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Antinociceptive activity of the HPLC- and MS-standardized hydroethanolic extract of *Pterodon emarginatus* Vogel leaves

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ABSTRACT

Several studies have demonstrated the analgesic and anti-inflammatory effects of fruit and seed extracts from *Pterodon emarginatus* Vogel (Fabaceae). The objective of this study was to evaluate the antinociceptive activity of the hydroethanolic extract of *P. emarginatus* leaves in mice and characterize its chemical composition using HPLC coupled to UV–vis diode array detection and mass spectrometry with electrospray ionization. Our results showed that the doses of 500 and 1000 mg/kg produced an antinociceptive effect, as observed in the hot plate test and writhing induced by acetic acid. The chromatographic profile and spectral mass data suggest the presence of di-*C*-glycosylflavones (e.g., vicenin-2 and schaftoside), *C*,*O*-glycosylflavones (e.g., chrysoeriol-8-*C*-glucosyl-2″-*O*-glucuronide-6-*C*-arabinoside) and luteolin-7-*O*-rutinoside as the main constituents. Lower levels of oleanane-type saponins, such as soyasaponin Bb and Be, and the saponin derivatives hederagenin and aglycone B, which are typical of Fabaceae family, were also found. From this study, it is suggested that the analgesic effect observed is not due to the terpenoids previously reported from fruit and seed extracts, but could be attributed to flavones and the hederagenin derivatives that were identified as main constituents of the hydroethanolic extract from the leaves.

Introduction

The genus *Pterodon* (Fabaceae/Leguminosae) is widely distributed over the savannahs of central and southern Brazil. *Pterodon emarginatus* Vogel (synonyms *Pterodon pubescens* Benth. and *Pterodon polygaliflorus* Benth.), also known as sucupira-branca, is widespread throughout the Goiás, Minas Gerais and São Paulo states in Brazil (Dutra et al. 2009; Hansen et al. 2010).

The fruits, seeds and seed oil from *P. emarginatus* are commercially available in the Brazilian medicinal flora market and are commonly used in folk medicine for their analgesic, anti-inflammatory, and anti-rheumatic properties (Sabino et al. 1999a; Coelho et al. 2005; Dutra et al. 2009; Hansen et al. 2010). The traditional use generally consists of macerating the plant in wine to form a hydroalcoholic extract. Several authors have investigated biological properties of *P. emarginatus* employing extracts from the fruit, seeds and bark.

The phytochemical analyses of *P. emarginatus* showed the presence of terpenoids (furane diterpene derivatives) (Coelho et al. 2005; Galceran et al. 2011) and phenolic compounds (flavonoids and isoflavones) (Dutra et al. 2009) in the fruits and seeds. Flavonoids, saponin heterosides (Bustamante et al. 2010) and triterpenes, including lupeol and betulin (Moraes et al. 2012), were identified in the bark.

Essential oil from *P. emarginatus* seeds exhibited antiulcerogenic, anti-inflammatory, and antinociceptive activity in animal models (Dutra et al. 2008a, 2009). The oleaginous extract of the seeds also showed antinociceptive and anti-inflammatory properties, which were thought to be attributed to furane diterpene derivatives of vouacapan and non-vouacapan compounds (Coelho et al. 2005; Galceran et al. 2011). Triterpenes were suggested to be responsible for the anti-inflammatory effect in the extract from the *P. emarginatus* bark (Moraes et al. 2012).

To our knowledge, only one study has been carried out using the leaves of sucupira-branca, and it identified the phytosteroids β-sitosterol and stigmasterol in the ethanolic extract and nine sesquiterpenics hydrocarbons (the main constituents being γ-muurolene and bicyclogermacrene) in the essential oil obtained by hydrodistillation (Santos et al. 2010). The authors discuss that the

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essential oil composition extracted from the leaves was different from that extracted from the fruit.

Although several studies have demonstrated the analgesic and anti-inflammatory effects of the fruit and seed extracts from *P. emarginatus*, there is no information regarding the properties of its leaves. The objective of this study was to characterize the chemical composition of the hydroethanolic extract of *P. emarginatus* leaves and to evaluate the antinociceptive activity of this extract in mice. For this purpose, the extract was analyzed by HPLC coupled to UV–vis diode array detection (HPLC-DAD) and mass spectrometry with electrospray ionization (HPLC–ESI-MS/MS).

Materials and methods

Plant material and preparation of the extract

Leaves of *Pterodon emarginatus* were collected in Goiatins, Tocantins State. A flowered voucher species was identified and deposited in the herbarium of Instituto de Botânica de São Paulo under number Rodrigues (1021). The botanical material was dried at room temperature and kept in amber glass flasks until extraction.

