400 mg/kg (n = 9) and diazepam (2 mg/kg, n = 7). All groups were treated orally 60 min before the first session (training session).

Influence upon core temperature

The treatments were administered immediately

Table 1. Performance of mice treated with ecdysterone 400 mg:kg and 800 mg:kg p.o. (ECD), diazepam 5 mg:kg p.o. (BZD) and saline + polysorbate 80 1% p.o (TWE) in the rota-rod test.

	Number of falls		Maximum time of permanence (s)	
	T0	T60	T0	T60
TWE	2.2 ± 0.9	1.2 ± 0.6	213 ± 25	225 ± 22
BZD	2.6 ± 0.9	9.0 ± 2.0*	189 ± 27	106 ± 28 *
ECD 400	0.7 ± 0.4	3.1 ± 1.3	263 ± 12	197 ± 31
ECD 800	1.7 ± 0.5	1.6 ± 0.7	221 ± 22	207 ± 22

T0: immediately before treating; T60: sixty minutes after treating. The data are reported as mean ± standard error (*different from T0. Two way repeated measures ANOVA; $F_{1,71} = 5.09$; $p < 0.05$).

Table 2. Behavior of mice treated with ecdysterone 400 mg:kg p.o. (ECD) and diazepam 2 mg:kg p.o. (BZD) and saline + polysorbate 80 1% p.o (TWE) in the elevated plus-maze test.

	Entries (%) Open arms	Time spent (%) Open arms	Entries (%) Enclosed arms	Time spent (%) Enclosed arms
ECD	15	07	85	93
BZD	47	55	53	45
TWE	13	13	87	87

Table 3. Effects of ecdysterone 100 mg:kg i.p. (ECD) and diazepam 1 mg:kg i.p (BZD) and saline + polysorbate 80 1% i.p. (TWE) on PTZ (80 mg/kg)-induced convulsions in mice.

Group	Latency (s)	Duration (s)	Deaths (30 min)
TWE	67.0 (57.8 - 78.3)	1.0 (1.0 - 1.0)	2
ECD	76.5 (58.0 - 97.0)	1.0 (1.0 - 1.0)	1
BZD	-	-	0

The data are expressed as median and interquartiles ranges.

Table 4. Performance of rats treated with ecdysterone 400 mg:kg. (ECD), diazepam 2 mg:kg p.o. (BZD) and saline + polysorbate 80 1% p.o (TWE) in the step-down inhibitory avoidance task.

	Training (s)	Test (s)
TWE	7.7 ± 1.7	91.1 ± 31.4*
ECD	8.7 ± 2.4	64.6 ± 27.3*
BZD	4.1 ± 1.1	10.6 ± 5.3

***** The data are reported as mean ± standard error (*different from Training session. Two way repeated measures ANOVA; $F_{1,71} = 5.09$; $p < 0.05$).

after the determination of basal temperature as described by Neves et al. (2003). Subsequent measurements were done 15, 30, 60 and 90 min after the drug injection. The following treatments were used: saline (i.p. n = 8), *P. glomerata* ethanol extract (500 mg/kg, i.p., n = 10), ecdysterone 100 mg/kg, i.p. (n = 8) and apomorphine (1 mg/kg, i.p., n = 9).

Statistical analysis

All statistical analyses were done using the Sigma Stat software (version 2.03; Jandel Scientific Corporation®). The specific test was selected accordingly with the experimental design of each animal model. They are specified in the corresponding results.

RESULTS AND DISCUSSION

Previous results by our group indicated that the ethanol extract of *P. glomerata* presented maximal effect at 500 mg/kg i.p. in the barbiturate sleeping time test (De-Paris et al., 2000). Based on these results, the bioguided fractionation strategy was proceeded using the same animal model as reference test.

In order to characterize the *P. glomerata* extract, HPLC quantification of ecdysterone in the EtOH extract was determined as 1.4% (w/w) in relation to the dried extract.

