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ScienceDirect
Pharmacology, Biochemistry and Behavior 86 (2007) 150–161
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Participation of dihydrostyryl-2-pyrones and styryl-2-pyrones in the central effects of *Polygala sabulosa* (Polygalaceae), a folk medicine topical anesthetic

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Received 28 July 2006; received in revised form 20 December 2006; accepted 22 December 2006
Available online 8 January 2007

Abstract

This study was undertaken to evaluate the psychopharmacological effects in mice of the hydroethanolic extract (HE), aqueous, hexane and ethyl acetate (EA) fractions, and 6-methoxy-7-prenyloxycoumarin, three dihydrostyryl-2-pyrones and three styryl-2-pyrones isolated from *Polygala sabulosa* (Polygalaceae), a folk medicine used as a topical anesthetic. In the elevated plus-maze test (EPM), the HE of *P. sabulosa* and its EA induced an increase in the percentage of time spent on, and in the frequency of entries into the open arms, as well as in the number of unprotected head-dipping, besides a reduction in protected stretch-attend postures, thus indicating an anxiolytic-like profile of action for this plant species. In the hypnosis test, HE and EA enhanced the duration of pentobarbital-induced sleep, a hypnosedative effect confirmed in ethyl ether-induced hypnosis. Moreover, both preparations reduced the duration of the first convulsion induced by pentylenetetrazol, besides decreasing the severity of the seizures. The dihydrostyryl-2-pyrones (**1**) and (**3**) as well as styryl-2-pyrones (**4**) and (**7**), centrally administered, showed a similar anxiolytic-like effect in the EPM test, while the dihydrostyryl-2-pyrone (**2**) and styryl-2-pyrone (**5**) were inactive at the doses used here. These results suggest that *P. sabulosa* is a herbal medicine which possesses anxiolytic-like, hypnosedative and anticonvulsant effects, and these central effects can be attributed to the presence of the dihydrostyryl-2-pyrone and styryl-2-pyrone compounds.
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Keywords: Phytomedicine; Polygalaceae; Dihydrostyryl-2-pyrones; Styryl-2-pyrones; Benzodiazepine-like action

1. Introduction

Anxiety disorders are the most prevalent of the psychiatric disorders (Lepine, 2002), with an average lifetime prevalence of 20–28% in different countries (Kesller et al., 2005; Wittchen and Jacobi, 2005), and thus demand considerable financial resources for their treatment. Currently, benzodiazepines and selective serotonin re-uptake inhibitors, among other antidepressant drugs, are often the first-choice therapy for anxiety. However, chronic administration of benzodiazepines may result in physiological dependence, and a severe withdrawal syndrome can occur with the abrupt end of treatment (O'Brien, 2005), while the antidepressants can cause several other adverse reactions (Khawam et al., 2006). Therefore, the development of new medications possessing anxiolytic effects without the drawbacks of benzodiazepines and antidepressants would be of great interest for the treatment of anxiety-related disorders. In this regard, medicinal plants could be an alternative and useful source of new medicines to treat mood disorders such as anxiety and depression (Beaubrum and Gray, 2000; Zhang, 2004).

Several plants have been reported to possess anxiolytic-like activity and have been used for this purpose in many countries. In particular, kava-kava (*Piper methysticum* Forst), a plant

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doi:10.1016/j.pbb.2006.12.019

species from the tropical Pacific island region, has been extensively used as a folk medicine to treat anxiety. Several studies showed that kava-kava has tranquilizing and anxiolytic properties (Smith et al., 2001; Rex et al., 2002; Garrett et al., 2003; Pittler and Ernst, 2003; Witte et al., 2005), those effects being attributed to kavalactones (Fig. 1), especially dihydrokavain (Smith et al., 2001). However, reports of alleged hepatotoxicity of commercial kava preparations caused alarm and forced several countries to remove these products from the market (Strahl et al., 1998; Escher et al., 2001; Russmann et al., 2001; Blumenthal, 2002; Brauer et al., 2003). Nevertheless, while the origins of kava toxicity remain to be elucidated they appear to be unrelated to kavalactones (Singh, 2005).

Polygala sabulosa A. W. Bennett (Polygalaceae), popularly named "timutu-pinheirinho", grows abundantly in the Southern Highlands of Brazil and has been used in regional folk medicine as a topical anesthetic. The phytochemical study of *P. sabulosa* revealed that the main difference between this and other species of the same genus is the presence of dihydrostyryl-2-pyrones and styryl-2-pyrones, compounds which present a structural skeleton very similar to that of the kavalactones found in kava-kava (Fig. 1). Due to these chemical constituents, it is likely that *P. sabulosa* presents central effects although its folk use is not related to psychopathologies. Thus, the present study investigated the in vivo actions of HE, aqueous, hexane or EA fractions, and 6-methoxy-7-prenyloxycoumarin, three dihydrostyryl-2-pyrones (**1**, **2** and **3**) and three styryl-2-pyrones (**4**, **5** and **7**) isolated from *P. sabulosa*, in mice evaluated in several validated behavioral models.

2. Material and methods

2.1. Animals

Male adult Swiss mice weighing 30–40 g were used in all experiments. Animals were maintained on a 12-h light–dark cycle (lights on at 7:00 a.m.) at constant room temperature (23 ± 2 °C). Mice were housed in groups (20 per cage) and had free access to food and water, except during the experiments. All animals were allowed to adapt to the laboratory conditions for at least one week before the behavioral assessment. Each animal was used just once. All experiments were conducted in accordance with the international standards of animal welfare recommended by the Brazilian Society of Neuroscience and Behavior (Act 1992) and approved by the local Committee for Animal Care in Research (#081CEUA and 23080.001156/2001–50/UFSC). The minimum number of animals and duration of observation required to obtain consistent data were employed.

2.2. Plant material

P. sabulosa was collected in Rancho Queimado (Santa Catarina State, Brazil), in February 2003, at the same place where it was previously collected and identified by Prof. Dr. Olavo de Araújo Guimarães. A voucher specimen (number 19640) was deposited in the Herbarium of the Department of Botany, Universidade Federal do Paraná (Curitiba, PR, Brazil).

2.3. Extraction and isolation

The whole plant was air-dried, ground to powder and extracted exhaustively at room temperature with 96% ethanol. The obtained extract was filtered and the solvent was removed under reduced pressure to give the HE, which was partitioned into *n*-hexane and ethyl acetate soluble fractions. The ethyl acetate fraction (EA) was subjected to successive silica gel column chromatography eluted with increasing amounts of ethyl acetate in *n*-hexane. The fractions obtained were then processed by flash chromatography or crystallization to afford the isolated compounds 6-methoxy-7-prenyloxycoumarin, three dihydrostyryl-2-pyrones (**1**, **2** and **3**) and three styryl-2-pyrones (**4**, **5** and **7**).

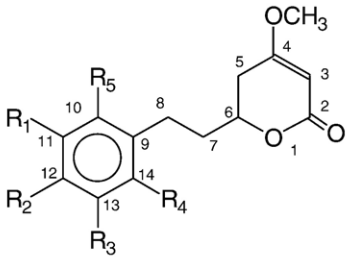
Detailed NMR spectroscopic analysis (^{1}H and ^{13}C) and comparison of the physical and spectroscopic data with those reported confirmed structures of 6-methoxy-7-prenyloxycoumarin, 4-methoxy-6-(11,12-methylenedioxydihydrostyryl)-2-pyrone (**1**), 4-methoxy-6-(11,12-methylenedioxy-14-methoxy-dihydrostyryl)-2-pyrone (**2**), 4-methoxy-6-(11,12-methylenedioxy-10,14-dimethoxy-dihydrostyryl)-2-pyrone (**3**), 4-methoxy-6-(11,12-methylenedioxystyryl)-2-pyrone (**4**), 4-methoxy-6-(11,12-methylenedioxy-14-methoxystyryl)-2-pyrone (**5**), 4-methoxy-6-(11,12-methylenedioxy-10,14-dimethoxystyryl)-2-pyrone (**6**) and 4-methoxy-6-(11,12-dimethoxystyryl)-2-pyrone (**7**), which we had previously described for this species (Pizzolatti et al., 2000, 2004).