The hydroethanolic extract was prepared by turbolysis using a semi-industrial blender. Approximately 100 g of plant powder (ground in a grinding mill using mesh 20) was extracted in 1000 ml of ethanol:water (v/v). The extract was then filtered with cotton and filter paper (weight 40 g/m^2), concentrated in a rotating evaporator and lyophilized under reduced pressure. The yield of dry extract was 15%. The dry extract was stored in amber flasks inside a desiccator at 4 °C and resuspended fresh with distilled water for use in pharmacological experiments.

Chemicals

Apigenin, vitexin, orientin, luteolin, thiobarbituric acid and pentobarbital sodium were purchased from Sigma–Aldrich (St. Louis, MO, USA), and their purities were greater 97% as determined by HPLC/DAD analysis. HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). PA-grade ethanol and acetic acid were purchased from Labsynth (Diadema, Brazil). Morphine was kindly donated by the Brazilian Federal Police, and its identification and purity were confirmed by HPLC. De-ionized water was prepared from distilled water using a Milli-Q system (Millipore, Waters, Milford, MA, USA).

Animals

Swiss albino mice (3–5 months, 35–50 g) were obtained from the biotery at the Department of Psychobiology at UNIFESP. The animals were kept in an isolated room with a 12 h light/dark cycle and a constant temperature (23 ± 2 °C) and provided water and food ad libitum, except during the experimentation period. Treatment with either extract or water was done by oral gavage; acetic acid, morphine and pentobarbital were diluted in saline and injected intraperitoneally (i.p.) at a volume of 0.1 ml per 10 g of body weight. All of the procedures were approved by the ethical animal use commission of UFABC (protocol 013/2011).

Reversed phase HPLC–DAD-ESI-MS/MS analysis

For reversed phase high-performance liquid chromatography analysis, lyophilized extracts were dissolved in water:methanol (80:20, v/v) at a concentration 5 mg/ml, filtered through a 0.45 μm filter and injected into the HPLC system (31.2 μl, final concentration of 78 μg/ml). The HPLC–DAD-ESI-MS system was composed of a DADSPD-M10AVP Shimadzu equipped with a photodiode array detector coupled to Esquire 3000 Plus quadrupole (Bruker Daltonics). The mass detector used was a quadrupole ion trap equipped with an atmospheric pressure ionization source via an electrospray ionization interface. The operation, acquisition and data analysis were controlled using SCL-10A VP software. Spectral UV data from the peaks were accumulated in the range 240–400 nm. Detection with the diode array was performed simultaneously at the following three wavelengths: 270, 350 and 530 nm. The mobile phases consisted of eluent A (0.1% aqueous formic acid) and eluent B (methanol). A reverse phase Zorbax C18 RP-18 column (4.6 mm × 250 mm, 5 μm) (Hewlett Packard) connected to a guard column and a gradient of 20–90% eluent B (v/v) over 50 min were used for separation with the following program: 0 min – 20% B in A; 10 min – 30% B in A, 20 min – 50% B in A; 30 min – 70% B in A; 40 min – 90% B in A; 45 min – 40% B in A, after which the initial conditions (20% B) were applied to re-equilibrate the column prior to another run. The flow rate was kept constant at 0.5 mL/min, and the temperature of the column was maintained at 28 °C. The ionization conditions were as follows: electrospray ionization was performed using an ion source voltage of −40 V and a capillary offset voltage of 4500 V. During the analyses, the cone voltage was varied between 40 and 200 V to produce the most stable signal. Helium was used as the collision gas and nitrogen as the nebulizing gas. Nebulization was aided with a coaxial nitrogen sheath gas provided at 27 psi. Desolvation was assisted with a counter current nitrogen flow set at a flux of 7.0 l/min and a capillary temperature of 325 °C. The full scan covered the range from *m/z* 100 to *m/z* 1000. Quantification of the flavones was carried out using vitexin as the standard, which was prepared at 0.1, 0.2, 0.3, 0.5 and 1.0 mg/ml, and analyzed by HPLC-DAD. Chromatographic analysis of each standard solution was performed in triplicate. The calibration curve was plotted as the peak area of the chromatogram against the standard compound. The data points were fitted into a best-fit line by linear regression method. The results were expressed as percentage (%) per 1 mg of the lyophilized extract. Constituents were characterized based on their retention behaviors, UV spectra, MS and MS/MS data compared to reference substances and literature data.

Initial pharmacological screening

Four groups of 3 mice each received orally water (control) or *P. emarginatus* extract at doses of 10, 100 or 1000 mg/kg and were placed in wire cages for behavioral observation as previously described by Carlini (2011). This qualitative test was used to verify the viable dose range for subsequent studies.