The fractions CAE, BUT and AQU were administered by intraperitoneal route at 500 mg/kg and tested on the barbiturate sleeping test. Only the pretreatment with CAE increased the sleeping time (Figure 2A) without any effect on the latency to sleep (data not shown). This lipophilic fraction kept its effect on the sleeping time when orally administered (1000 mg/kg, Figure 2B). Following, ecdysterone, the main substance isolated from CAE, was tested. Ecdysterone 100 mg/kg i.p. caused a decrease in the latency and an increase on the sleeping time (Figures 3A and 3B). The same treatment did not alter the core temperature of mice (data not shown). These results indicate a depressant effect of ecdysterone. Thus, the effect of ecdysterone *per os* (100, 200, 400 and 800 mg/kg) on the pentobarbital-sleeping time was evaluated. Ecdysterone 400 mg/kg and 800 mg/kg, p.o., increased the sleeping time (Figure 4) but it did not change the latency to sleep in any tested dose (data not shown).

These results suggest that ecdysterone is responsible for the depressant effect of the ethanol extract on the barbiturate sleeping time test. The differences between the activities of the ethanol extract and ecdysterone when administered orally could be a consequence of the low systemic levels obtained for ecdysterone after the ethanol extract administration due to the drug poor bioavailability by this route in addition to its low content in this extract (1.4%).

Ecdysterone is a steroid molecule which is extensively studied as an insect's hormone. More recently some biological activities in mammals have been reported on normal and tumor cellular metabolism (Wu & Wang., 2003, Konovalova et al., 2002). Ecdysterone also improved the learning and memory in the Morris Water Maze and increased the expression of c-fos into the hippocampus of rats (Yang et al., 2004). Thus, it is plausible that ecdysterone represents a type of neuractive steroid (NAS).

The term neuroactive steroid (NAS) refers to steroids which, independent of their origin, are capable of modifying neural activities. NAS are involved in several psychiatric disorders, including depression syndromes, stress responses, anxiety disorders and memory processes and pre-menstrual syndrome (Amin et al., 2006). The neurosteroid dehydroepiandrosterone cause an increase in the sleep time induced by ethanol or pentobarbital (Melchior & Ritzman, 1992). Some NAS have been shown to exert hypnotic, sedative and anticonvulsive effects, mainly through $GABA_A$ receptor modulation (for review see Dubrovsky, 2005).

Thus the hypothesis of ecdysterone presenting effects on GABA system was investigated by testing the drug on animal models of anxiety, convulsions and memory which are recognized as useful tools to detect benzodiazepine-like or GABAergic drugs (Izquierdo &

Medina, 1997, Rodgers & Dalvi, 1997, Cooper et al., 1996): elevated plus-maze, PTZ-induced convulsions test and step-down inhibitory avoidance.

In addition, since substances with hypnotic-sedative action have a great potential to interfere with motor activity parameters the effect of ecdysterone (400 and 800 mg/kg v.o.) was evaluated in the rota-rod test. It did not alter any parameter evaluated (Table 1), demonstrating that it does not impair the motor coordination of the animals and does not manifest neurotoxic effects as well.

None of the parameters evaluated in the plus-maze test were modified by the pre-administration of ecdysterone (400 mg/kg v.o.) (Table 2). Ecdysterone (100 mg/kg i.p.) still did not protect the mice from the PTZ-induced convulsions (Table 3). Additionally, ecdysterone (400 mg/kg v.o.) did not incite any change in the step-down inhibitory avoidance rat's performance (Table 4). This test is a classical model to take measurements of memory with a strong aversive component. Thus, this result point out that probably ecdysterone is not the substance responsible for the amnesic effect previously reported to the ethanol extract (De-Paris et al., 2000).

With the results obtained so far it can be assumed that the liphofilic fraction from *P. glomerata* when acutely administered to adult rodents present a hypnotic effect that could be attributed to ecdysterone. This effect seems not to be mediated by the GABAergic system since ecdysterone was ineffective in animals models considered predictive of benzodiazepine-like or GABAergic effects.

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