2.4. Drugs and solvents

Ethyl ether (F. Maia Indústria & Comércio Ltda., Cotia, São Paulo, SP, Brazil), sodium pentobarbital (Lab. Abbott, São Paulo, SP, Brazil), pentylenetetrazol (Sigma Chemical Co., St. Louis, MO, USA) were used in the experimental paradigms. Diazepam (Dienpax®, Sanofi-Winthrop Lab., São Paulo, SP, Brazil) was used as a reference drug (positive control). Drugs were dissolved/suspended in saline (0.9% NaCl) immediately before intraperitoneal (i.p.) injection in a volume of 0.1 ml/10 g. HE, the three fractions and 6-methoxy-7-prenyloxycoumarin of *P. sabulosa* were freshly suspended in 10% Tween-80 and tap water, before each pharmacological test. Dihydrostyryl-2-pyrones and styryl-2-pyrones compounds were initially dissolved in 100% dimethylsulfoxide (DMSO) and subsequently diluted in sterile phosphate-buffered saline (PBS) (pH 7.4) to a final concentration of 0.8% DMSO and injected directly into the lateral ventricles of mice as described in the following section.

2.5. Treatments

Animals received, through an intragastric cannula (per os route, p.o.), HE, fractions or 6-methoxy-7-prenyloxycoumarin of *P. sabulosa* (250, 500 or 1000 mg/kg) in a constant volume of 0.1 ml/10 g. Control mice were treated by the same route and volume with vehicle, and the standard anxiolytic diazepam (DZP 0.75 to 2.5 mg/kg, i.p.) was used as the positive control drug.



Compound	R_1	R_2	R_3	R_4	R_5	C5-C6	C7-C8
DST (**1**)	OCH_2O		H	H	H	=	
DST (**2**)	OCH_2O		H	OCH_3	H	=	
DST (**3**)	OCH_2O		H	OCH_3	OCH_3	=	
STY (**4**)	OCH_2O		H	H	H	=	=
STY (**5**)	OCH_2O		H	OCH_3	H	=	=
STY (**6**)	OCH_2O		H	OCH_3	OCH_3	=	=
STY (**7**)	OCH_3	OCH_3		H	H	=	OCH_3
Methysticin*	H	OCH_2O		H	H		=
Dihydromethysticin*	H	OCH_2O		H	H		
Kavain*	H	H	H	H	H		H
7,8-dihydrokavain*	H	H	H	H	H		H
5,6-dihydrokavain*	H	H	H	H	H	=	H
Yangonin*	H	OCH_3	H	H	H	=	H

Fig. 1. Chemical structure of dihydrostyryl-2-pyrones and styryl-2-pyrones identified in *Polygala sabulosa* and kavalactones found in *Piper methysticum*. *Main kavalactones (Bilia et al., 2004). Abbreviations: DST = dihydrostyryl-2-pyrone, STY = styryl-2-pyrone.

The isolated compounds dihydrostyryl-2-pyrones (**1**), (**2**) or (**3**), and styryl-2-pyrones (**4**), (**5**) or (**7**) (0.3 fmol–25 pmol) were injected into one of the brain's lateral ventricles (i.c.v.) in a constant volume of 2 μl. Another group of animals received i.c.v. injections of DZP (7–20 ηmol), as the standard anxiolytic drug (Teixeira et al., 1996). Control mice were similarly treated with vehicle alone (0.8% DMSO in PBS) and tested in parallel with the treated animals. All i.c.v. injections were carried out using the "free-hand" technique as described by Laursen and Belknap (1986) and previously employed by our group (Ribeiro and De Lima, 1998). In brief, under light ether anesthesia (i.e. just sufficient for loss of the postural reflex), a 27 gauge needle attached to a 5 μl Hamilton syringe was inserted perpendicularly 3 mm deep through the skull, at a position 2 mm lateral to the midline on a line drawn through the anterior base of the ears. Each animal received only one i.c.v. injection. Upon termination of the experiment, each mouse was decapitated and its brain examined freshly. Results from mice presenting misplacement of the cannula or any signs of cerebral hemorrhage were excluded from the statistical analysis (less than 5% of the animals overall).

2.6. Behavioral tests

2.6.1. Elevated plus-maze test (EPM)

The putative anxiolytic-like activity of extract, fractions and compounds isolated from *P. sabulosa* was assessed using the EPM, as proposed by Lister (1987). This experimental paradigm shows several advantages over other tests in measuring anxiety (Dawson and Tricklebank, 1995). The test is based on the natural aversion of rodents to open spaces. Briefly, the EPM apparatus was made of clear Plexiglas and consisted of two opposed open arms (30 × 5 × 0.25 cm) and two opposed closed arms (30 × 5 × 15 cm), all extending from a central platform (5 × 5 cm), elevated 45 cm from the floor. The apparatus was placed in a small closed room lit by a 15 W red light. Sixty minutes after the several oral treatments (for DZP i.p., 30 min), each mouse was placed on the central platform, facing a closed arm, and observed for a 5-min period. The frequencies of entries into either open or enclosed arms, as well as the time spent in each arm type were recorded. An entry was scored as such only when the animal placed all 4 limbs into any given arm. The ratios "time spent in the open arms/time spent in all (i.e. open and closed) arms" and "frequency of entries into open arms/total entries into all arms" were calculated and multiplied by 100, to yield the percentage of time spent in and of frequency of entries into open arms, respectively. Both parameters are considered to reflect fear-induced inhibition from entering the open arms and drugs with anxiolytic activity usually increase the time spent in and/or the frequency of entries into open arms, whereas the reverse holds true for anxiogenic-like drugs. Furthermore, the number of entries into enclosed arms was used as an index of general activity (Rodgers and Dalvi, 1997).

2.6.2. Pentobarbital-induced hypnosis

To evaluate the potentiation of the barbiturate hypnosis, sodium pentobarbital was administered i.p. at the dose of 50 mg/kg, 1 h after the different treatments. The latency to the loss of righting reflex (in s) and the duration of sleep (in min) were recorded for each animal (Carlini et al., 1986), with a cutoff of 3 h and 10 h to evaluate the effects of HE and EAF, respectively.

2.6.3. Ethyl ether-induced hypnosis

Groups of mice were treated p.o. with the different preparations of *P. sabulosa* or vehicle and 1 h later the animals