Pentobarbital induced sleeping time

Three groups of 10 mice each were orally treated with either water (control) or *P. emarginatus* extract at doses of 500 and 1000 mg/kg. After one hour, the mice received sodium pentobarbital (50 mg/kg, i.p.) and the latency to sleeping and time between the loss and the recovery of the rightening reflex were recorded (Bezerra et al. 2011).

Spontaneous motor activity

Three groups of 9–10 mice each were orally treated with either water (control) or *P. emarginatus* extract at doses of 500 and 1000 mg/kg. The animals were then placed individually in boxes fitted with photoelectric cells immediately after administration of extract, and the ambulation was recorded cumulatively at 10, 30, 60, 90, 120 and 180 min (Bezerra et al. 2011).

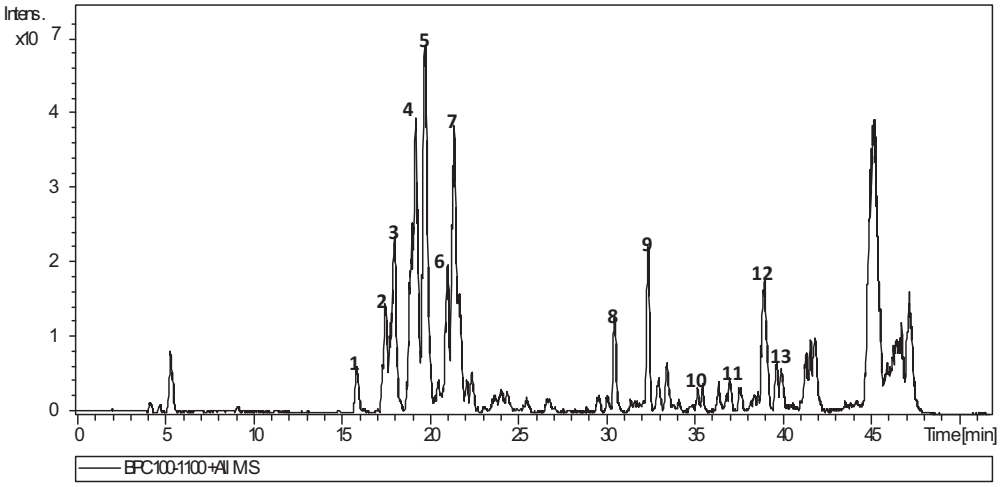


Fig. 1. Total ion current chromatogram of the hydroethanolic extract from *P. emarginatus* leaves obtained using the ESI-MS in negative ion mode. The following compounds are proposed for each peak: (1) lucenin-2; (2) vicenin-2; (3) luteolin 8-*C*-glucosyl-6-*C*-arabinoside; (4) schaftoside; (5) chrysoeriol-8-C-glucosyl-2″-O-glucuronide-6-C-arabinoside; (6) schaftoside-2″-*O*-rhamnoside; (7) luteolin-7-*O*-rutinoside; (8) myricetin-3-*O*-glucoside; (9) myricetin-3-*O*-acetylglucoside; (10) soyasaponin Be; (11) soyasaponin Bb; (12) aglycone B-3-*O*-rhamnosyl galactosyl glucuronide; (13) hederagenin-3-*O*-rhamnosyl galactosyl glucuronide.

Motor coordination (rota-rod)

Mice were previously selected for the rota-rod based on their capacity of remain on a revolving bar at 12 rpm for 60 s for at least one of three trials. Three groups of 10 animals each were orally treated with either water (control) or *P. emarginatus* extract at doses of 500 and 1000 mg/kg and evaluated on the device before (basal) and 30, 60 and 120 min after administration. The amount of time spent on the rod (maximum of 60 s) was recorded (Bezerra et al. 2011).

Writhing test

Four groups of 5–8 mice were orally treated with water (control), *P. emarginatus* extract at doses of 500 and 1000 mg/kg, or morphine (10 mg/kg, i.p. – positive control). At 30 min after drug administration, the animals were injected with 0.8% acetic acid (i.p.), and the number of writhes was counted over a period of 10 min (Pires et al. 2009).

Hot plate test

The hot plate consists of putting mice on a plate kept at a constant temperature (55 ± 1 °C) and evaluating the latency to respond to the thermal stimulus, as described by Pires et al. (2009). A cut-off time of 30 s was used to avoid damage to the animals' paws. Four groups of mice ($n = 5–7$) were evaluated before (basal) and at 30 and 60 min after oral gavage with either water (control), *P. emarginatus* extract at doses of 500 and 1000 mg/kg or morphine (10 mg/kg, i.p – positive control).

Statistical analysis

The animal behaviors observed upon initial pharmacological screening were qualitatively analyzed. The data regarding motor coordination were analyzed by Kruskal–Wallis followed by the Mann–Whitney test when necessary. The spontaneous motor activity, sleeping time, the number of writhes and response latency to thermal stimulus were analyzed by either one-way analysis of variance (ANOVA) or two-way ANOVA (for repeated measures), followed by Duncan's test when necessary. The results are expressed as the mean ± standard error of mean (SEM). The analysis was performed using the Statistica® program, and a level of 5% ($p < 0.05$) was considered to be statistically significant.

Results

Reversed phase HPLC-DAD-ESI-MS/MS analysis

Fig. 1 shows the total ion current chromatogram of *P. emarginatus* leaf extract obtained using ESI-MS in the negative ion mode, while Table 1 summarizes the information regarding the main peaks obtained during RPHPLC-DAD-ESI-MS/MS analyses. None of the compounds found in the hydroethanolic extract was previously reported in this genus. Except for di-*C*,*O*-glycosylflavones, such as, chrysoeriol-8-*C*-glucosyl-2″-*O*-glucuronide-6-*C*-arabinoside and schaftoside-2″-*O*-rhamnoside, that are uncommon, the others constituents are known and their MS data are reported in references cited in Table 1. Supplementary information showing the ESI-MS/MS spectra in negative and positive ion mode of compounds **1**–**13** is provided as Supplementary material.

Based on the HPLC-DAD chromatogram and the calibration curve using vitexin as standard, the breakdown of the extract was as follows: 3% lucenin-2 (**1**), 5% vicenin-2 (**2**), 8% luteolin-8-*C*-glucosyl-6-*C*-arabinoside (**3**), 15% schaftoside (**4**), 20% chrysoeriol-8-*C*-glucosyl-2″-*O*-glucuronide-6-*C*-arabinoside (**5**), 8% schaftoside-2″-*O*-rhamnoside (**6**) and 28% luteolin-7-*O*-rutinoside (**7**). Myricetin-3-*O*-glucoside (**8**), myricetin-3-*O*-acetylglucoside (**9**) and saponins **10–13** were not quantified.

Initial pharmacological screening

Treatment of mice with *P. emarginatus* extract at doses of 10, 100 and 1000 mg/kg (oral) did not induce any sign of toxicity in the animals. The mice receiving 1000 mg/kg of the extract appeared less active at 15–60 min after treatment (qualitative observation). This test implied that the doses administered do not interfere with animal's behavior.

Pentobarbital-induced sleeping time

Oral administration of the *P. emarginatus* extract at a dose of 1000 mg/kg increased the sleeping time induced by pentobarbital

Table 1
Results of the extract analysis obtained by RPHPLC-DAD-ESI-MS/MS. Retention times (Rt), wavelengths of maximum absorbance (λ_{max}), name and structure proposed for the constituents found in the hydroethanolic extract from *Pterodon emarginatus* Vogel leaves.

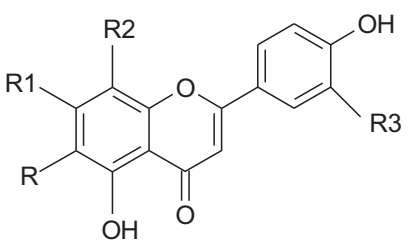
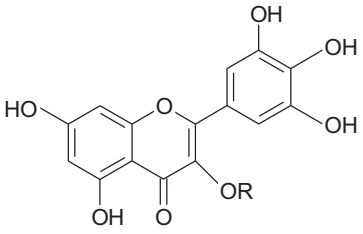
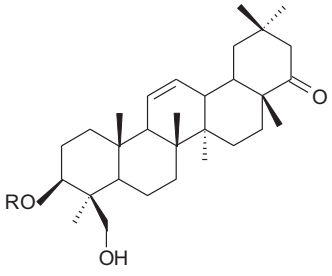
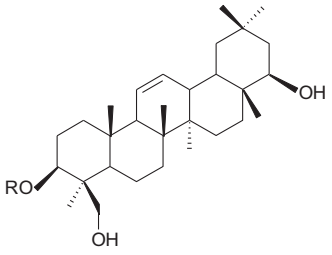
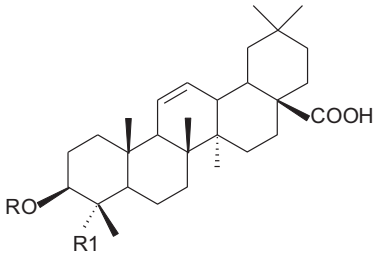
	RT (min)	UV λ_{max} (nm)	Name	Structure proposed	References[a]
1	15.9	ND	Lucenin-2		Ferreres et al. (2008), Dinelli et al. (2009), Lin and Harnly (2010), Wohlmuth et al. (2010) and Abranko et al. (2011)
2	17.5	270, 348	Vicenin-2		
3	17.9	269, 350	Luteolin 8-*C*-glucosyl-6-*C*-arabinoside		
4	19.7	268, 348	Schaftoside		
5	20.8	270, 346	Chrysoeriol-8-*C*-glucosyl-2″-*O*-glucuronide-6-*C*-arabinoside		
6	21.6	270, 349	Schaftoside-2″-*O*-rhamnoside		
7	22.4	272, 347	Luteolin-7-*O*-rutinoside		
				(1) R = R2 = glu; R1 = R3 = OH	
				(2) R = R2 = glu; R1 = OH; R3 = H	
				(3) R = ara; R1 = R3 = OH; R2 = glu	
				(4) R = glu; R1 = OH; R2 = ara; R3 = H	
				(5) R = ara; R1 = OH; R2= glu-2¨-O-glcUA; R3 = OMe	
				(6) R = glu-2”-O-rha; R1 = OH; R2 = ara; R3 = H	
				(7) R1 = O-rut; R = R2 = H; R3 = OH	
8	30.4	265–355	Myricetin-3-*O*-glucoside		Fracassetti et al. (2013)
9	32.9	265–355	Myricetin-3-*O*-acetylglucoside		
				(8) R = glu	
				(9) R = acetylglu	
10	35.3	ND	Soyasaponin Be		Jin et al. (2007) and Tava et al. (2011)
				R = rha-gal-glcUA	
11	36.3	ND	Soyasaponin Bb		Sagratini et al. (2009), Pollier et al. (2011) and Tava et al. (2011)
				R = rha-gal-glcUA	