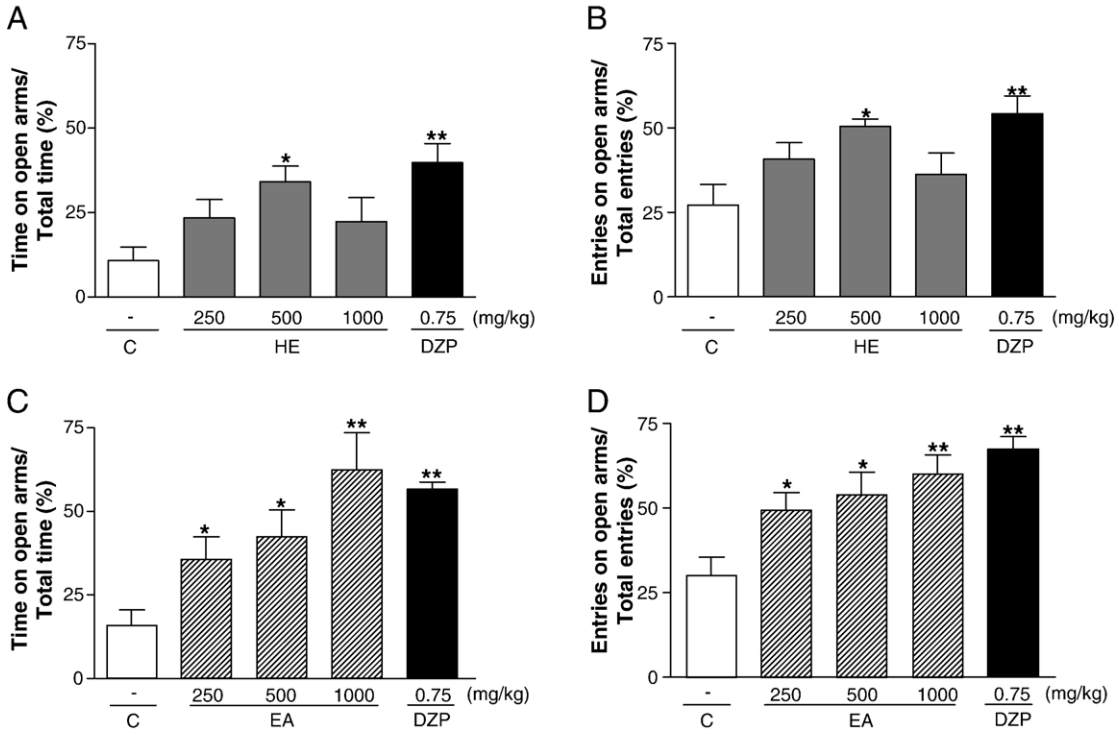


Fig. 2. Effects of the hydroethanolic extract (HE) or ethyl acetate fraction (EA) (250–1000 mg/kg, p.o.) of *Polygala sabulosa* on the behavior of mice evaluated in the elevated plus-maze test, recorded for 5 min, 1 h after p.o. administration. Diazepam (DZP 0.75 mg/kg, i.p.) was used as the standard anxiolytic drug. Percentage of time spent on open arms (panels A and C) and percentage of entries on open arms (panels B and D) are shown. Each value represents the mean ± S.E.M. of 6–10 animals. $^{*}P \leq 0.05$ and $^{**}P \leq .01$ as compared to control (one-way ANOVA followed by Dunnett's test).

were placed in an ethyl ether (5 mL, by 5 min of saturation) saturated glass cage (30 × 20 cm) as described by Vieira (2001). The latency to the loss of righting reflex and the duration of sleep (in s) were recorded using a stopwatch. Sleeping-time was measured by the loss of the righting reflex, with the recovery of this reflex being considered the hypnosis endpoint as previously described (Carlini et al., 1986). DZP (0.75 or 1 mg/kg, i.p.) was used as the positive control drug (standard anxiolytic/hypnose-dative compound) in both assays (pentobarbital and ethyl ether-induced sleep).

2.6.4. Pentylenetetrazol (PTZ)-induced convulsions

PTZ at 80 mg/kg was administered i.p. to groups of mice pretreated (1 h) with water, HE or EA of *P. sabulosa*. The latency (in s) to the first convulsive episode (clonic or tonic/clonic convulsion) as well as its duration was recorded. The severity of convulsions was also registered up to 30 min, using the following scale: grade 0 (no response), grade 1 (myoclonic body jerks), grade 2 (generalized clonic convulsion), grade 3 (generalized clonic jerks with loss of righting reflex), grade 4 (generalized convulsion with tonic extension), grade 5 (generalized convulsion with tonic extension and death) (Swinyard et al., 1952; Czuczwar and Frey, 1986).

2.6.5. Rota-rod test

Pre-selected mice (animals that stayed for at least 1 min on the rotating bar in a session of 2 min, 24 h before testing) were placed on the horizontal rotating bar (diameter 2.5 cm, 12 r.p.m.) of the rota-rod apparatus (in-house built), 1 h after the treatments. Total time spent on the rotating bar during a 1 min session was registered using a stopwatch, and the number of falls during the session was also recorded (Dunham and Miya, 1957).

2.6.6. Rectal temperature

The temperature was measured by inserting a thermistor probe 2 cm into the rectum of the animals. Digital recording of the temperature was made with an accuracy of 0.1 °C by means of a Dixtal® digital thermometer. The probe, dipped into silicon oil before inserting, was held in the rectum till a stable rectal temperature was measured for 20 s. The rectal temperature was recorded twice, before (T_1) and 1 h after the treatments (T_2). The temperature variation (ΔT) was estimated as $\Delta T = T_2 - T_1$ for each animal (Carlini et al., 1983).

2.7. Experimental procedures

In Experiment 1, mice were treated with HE (250, 500, and 1000 mg/kg, p.o.) 1 h before their submission to the EPM test, pentobarbital-induced sleeping-time, PTZ-induced convulsions, rota-rod test or rectal temperature measurement.

In Experiment 2, mice were treated with HE (500 mg/kg, p.o.), aqueous and hexane fractions as well as the 6-methoxy-7-prenyloxycoumarin (250, 500 and 1000 mg/kg, p.o.) or EA obtained from *P. sabulosa* (125, 250, 350, 500, 750 and

Table 1
Effects of p.o. administration of the hydroalcoholic extract (HE) or ethyl acetate fraction (EA) of *Polygala sabulosa* on several behavioral parameters evaluated in the elevated plus-maze (EPM) test or in the pentylenetetrazol-induced convulsions (PTZ)

Drugs	Dose (mg/kg)	EPM				PTZ	
		uHD	pSAP	OAEA	*N*	Severity	*N*
C	–	6.00±1.50	12.44±1.65	2.00±0.87	9	20.00±1.73	13
HE	250	12.11±2.86	4.67±1.47**	2.78±0.91	9	16.25±2.02	8
HE	500	15.87±1.83*	3.25±1.11**	5.38±0.78	8	13.86±1.67*	14
HE	1000	9.78±2.25	4.78±0.94**	2.67±0.82	9	13.11±1.45*	9
DZP	0.75	17.89±2.57**	6.00±1.24**	13.22±2.63**	9	1.60±0.51**	5
DZP	1	–	–	–	–	0.00±0.00**	5
C	–	4.70±1.58	16.80±1.37	2.33±0.99	10	10.77±1.76	13
EA	250	12.25±3.44	7.25±1.84**	5.38±1.03	8	12.13±2.89	8
EA	500	19.25±4.15**	7.12±2.36**	8.00±2.19*	8	4.29±1.48*	7
EA	1000	25.43±4.78**	3.86±1.74**	8.80±2.65**	7	4.00±1.11*	7
DZP	0.75	23.83±2.59**	4.17±0.98**	16.50±1.48**	6	2.00±0.58**	5
DZP	1	–	–	–	–	0.00±0.00**	5

Each value represents the mean±S.E.M. for 6–10 animals (EPM test) or 5–14 (PTZ-induced convulsions) (*N* = number of animals). *$P<0.05$ or **$P<0.01$ as compared to respective control group. Data analyzed by one-way ANOVA followed by Dunnett's test. Abbreviations: C = control, HE = hydroethanolic extract, EA = ethyl acetate fraction, DZP = diazepam, uHD = unprotected head-dipping, pSAP = protected stretch-attend postures, OAEA = open-arms end activity.

1000 mg/kg, p.o.) and after 1 h they were subjected to ethyl ether-induced hypnosis. This test was used in order to bio-guide the purification and the isolation of the active constituents of *P. sabulosa*. Mice were treated either with vehicle or DZP (0.75 mg/kg or 1 mg/kg, i.p.) as control and positive control, respectively.