Table 1 (*Continued*)

	RT (min)	UV λ_{max} (nm)	Name	Structure proposed	References[a]
12	38.1	ND	Aglycone B-3-*O*-rhamnosyl galactosyl glucuronide	 **(12)** R = rha-gal-glcUA; R1 = COH (aldehyde) **(13)** R = rha-gal-glcUA; R1 = CH_2OH (alcohol)	Sagratini et al. (2009), Pollier et al. (2011) and Tava et al. (2011)
13	39.5	ND	Hederagenin-3-*O*-rhamnosyl galactosyl glucuronide		

ND: not determined.

[a] The references cited in this table, correspond to MS data used to compare the MS data obtained in this study with literature data.

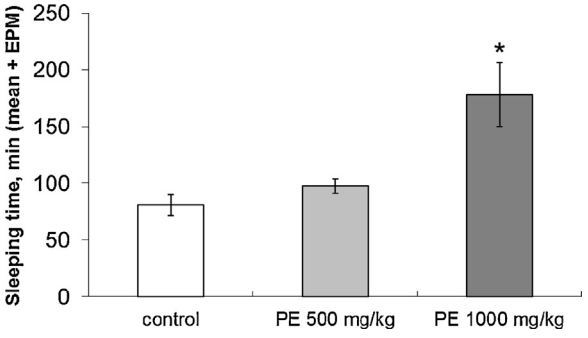


Fig. 2. Sleeping time (min, mean ± SEM) of mice orally treated with water (control group) or *Pterodum emarginatus* (PE) leaf extract at doses of 500 and 1000 mg/kg. *$p < 0.05$ compared to the control group (ANOVA followed by Duncan's test, $n = 10$).

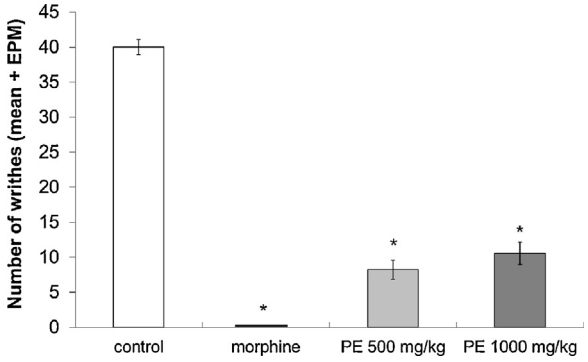


Fig. 3. Number of writhes (mean ± SEM) recorded in mice orally treated with water (control group), *Pterodum emarginatus* (PE) leaf extract at doses of 500 and 1000 mg/kg or morphine (10 mg/kg, i.p.). *$p < 0.05$ compared to the control group (ANOVA followed by Duncan's test, $n = 5–8$).

in mice [$F(2,27) = 8.89$; $p < 0.005$ – ANOVA/Duncan] (Fig. 2). However, the dose of 500 mg/kg did not alter the effect of pentobarbital on the animals.

Spontaneous motor activity and motor coordination

None of the doses administered affected either the ambulation or motor coordination of the mice on the corresponding tests (data not shown).

Writhing test

Both administered doses of the *P. emarginatus* extract significantly decreased the number of writhes induced by acetic acid [$F(3,22) = 156.15$; $p < 0.001$ – ANOVA/Duncan] (Fig. 3). Treatment with morphine (10 mg/kg, i.p.) also prevented the abdominal contortions in the corresponding treated animals.

Hot plate test

The response time of the mice to the thermal stimulus using a hot plate before treatment (basal) and after either 30 or 60 min are shown in Table 2. There was a significant difference among the groups [$F(3,20) = 3.504$; $p < 0.05$ – ANOVA) as well as temporally within each group [$F(6,40) = 5.365$; $p < 0.001$ – two-way ANOVA). Treatment with morphine (10 mg/kg, i.p.) increased the latency of response of the mice ($p < 0.05$ – ANOVA/Duncan) at 30 min but not at 60 min. Treatment with the *P. emarginatus* extract at 500 mg/kg produced a similar effect to that of morphine, i.e., a significant increase in the response time at 30 min.

Discussion

Phytochemical studies of the *Pterodon* genus have revealed the presence of isoflavones (Hansen et al. 2010), diterpenes (Coelho et al. 2005) and furane-diterpenes (Galceran et al. 2011). Only one study evaluated the chemical composition of the essential oil and ethanolic extract of *P. emarginatus* leaves (Santos et al. 2010), which revealed the presence of the phytosterols β-sitosterol and stigmasterol in the ethanolic extract and γ-muurolene and bicyclogermacrene as the main terpenoids in the essential oil. Interestingly, the hydroethanolic extract evaluated in our study was found to have flavones and saponins as the main constituents, whereas terpenoids were not detected. It is important to remember that we only evaluated the hydroethanolic extract (polar extract) in

Table 2
Response time (s, mean ± SEM) to the thermal stimulus for mice evaluated before (basal) and after 30 and 60 min of oral treatment with water (control group), *Pterodum emarginatus* (PE) leaf extract at doses of 500 and 1000 mg/kg or morphine (10 mg/kg, i.p.).

Group	Dose (mg/kg)	Response time, s (mean ± SEM)		
		Basal	30 min	60 min
Control	–	12.8 ± 1.7	13.0 ± 1.1	12.0 ± 0.4
Morphine	10	9.8 ± 0.9	21.6 ± 1.3*,#	15.4 ± 1.6#
P. emarginatus	500	10.0 ± 1.2	17.1 ± 1.3*,#	12.1 ± 0.6
P. emarginatus	1000	10.1 ± 0.8	14.3 ± 0.7#	15.6 ± 1.4#

* $p < 0.05$ compared to the control group (ANOVA followed by Duncan's test).
# $p < 0.05$ compared to the corresponding basal response (two-way ANOVA followed by Duncan's test, $n = 5–7$).

this study, which better represents the popular preparation in wine or "cachaça". According to Dutra et al. (2008b), the extraction carried out in *P. emarginatus* seeds using ethanol/water (70:30, under reflux) was the ideal condition for the detection of phenolic constituents. On the other hand, these constituents were not detected in essential oil, hexanic, and other apolar fractions of *P. emarginatus* seeds (Dutra et al. 2008b).

The constituents found in the hydroethanolic extract of *P. emarginatus* leaves were suggested based on their retention time, UV-spectra and ESI-MS/MS spectra in negative and positive ion mode (see Fig. 1, Table 1 and supplementary material).

The UV–vis spectra of flavones **1–7** (Table 1) exhibited band I (330–345 nm) and band II (264–272 nm) with similar intensities. Compound **1** and compound **2** were characterized as lucenin-2 and vicenin-2 based on UV and ESI-MS spectra, that showed deprotonated molecule at *m/z* 609 and *m/z* 593, respectively, as well as the MS/MS fragmentation pattern, identical to those previously described by Abranko et al. (2011). For compounds **3** and **4** the ESI-MS spectra showed deprotonated molecules at *m/z* 579 and *m/z* 563, respectively. A 6-*C*-pentosyl-8-*C*-hexosyl substitution was indicated for compound **3**, due to the increased abundance of the fragment ion at *m/z* 489 (base peak), in relation to the fragment ion at *m/z* 459, according to Lin and Harnly (2010). For compound **4**, the higher intensity of the fragment ion at *m/z* 443 (100%) relative to fragment ion at *m/z* 473 was indicative of a 6-*C*-hexosyl-8-*C*-pentosyl substitution (Wohlmuth et al. 2010). Based on literature data (Lin and Harnly 2010; Wohlmuth et al. 2010) compound **3** was tentatively identified as luteolin-6-*C*-arabinoside-8-*C*-glucoside, while compound **4** as apigenin-6-*C*-glucoside-8-*C*-arabinoside, also known as schaftoside. The isomer isoschaftoside, was also obtained at low content.