In Experiment 3, mice were treated with the EA (250, 500, and 1000 mg/kg, p.o.) 1 h before their submission to the EPM, pentobarbital-induced hypnosis, PTZ-induced convulsions, rota-rod test or rectal temperature measurement.

In the fourth experiment, mice received i.c.v. injections of vehicle or the dihydrostyryl-2-pyrones (**1**), (**2**) and (**3**) or styryl-2-pyrones (**4**), (**5**) and (**7**), at doses of 0.3 fmol to 25 pmol, and, 5 min after postural recovery, they were submitted to the EPM test. The styryl-2-pyrone compound (**6**) was not tested due to an extremely low yield in the chemical extraction process which resulted in an insufficient quantity to allow in vivo testing.

2.8. Statistical analysis

All results are presented as mean±S.E.M. and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test, with treatment being the independent variable. All statistical analysis was carried out using *Statistica*® version 6.0. Differences between treated and control groups were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. Effects of the treatment with HE of P. sabulosa

3.1.1. Elevated plus-maze

As shown in Fig. 2, p.o. administration of HE 500 mg/kg induced a significant increase in the percentage of time spent (Fig. 2A) on open arms and in the frequency (Fig. 2B) of entries into open arms [$F(4,39)=4.16$, $F(4,39)=4.34$, respectively,

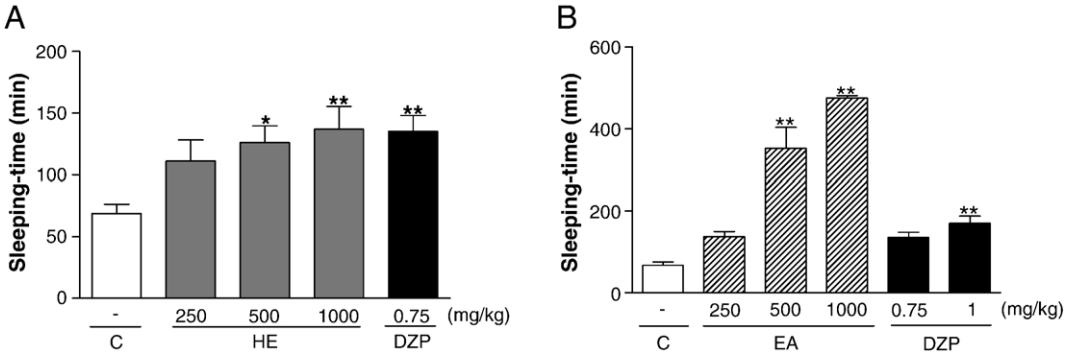


Fig. 3. Effects of the hydroethanolic extract (HE) (panel A) or ethyl acetate fraction (EA) (panel B) (250–1000 mg/kg, p.o.) of *Polygala sabulosa* on the sleeping-time induced by sodium pentobarbital (50 mg/kg, i.p.). Diazepam (DZP 0.75–1 mg/kg, i.p.) was used as the standard hypnosedative drug. Each value represents the mean± S.E.M. of 6–10 animals. *$P \leq 0.05$ and **$P \leq 0.01$ as compared to control (one-way ANOVA followed by Dunnett's test).

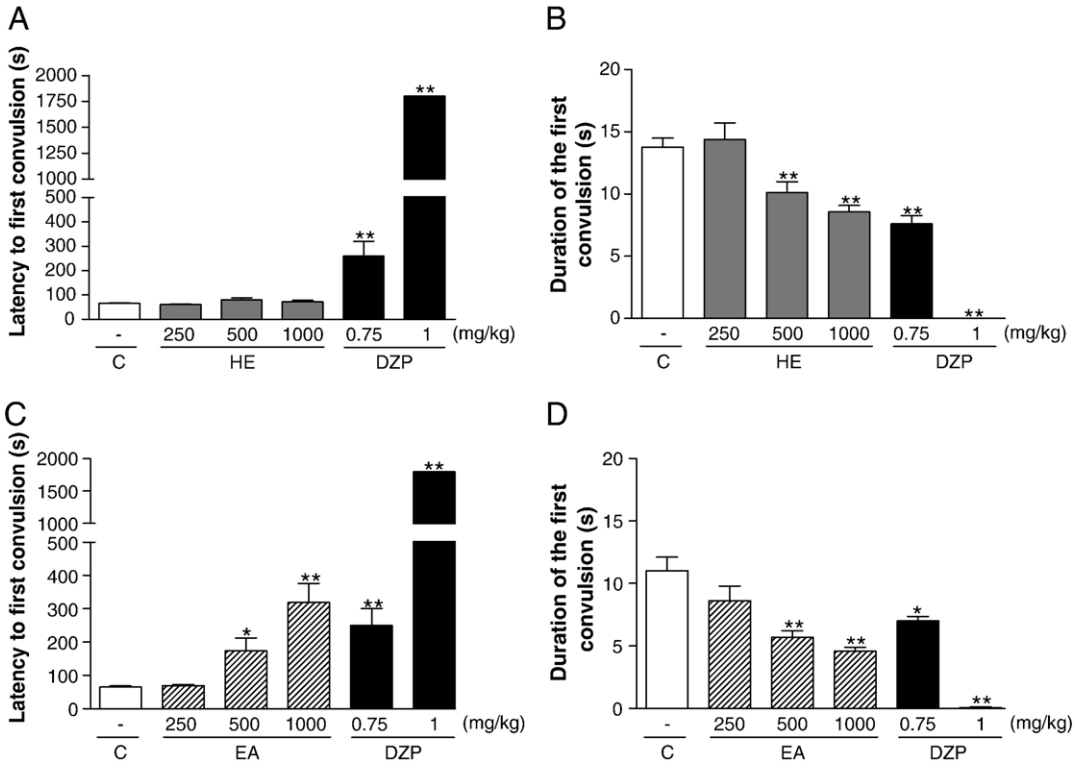


Fig. 4. Effects of the hydroethanolic extract (HE) or ethyl acetate fraction (EA) (250–1000 mg/kg, p.o.) of *Polygala sabulosa* on the latency to the first convulsive episode (panels A and C) and duration of the first convulsion (panels B and D) induced by pentylenetetrazol (PTZ 80 mg/kg, i.p). Diazepam (DZP 0.75–1 mg/kg, i.p.) was used as the standard anticonvulsant drug. Each value represents the mean ± S.E.M. of 5–14 animals. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ as compared to control (one-way ANOVA followed by Dunnett's test).

$P<0.05$], as well as a significant increase in the incidence of unprotected head-dipping behavior [$F(4,39)=4.38$, $P<0.05$] and a decrease in the number of protected stretch-attend postures [$F(4,39)=7.53$, $P<0.01$] (Table 1). The dose–response curves for these effects were typically bell-shaped with no significant changes detected at 1000 mg/kg ($P>0.05$). Altogether, these behavioral changes promoted by HE were similar to those produced by DZP, the standard anxiolytic compound ($P<0.01$). The other behavioral parameters evaluated in the EPM, such as entries on enclosed arms and rearing (data not shown), as well as open-arms end activity were not significantly altered by any dose of HE ($P>0.05$), whereas DZP was able to significantly increase the open-arms end activity [$F(4,39)=10.97$, $P<0.01$] (Table 1).

3.1.2. Pentobarbital-induced hypnosis

The oral treatment of mice with HE, at doses of 250, 500 or 1000 mg/kg, 1 h before the sodium pentobarbital injection

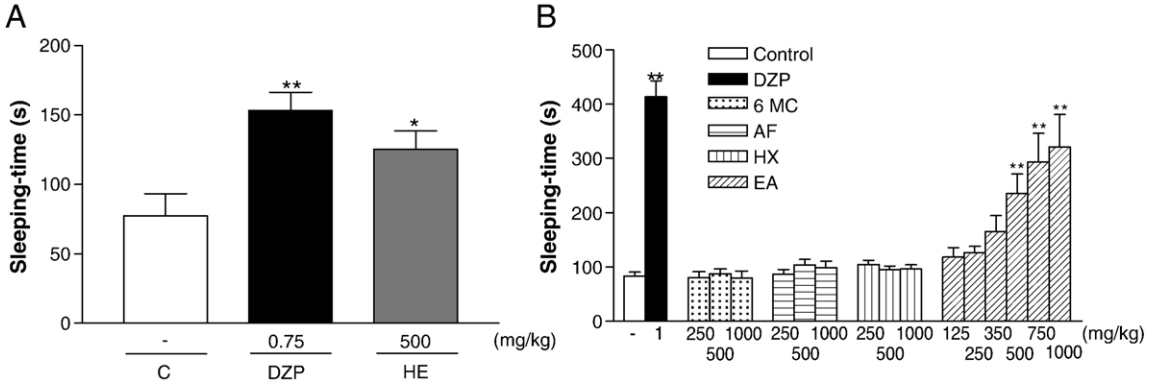


Fig. 5. Effects of the hydroethanolic extract (HE) (500 mg/kg, p.o) (panel A) or aqueous (AF) and hexane (HX) fractions, 6-methoxy-7-prenyloxycoumarin (6 MC) (250–1000 mg/kg, p.o.), or ethyl acetate fraction (EA) (125–1000 mg/kg, p.o.) (panel B) obtained from *Polygala sabulosa* on the sleeping-time induced by ethyl ether. Diazepam (DZP 0.75–1 mg/kg, i.p.) was used as the standard hypnosedative drug. Each value represents the mean ± S.E.M. of 6–11 animals. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ as compared to control (one-way ANOVA followed by Dunnett's test).

Table 2
Effects of p.o. administration of the ethyl acetate fraction (EA) of *Polygala sabulosa* on several behavioral parameters evaluated on pentobarbital-induced hypnosis (PB), on rota-rod test (RR) and the rectal temperature (RT)

Drugs	Dose (mg/kg)	PB	*N*	RR		*N*	RT	*N*
		Latency to the loss of righting reflex (s)		Time on the rotatory bar (s)	Number of falls		ΔT (°C)	
C	–	240.02±32.76	10	59.33±0.44	0.22±0.15	9	−0.15±0.21	10
EA	250	213.40±5.17	8	59.25±0.49	0.25±0.16	8	−0.81±0.28	8
EA	500	177.00±10.17*	7	59.00±0.50	0.33±0.17	9	−1.23±0.34*	7
EA	1000	152.70±25.59*	6	55.00±1.66*	1.67±0.55*	9	−2.00±0.31**	7
DZP	0.75	196.40±10.82	9	59.33±0.44	0.22±0.15	9	–	–
DZP	1	159.30±13.48*	6	–	–	–	–	–
DZP	1.5	–	–	–	–	–	−1.47±0.18**	6
DZP	2.5	–	–	53.33±1.86**	2.22±0.62**	9	–	–

Each value represents the mean±S.E.M. for 6–10 animals (*N* = number of animals). *$P<0.05$ or **$P<0.01$ as compared to respective control group. Data analyzed by one-way ANOVA followed by Dunnett's test. Abbreviations: C = control, EA = ethyl acetate fraction, DZP = diazepam. The rectal temperature was recorded twice, before (T_1) and 1 h after (T_2) the treatments. The temperature variation (ΔT) was estimated as $\Delta T = T_2 - T_1$, in each animal.

did not modify the latency to induce sleep (C=205.55±7.39, HE_{250}=199.50±14.27, HE_{500}=217.50±14.23, HE_{1000}=195.89±15.07, $DZP_{0.75}$=224.00±30.58; $P>0.05$), but it was able to significantly increase the duration of the hypnosis induced by this extract, at 500 and 1000 mg/kg [$F(4,40)=3.69$, $P<0.05$ and $P<0.01$, respectively], as depicted in Fig. 3A, a sedative effect similar to that produced by DZP ($P<0.01$). This sedative action of HE was confirmed in the hypnosis induced by ethyl ether, a sleep-inducer devoid of liver metabolism (Fig. 5A).

3.1.3. PTZ-induced convulsions and complementary tests

As shown in Fig. 4, HE (250 to 1000 mg/kg), given p.o., did not alter the latency to the first convulsive episode ($P>0.05$) (Fig. 4A), but the highest doses (500 and 1000 mg/kg) significantly decreased the duration of the first convulsion [$F(5,48)=24.16$, both $P<0.01$] (Fig. 4B), as well as the severity of seizures [$F(5,48)=15.76$, both $P<0.05$] (Table 1). Both doses of DZP (0.75 and 1 mg/kg) significantly increased the latency (Fig. 4A) to and reduced the duration of the first convulsion (Fig. 4B) as well as the severity of convulsions ($P<0.01$) (Table 1). In fact, the highest dose of DZP (1 mg/kg; i.p.) completely protected the mice from PTZ-induced convulsions when compared to the control group ($P<0.01$) (Fig. 4 and Table 1).

Groups of mice were treated with HE (250 to 1000 mg/kg, p.o) and evaluated by rota-rod and rectal temperature (data not shown). None of the doses of HE used was able to change either the time spent on the rotatory bar and the number of falls on the rota-rod test or the rectal temperature. DZP (2.5 mg/kg, i.p), in turn, promoted a decrease in time spent on the bar and an increase in the number of falls from the rota-rod, and produced a reduction of rectal temperature at 1.5 mg/kg.

3.2. Bio-guide testing of P. sabulosa on the ethyl ether-induced hypnosis

The effect of p.o. administration of HE at the intermediate dose (500 mg/kg) on ethyl ether-induced sleep is shown in Fig. 5. This treatment did not modify the latency to the loss of the righting reflex (data not shown). However, it significantly increased the duration of the hypnosis induced by ethyl ether [$F(2,17)=7.15$, $P<0.05$] (Fig. 5A). Treatment with DZP (0.75 mg/kg; i.p.; standard anxiolytic/hypnosedative drug), used as a positive control in this assay, did not alter the latency to the loss of the righting reflex (data not shown), but did promote an increase in the sleeping time ($P<0.01$) (Fig. 5A).

Fig. 5B shows the effect of the aqueous, hexane or EA fractions and 6-methoxy-7-prenyloxycoumarin of *P. sabulosa* on the ethyl ether-induced sleep. None of the treatments (250 to 1000 mg/kg), except EA (500 to 1000 mg/kg), significantly changed the latency to and the duration of hypnosis induced by ethyl ether (data not shown). EA, on the other hand, did not modify the latency (data not shown), but significantly increased the duration of the hypnosis in a similar way to DZP [$F(16,116)=13.49$, $P<0.01$] (Fig. 5B).

3.3. Effects of the treatment with the EA of P. sabulosa

3.3.1. Elevated plus-maze

As shown in Fig. 2, the oral administration of EA (250, 500 and 1000 mg/kg) induced a significant increase in the percentage of time spent on open arms [$F(4,34)=6.92$] (Fig. 2C), in a dose-dependent manner with significant effects at 250 ($P<0.05$), 500 ($P<0.05$) and 1000 mg/kg ($P<0.01$). Moreover, the highest doses of EA (500 and 1000 mg/kg) promoted a significant increase in the amount of unprotected head-dipping behavior [$F(4,34)=6.96$, both $P<0.01$] and in the open-arms end activity [$F(4,34)=11.56$, $P<0.05$ and $P<0.01$, respectively] (Table 1). All doses also promoted an increase in the frequency of entries into open arms [$F(4,34)=6.20$] (Fig. 1D), with significant effects at 250 ($P<0.05$), 500 ($P<0.05$) and 1000 mg/kg ($P<0.01$), as well as in the number of protected stretch-attend postures [$F(4,34)=10.06$, $P<0.01$] (Table 1). These behavioral changes were similar to those promoted by the positive control DZP 0.75 mg/kg ($P<0.01$) (Fig. 1C, D and Table 1). The other behavioral parameters evaluated in the EPM, such as entries on enclosed arms and rearing (data not shown), were not significantly altered by any doses of EA or DZP used here ($P>0.05$).