Di-*C*,*O*-glycosylflavones, such as compounds **5** and **6**, are flavonoids uncommon in plants. For compound **5**, the ESI-MS spectrum showed deprotonated molecule at *m/z* 769, which after fragmentation produced a fragment ion at *m/z* 575, which corresponded to the loss of 194 u (glucuronic acid – 176 u and water moieties). This fragment ion is characteristic of an *O*-glycosylation on the hydroxyl group on the 2″ position of the C-glycosylation sugar in "*O*-glycosilated-*C*-glycosyl derivatives" (Ferreres et al. 2008; Dinelli et al. 2009). The base peak at *m/z* 545 corresponding to the loss of 30 u (methoxy group) from fragment ion at *m/z* 575 was indicative of the presence of a methyl group on the flavone moiety (chrysoeriol) in compound **5**. For compound **6**, the ESI-MS spectrum displayed deprotonated molecule at *m/z* 709, which after fragmentation showed a fragment ion at *m/z* 545, attributed to the loss of rhamnose (146 u) moiety and water (Ferreres et al. 2008; Dinelli et al. 2009). Compound **5** was tentatively characterized as chrysoeriol-8-*C*-glucosyl-2″-*O*-glucuronide-6-*C*-arabinoside, and compound **6** as schaftoside-2″-*O*-rhamnoside, according to literature data (Ferreres et al. 2008; Dinelli et al. 2009).

For compound **7**, the ESI-MS spectrum exhibited deprotonated molecule at *m/z* 593, which after fragmentation produced a fragment ion at *m/z* 285, suggesting the presence of rutinoside moiety and luteolin as aglycone. Compound **7** was tentatively characterized as luteolin-7-*O*-rutinoside, according to literature data (Wohlmuth et al. 2010). Compounds **8** and **9** exhibited UV spectra typical of flavonols (265–355 nm) and the respective ESI-MS spectra showed deprotonated molecules at *m/z* 479 and *m/z* 521 in the negative mode and sodiated molecules at m/z 503 and m/z 545 in the positive mode, respectively (ESI-MS/MS spectra in supplementary material). According to Fracassetti et al. (2013) myricetin-*O*-glycosides with a blocked hydroxyl at 3 position of aglycone shows UV maximum absorption at 356 nm. Compound **8** was tentatively characterized as myricetin-3-*O*-glucoside, and compound **9** as myricetin-3-*O*-acetylglucoside, according to literature data (Fracassetti et al. 2013).

For saponin **10** and **11**, the ESI-MS spectra displayed deprotonated molecules at *m/z* 939 and *m/z* 941, respectively. The fragmentation pattern exhibited in ESI-MS/MS spectra of these compounds was similar (supplementary material), producing fragment ions corresponding to the loss of water, carbon dioxide moiety and rhamnose. For compound **10**, the fragment ions produced were *m/z* 921 (base peak), *m/z* 877 and *m/z* 793; while that for compound **11** were *m/z* 923 (base peak), *m/z* 879 and *m/z* 795, respectively. For compound **10**, the aglycone is soyasapogenol E (MM – 456), having a keto group at position 22, which is formed by the oxidation of OH group from soyasapogenol B (Jin et al. 2007; Sagratini et al. 2009; Pollier et al. 2011). The aglycone of compound **11** is soyasapogenol B, a triterpenic oleanolic type triol with molecular mass (MM) 458 u.

The most common sugar residues in saponin compounds are hexoses (glucose, galactose), pentoses (arabinose, xylose), methylpentoses (furanose, quinovose, rhamnose), and uronic acids (glucuronic acid, galacturonic acid), which are linked to aglycone through the ether glycosidic bonds generally in 3-O position (Tava et al. 2011). Saponin **10** was tentatively characterized as soyasaponin Be, based on MS data published by Jin et al. (2007), while saponin **11** was tentatively identified as soyasaponin Bb, which is in agreement with previously published data (Jin et al. 2007; Sagratini et al. 2009; Pollier et al. 2011). Soyasaponin Be and soyasaponin Bd were typical of soya bean (*Glycine max*, Fabaceae) (Jin et al. 2007; Sagratini et al. 2009).