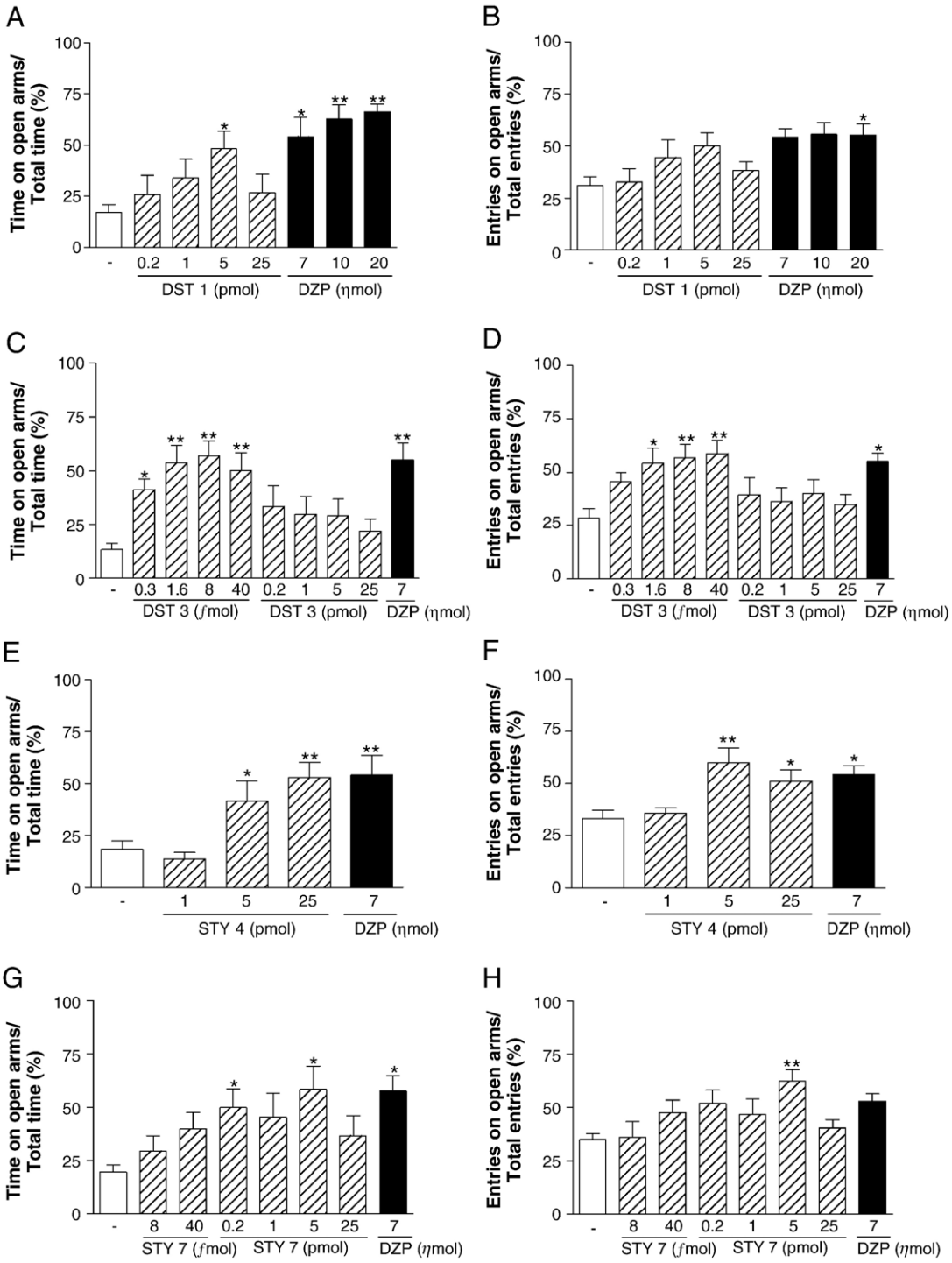


Fig. 6. Effects of the central administration of dihydrostyryl-2-pyrones (**1**) (0.2–25 pmol) or (**3**) (0.3 fmol–25 pmol), and styryl-2-pyrones (**4**) (1–25 pmol) or (**7**) (8 fmol–25 pmol), isolated from *Polygala sabulosa* on the behavior of mice evaluated in the elevated plus-maze test, 5 min after the i.c.v. administration. Diazepam (DZP 7–20 ηmol, i.c.v.) was used as the standard anxiolytic drug. Percentage of time spent on open arms (panels A, C, E and G) and percentage of entries on open arms (panels B, D, F and H) are shown. Each value represents the mean ± S.E.M. of 6–10 animals. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ as compared to control (one-way ANOVA followed by Dunnett's test).

3.3.2. Pentobarbital-induced hypnosis

Only the highest dose of EA (1000 mg/kg, p.o.) significantly reduced the latency to the loss of the righting reflex [$F(4,31)=2.57$, $P<0.05$] (Table 2). Nevertheless, EA, at 500 and 1000 mg/kg, increased the duration of the hypnosis induced by pentobarbital ($P<0.01$), as depicted in Fig. 3B, a hypnosedative-like action even more evident than that produced by DZP 1 mg/kg [$F(4,31)=48.45$; $P<0.01$]. This sedative action of EA was confirmed in the ethyl ether-induced hypnosis (Fig. 5B).

3.3.3. Pentylenetetrazol (PTZ)-induced convulsions and complementary tests

As shown in Fig. 4C, EA (500 or 1000 mg/kg, p.o.) significantly increased the latency to the first convulsive episode induced by PTZ (80 mg/kg, i.p.) [$F(5,41)=423.20$, $P<0.05$ and $P<0.01$, respectively]. Both doses also reduced the duration of the first convulsion [$F(5,41)=16.17$, $P<0.01$] (Fig. 4D), as well as the severity of the seizures [$F(5,41)=7.01$, $P<0.05$], similar to DZP (Table 1). The highest dose of DZP (1 mg/kg, i.p.) completely protected mice from the convulsions induced by PTZ ($P<0.01$) (Fig. 4 and Table 1).

EA, at the highest dose (1000 mg/kg, p.o.), was able to significantly reduce the time spent on the rotatory bar [$F(5,47)=5.91$, $P<0.05$] and increased the number of falls [$F(5,47)=5.91$, $P<0.05$] in a similar way to DZP 2.5 mg/kg ($P<0.01$) (Table 2). Rectal temperature was decreased by EA [$F(4,33)=8.03$] at 500 ($P<0.05$) and 1000 mg/kg ($P<0.01$), similarly to DZP 1.5 mg/kg ($P<0.01$) (Table 2).

*3.4. Effects of dihydrostyryl-2-pyrones (**1**), (**2**) and (**3**), and styryl-2-pyrones (**4**), (**5**) and (**7**) in the elevated plus-maze (EPM)*

Central injection of dihydrostyryl-2-pyrone (**1**) 5 pmol promoted a significant increase in the percentage of time spent in the open arms of EPM [$F(7,59)=5.23$, $P<0.05$] (Fig. 6A) and in the amount of unprotected head-dipping [$F(7,59)=6.91$, $P<0.01$] as well as a decrease in the protected stretch-attend postures ($F(7,59)=7.02$, $P<0.01$] (Table 3). This compound, at the same dose, presented a trend to increase ($P=0.06$) the frequency of entries into the open arms of the EPM (Fig. 6B). These behavioral alterations indicate an anxiolytic-like action such as that presented by DZP (7–20 ηmol) (Fig. 6A, B and Table 3).

Dihydrostyryl-2-pyrone (**3**) (0.3 to 40 fmol) increased the percentage of time spent in the open arms of the EPM [$F(9,109)=4.65$, $P<0.05$] (Fig. 6C), with significant effects at 0.3 fmol ($P<0.05$), 1.6, 8 and 40 fmol ($P<0.01$). It also enhanced the frequency of entries in these arms [$F(9,109)=3.30$] (Fig. 6D), at 1.6 ($P<0.05$), 8 and 40 fmol ($P<0.01$), the amount of unprotected head-dipping [$F(9,109)=4.76$] (Table 3), at 0.3 ($P<0.05$), 1.6 ($P<0.01$), 8 ($P<0.01$) and 40 fmol ($P<0.05$), and the open-arms end activity [$F(9,109)=3.10$], at 1.6 ($P<0.01$), 8 ($P<0.01$) and 40 fmol ($P<0.05$) (Table 3).

Styryl-2-pyrone (**4**) (5 and 25 pmol) induced an effect similar to those presented by DZP 7 ηmol, i.e. a significant increase in the percentage of time spent [$F(4,34)=7.65$, $P<0.05$ and $P<0.01$, respectively] (Fig. 6E) and in the frequency of entries [$F(4,34)=5.64$, $P<0.01$ and $P<0.05$, respectively] (Fig. 6F) in the open arms of the EPM as well as a reduction in the protected stretch-attend postures [$F(4,34)=20.85$, both $P<0.01$] (Table 3). Moreover, the highest dose promoted an increase in the unprotected head-dipping [$F(4,34)=4.36$, $P<0.05$] and in the open-arms end activity [$F(4,34)=4.89$, $P<0.05$] (Table 3).

As shown in Fig. 6, i.c.v. administration of styryl-2-pyrone (**7**) (0.2 and 5 pmol) caused a significant increase in the percentage of time spent on open arms [$F(7,77)=2.81$, both $P<0.05$] (Fig. 6G), in the frequency of open arms entries at 5 pmol [$F(7,77)=2.57$, $P<0.01$] (Fig. 6H), in the amount of unprotected head-dipping at 0.2 to 5 pmol [$F(7,77)=3.17$, $P<0.05$], and a decrease in the protected stretch-attend postures at 5 pmol [$F(7,77)=4.82$, $P<0.05$] (Table 3).

The other behavioral parameters evaluated in the EPM test were not affected by these treatments (data not shown). Data obtained after i.c.v. treatment with dihydrostyryl-2-pyrone (**2**) and styryl-2-pyrone (**5**) were not statistically different from the control values (data not shown).

Table 3
Effects of i.c.v. administration of dihydrostyryl-2-pyrones (**1**) or (**3**) and styryl-2-pyrones (**4**) or (**7**), isolated from *Polygala sabulosa*, on the other behavioral parameters evaluated in the elevated plus-maze (EPM) test in mice

Drugs	Dose/ site	EPM			
		uHD	pSAP	OAEA	*N*
C	–	7.75±2.77	13.83±2.28	0.75±0.37	12
DST (**1**)	0.2 pmol	14.22±5.34	14.22±2.54	2.33±0.85	9
DST (**1**)	1 pmol	11.56±2.98	7.11±1.44*	1.67±0.90	9
DST (**1**)	5 pmol	27.73±5.94**	5.09±1.55**	5.18±1.59**	11
DST (**1**)	25 pmol	10.63±3.88	8.62±1.60	1.88±1.01	8
DZP	7 ηmol	30.33±7.14*	2.50±0.76**	5.50±1.20*	6
DZP	10 ηmol	36.60±7.65**	4.60±1.29*	5.80±0.66*	5
DZP	20 ηmol	41.43±2.36**	0.71±0.47**	10.43±1.00**	7
C	–	7.13±2.08	16.60±2.22	0.60±0.34	15
DST (**3**)	0.3 fmol	23.00±2.10*	11.30±2.53	4.00±0.60	10
DST (**3**)	1.6 fmol	30.70±5.24**	7.80±2.49*	5.90±1.08**	10
DST (**3**)	8 fmol	26.85±4.43**	7.31±1.48**	5.31±±1.18**	13
DST (**3**)	40 fmol	23.71±4.58*	8.71±2.11*	4.21±1.14*	14
DST (**3**)	0.2 pmol	15.00±5.42	14.44±2.77	4.44±1.40	9
DST (**3**)	1 pmol	11.67±3.52	12.13±1.55	2.53±0.94	15
DST (**3**)	5 pmol	10.62±3.16	13.00±2.07	2.46±0.96	13
DST (**3**)	25 pmol	12.62±3.50	13.92±2.04	2.08±0.85	13
DZP	7 ηmol	30.71±6.05**	2.57±0.65**	5.29±1.04*	7
C	–	8.18±3.00	11.91±1.33	0.82±0.40	11
STY (**4**)	1 pmol	8.17±2.02	12.83±1.45	1.17±0.60	6
STY (**4**)	5 pmol	18.57±6.25	3.14±0.99**	2.86±1.18	7
STY (**4**)	25 pmol	22.89±3.77*	3.22±0.52**	4.22±1.05*	9
DZP	7 ηmol	30.33±7.14**	2.50±0.76**	5.50±1.20**	6
C	–	8.47±2.22	13.53±1.37	1.27±0.42	15
STY (**7**)	8 fmol	10.75±3.26	15.50±3.13	2.50±1.35	8
STY (**7**)	40 fmol	19.82±4.58	10.27±2.25	4.45±1.53	11
STY (**7**)	0.2 pmol	23.00±5.07*	7.94±2.05	4.62±1.25	16
STY (**7**)	1 pmol	25.80±6.47*	7.50±1.48	5.20±1.54	10
STY (**7**)	5 pmol	26.88±3.55*	3.38±1.16*	3.50±1.10	8
STY (**7**)	25 pmol	17.11±3.87	7.44±2.20	2.50±1.02	9
DZP	7 ηmol	33.25±5.58**	1.88±0.69*	6.88±1.27**	8

Each value represents the mean±S.E.M. for 5–15 animals (*N* = number of animals). *$P<0.05$ or **$P<0.01$ as compared to respective control group. Data analyzed by one-way ANOVA followed by Dunnett's test. Abbreviations: C = control, DST = dihydrostyryl-2-pyrone, STY = styryl-2-pyrone, DZP = diazepam, uHD = unprotected head-dipping, pSAP = protected stretch-attend postures, OAEA = open-arms end activity.

4. Discussion

Several medicinal plants are traditionally endowed with anxiolytic or sedative properties and have been used with great acceptance by the population of many countries. Herbal medicine has played a key role in world health and represents

an alternative source for new lead molecules and also for the development of standardized phytomedicines with proven efficacy in neuropsychiatric practice, particularly in the treatment of anxiety, a disorder without effective therapies devoid of adverse reactions. In this regard, kava-kava is one example of a plant species that has been extensively used in traditional medicine and was commonly prescribed or available as an over-the-counter herbal medicine in Europe and United States as a mild anxiolytic and sedative agent. However, kava-kava as a phytomedicine was banned from the market due to an alleged hepatotoxicity, including cases requiring liver transplant and resulting in death (Centers for Disease Control and Prevention, 2003; Currie and Clough, 2003), although the underlying mechanism of such a toxic effect is still unknown.