The ESI-MS spectra of saponin **12** and **13** exhibited deprotonated molecules at *m/z* 953 and *m/z* 955, respectively. The fragmentation pattern, which is similar to saponins **10** and **11**, produced fragment ions attributed to the loss of water at *m/z* 935 and *m/z* 937 (base peak), the loss of carbon dioxide at *m/z* 891 (base peak) and *m/z* 893, and the loss of rhamnose at *m/z* 807 and *m/z* 809 for saponins **12** and **13**, respectively. The aglycone of saponin **13** is a triterpenic acid with MM 472 u known as hederagenin, while for saponin **12**, the aglycone is a triterpenoid known as aglycone B (3-hydroxy-23-oxoolean-12-en-28-oic acid) with MM 470 u, which contains a C-23 formyl group in the aglycone core. According to Pollier et al. (2011), aglycone B is formed through the enzymatic oxidation of hederagenin. The presence of a formyl group in aglycone B can explain the increased polarity (minor Rt in reversed phase) of saponin **12** in comparison with hederagenin glycoside (saponin **13**). Saponin **13** was tentatively characterized as hederagenin-3-*O*-rhamnosyl galactosyl glucuronide and saponin **12** as aglycone B-3-*O*-rhamnosyl galactosyl glucuronide by comparison with previously published data (Jin et al. 2007; Sagratini et al. 2009; Pollier et al. 2011). Hederagenin and aglycone B derivatives were found in *Medicago trunculata*, a species also from Fabaceae family (Pollier et al. 2011). The ESI-MS/MS spectra of these saponins are shown in supplementary material.

Several pre-clinical studies have supported the folk use of *Pterodon emarginatus* fruits and seeds as analgesic, anti-inflammatory, anti-rheumatic, among others (Carvalho et al. 1999; Sabino et al. 1999a; Coelho et al. 2005; Dutra et al. 2008a, 2009; Galceran et al. 2011). In the most of these studies, terpenoids (furanoditerpenoids) were related as the main responsible for the biological effects. According to Santos et al. (2010), the chemical composition of *P. emarginatus* leaves is different from that observed for fruits and seeds. This is the first study to evaluate the analgesic activity of the hydroethanolic extract of *P. emarginatus* leaves. Our results suggest that this extract has a mild analgesic effect at doses of 500 and 1000 mg/kg, as evaluated in acetic acid-induced writhing and hot plate tests in mice. Although these doses are high, they correspond to the same doses employed by other authors,

which showed the capability of this extract to produce the desired biological effects (Carvalho et al. 1999; Dutra et al. 2009; Moraes et al. 2009). Additionally, these doses did not alter either the locomotor activity or motor coordination of the mice, and no signs of toxicity in the initial pharmacological screening were observed. In the pentobarbital-induced sleeping test, the dose of 1000 mg/kg increased the sleeping time of mice, suggesting a depressant-like effect that is characteristic of analgesic drugs.

In a study carried out by Carvalho et al. (1999), the ED_{50} for carrageenan-induced edema in rats was 500 mg/kg (oral) for the hexanic crude extract of *P. emarginatus* fruit, whereas the 50% lethal dose was 4.02 g/kg (eight times higher). Toxicological studies carried out with *P. emarginatus* (syn. *P. pubescens*) seed oil (extracted by ethanol) showed that acute oral administration at doses ranging from 2 to 8 g/kg did not produce animal mortality, which was also observed by the lack of lesions upon histopathological analysis of the tissues (Sabino et al. 1999b). The authors also evaluated the in vitro mutagenic and cytotoxic effects of the extract, and the results indicated that the preparation was neither cytotoxic nor mutagenic. These results corroborate with our data, which showed that the hydroethanolic extract of *P. emarginatus* leaves has low toxicity.

The majority the studies that focus on *P. emarginatus* have used apolar extracts or essential oils from the fruits or seeds. Furanoditerpene derivatives have been described as the main responsible for the analgesic and anti-inflammatory effects of the species (Coelho et al. 2005; Galceran et al. 2011), but these constituents were not found in our hydroethanolic extract. Based on the proposed phytochemical constituents and literature data, we suggest that several flavones and saponins are the possible responsible for the analgesic effect observed in this study. Some studies have demonstrated the antinociceptive properties of the sapogenins hederagenin and hederagenin monodesmosides by inhibiting the effects of rheumatoid arthritis (Choi et al. 2005). Several flavonoids and other phenolic compounds have been described as antioxidants, analgesics and anti-inflammatory agents. For example, vicenin-2 was able to reduce the number of writhes induced by acetic acid (Gorzalczany et al. 2011), whereas schaftoside exerted an anti-inflammatory effect in a model of LPS-induced mouse lung inflammation (De Melo et al. 2005). An anti-inflammatory effect in the carrageenan-induced paw edema model and antinociceptive activity in the p-benzoquinone-induced writhing reflex in mice were described for luteolin derivatives (Tatli et al. 2008). Myricitrin also exhibited an antinociceptive effect, thus abolishing or reducing the nociceptive response induced by intraplantar injection of several chemical algogens into the hind paw of mice (Cordova et al. 2011). These different flavones were found in the present extract and may be responsible for these effects.

In conclusion, the antinociceptive effect observed in this study is not due to the terpenoids previously reported in extracts from fruits and seeds, but could be attributed to flavones and the hederagin derivatives identified in the hydroethanolic extract of *P. emarginatus* leaves.

Conflict of interest

No conflict to disclose.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2014.04.009.

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