In this paper, we reported the psychopharmacological effects on mouse behavior of HE of *P. sabulosa*, its fractions (aqueous, hexane and ethyl acetate), the 6-methoxy-7-prenyloxycoumarin and six isolated compounds (dihydrostyryl-2-pyrones and styryl-2-pyrones) isolated from *P. sabulosa*, compounds which present a structural skeleton similar to the kavalactones found in kava-kava, using several well validated animal models for the first time to the best of our knowledge. These central actions of *P. sabulosa* and its active constituents were compared to those produced by DZP, a GABA/benzodiazepine agonist commonly used as an anxiolytic drug.

The anxiolytic-like activity of HE and its EA were investigated in the EPM test. This experimental paradigm is based on the fact that open spaces are extremely aversive to rodents (Pellow and File, 1986). HE of *P. sabulosa* and its EA induced an increase in the percentage of time spent and in the frequency of entries into the open arms, as well as in the amount of head-dipping, besides a reduction in stretch-attend postures. The alterations promoted in these behavioral parameters, valid indexes of anxiety (Cruz et al., 1994; Setem et al., 1999), indicate an anxiolytic-like profile of action for this plant species and its fraction which is rich in dihydrostyryl-2-pyrones and styryl-2-pyrones compounds.

Some of the structurally related dihydrostyryl-2-pyrones and styryl-2-pyrones isolated from *P. sabulosa*, present in the EA, also promoted a clear anxiolytic-like effect when centrally administered in mice. Dihydrostyryl-2-pyrones (**1**) and (**3**), and styryl-2-pyrones (**4**) and (**7**) produced a significant increase in the exploration of open-arms parameters as well as a reduction in risk assessment behaviors in the EPM test. Compound (**1**) showed a less-evident effect, whereas dihydrostyryl-2-pyrones (**3**) and styryl-2-pyrones (**7**) were the most potent compounds, with effects similar to the standard anxiolytic drug DZP. Compounds (**2**) and (**5**), on the other hand, showed no effect at all in the same experimental paradigm. These actions observed in the EPM indicate a quite selective anxiolytic-like effect and were not merely a result of a general stimulation of locomotor activity, since the compounds isolated from *P. sabulosa*, as well as the HE or EA, did not alter the entries into the enclosed arms, a well accepted index of motor activity (Rodgers and Dalvi, 1997).

It is noteworthy that the dose–response curves for the effects of HE and some isolated compounds, in several behavioral parameters evaluated on the EPM, were bell-shaped. This is a common feature in most herbal remedies since they possess various unknown active principles that could interact in different ways (Carvalho-Freitas and Costa, 2002). Moreover, this kind of biphasic curve is also quite common among benzodiazepine agonists (Davies and Steinberg, 1984; Henberg and Williams, 1983; Soderpalm et al., 1991; Ruarte and Alvarez, 1999; Walters et al., 2000; Wang et al., 2003) and can be attributed to their sedative effect at high doses which leads to a reduction in maze exploration.

We also showed that the HE and EA potentiate barbiturate-induced hypnosis. Sodium pentobarbital induces sleep, in rodents and humans, by acting at its own receptor sites on the GABA/receptor ionophore complex (Koch-Weser and Greenblatt, 1974). Thus, it is generally accepted that the depressant effects of some drugs can be evaluated by pentobarbital assay in laboratory animals (Lancel, 1999). In our study, HE increased the duration of sleep while EA also promoted a reduction in the latency to the loss of the righting reflex as well as a prolongation of sleeping-time, suggesting a central nervous system depressant effect of *P. sabulosa*. Interestingly, compared to HE and DZP effects, the actions of EA seem very robust, indicating that this fraction contains active(s) principle(s) which could be of potential therapeutic interest.

HE and the three fractions of *P. sabulosa* were also tested on ethyl ether-induced sleeping-time. This test was used in order to confirm the putative hypnosedative effect of HE (such as observed in the pentobarbital assay) and to bio-guide the phytochemical analysis of *P. sabulosa*. Prolongation of the sleeping-time induced by hypnotic drugs such as barbiturates may be due to pharmacokinetic interactions (Gyamfi et al., 2000), since several drugs can interact with the cytochrome P450 complex, an action which can promote a potentiation of the central depressant effect of barbiturates, as already described by Vieira (2001), without any central activity. Since ethyl ether is not metabolized in the liver, the enhanced duration of sleep observed after HE treatment confirms the latter's sedative action. Moreover, among the three plant fractions (hexane, aqueous and ethyl acetate fractions) and the 6-methoxy-7-prenyloxycoumarin, only EA showed a significant effect in this test, indicating that this fraction contains the active(s) principle (s) responsible for the central effects of *P. sabulosa* which could be attributed to dihydrostyryl-2-pyrone and styryl-2-pyrone compounds, the main components of this fraction.

The PTZ-convulsion assay is the main acute experimental model used in preliminary screening to test the potential of anticonvulsant drugs (De Deyn et al., 1992). PTZ is believed to exert its action by antagonizing the $GABA_A$–receptor complex (Wilson and Escueta, 1974). HE pretreatment reduced the duration of the first convulsion as well as the severity of convulsive episodes at the highest dose, suggesting a partial anticonvulsant effect comparable to the $GABA_A$ agonist DZP which caused total inhibition of convulsions at 1 mg/kg. Moreover, besides reducing the duration of the first convulsion and the severity score, EA promoted an increase in the latency to the first convulsive episode induced by PTZ, also showing a protective effect.

Changes in motor performance and in body temperature could account for some of the previous results. In order to discount any relationship between the anxiolytic, sedative and anticonvulsant effects of HE or EA and changes promoted in motor performance or body temperature, mice were examined on the rota-rod and their body temperature was measured. HE treatment did not modify the performance of the animals on the rotatory bar, which depends essentially on their motor function, and did not produce any alteration in the animals' body temperature. Thus, the central effects of HE cannot be attributed to a decrease in motor performance or to hypothermia. Interestingly, similar doses of EA, which protected mice from convulsions, produced a significant hypothermia that could contribute to its anticonvulsant action. Liu et al. (1993) and Lundgren et al. (1994) suggested that hypothermia may be useful in reducing seizures and associated brain damage and that hyperthermia should be avoided in *status epilepticus*. The beneficial effect of hypothermia has been demonstrated in the PTZ-induced convulsions model (Rauca et al., 2000) and hypothermia is a common effect of benzodiazepines (Frosini et al., 2004). Moreover, the reduction of time spent on the rotatory bar and the increase in falls from the rota-rod, similar to DZP 2.5 mg/kg, strengthens the evidence for central depressant effects induced by EA.

In summary, the present results show that the crude extract (HE) and the ethyl acetate (EA) fraction obtained from *P. sabulosa* promote an anticonvulsant, hypnosedative and anxiolytic-like effect and that these effects are more evident for EA. The purification process was effective in concentrating the active(s) principle(s) responsible for the central action of *P. sabulosa* since its EA was at least twice as potent in promoting these effects and was the only fraction, of the three studied here, that showed central activity, reinforcing the idea that the active (s) principle(s) responsible for these actions are dihydrostyryl-2-pyrones and styryl-2-pyrones, the only constituents of this fraction. The anxiolytic-like effects are also observed after the central treatment with dihydrostyryl-2-pyrones (**1**) and (**3**) and styryl-2-pyrones (**4**) and (**7**), isolated from the EA of *P. sabulosa*. These effects may involve the GABAergic system since they are similar to those seen after treatment with DZP. Thus, presently we are further investigating the underlying mechanism(s) of action in in vivo and in vitro systems to better understand the central actions of *P. sabulosa*. Acute and chronic studies are also being carried out to evaluate the toxicological potential of this plant and to guarantee its safe use.

Acknowledgments

F. S. Duarte is a recipient of a Ph.D. scholarship from the Brazilian National Research Council (CNPq), which also provided a research grant to T. C. M. De Lima. The authors wish to thank Dr. Gareth Cuttle for the final English revision of the text.

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