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Chemical composition, antinociceptive and anti-inflammatory effects in rodents of the essential oil of *Peperomia serpens* (Sw.) Loud

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ABSTRACT

Ethnopharmacological relevance: Peperomia serpens (Piperaceae), popularly known as "carrapatinho", is an epiphyte herbaceous liana grown wild on different host trees in the Amazon rainforest. Its leaves are largely used in Brazilian folk medicine to treat inflammation, pain and asthma.
Aim of the study: This study investigated the effects of essential oil of *Peperomia serpens* (EOPs) in standard rodent models of pain and inflammation.
Materials and methods: The antinociceptive activity was evaluated using chemical (acetic acid and formalin) and thermal (hot plate) models of nociception in mice whereas the anti-inflammatory activity was evaluated by carrageenan- and dextran-induced paw edema tests in rats croton oil-induced ear edema, as well as cell migration, rolling and adhesion induced by carrageenan in mice. Additionally, phytochemical analysis of the EOPs has been also performed.
Results: Chemical composition of the EOPs was analyzed by gas chromatography and mass spectrometry (GC/MS). Twenty-four compounds, representing 89.6% of total oil, were identified. (*E*)-Nerolidol (38.0%), ledol (27.1%), α-humulene (11.5%), (*E*)-caryophyllene (4.0%) and α-eudesmol (2.7%) were found to be the major constituents of the oil. Oral pretreatment with EOPs (62.5–500 mg/kg) significantly reduced the writhing number evoked by acetic acid injection, with an ED_{50} value of 188.8 mg/kg that was used thereafter in all tests. EOPs had no significant effect on hot plate test but reduced the licking time in both phases of the formalin test, an effect that was not significantly altered by naloxone (0.4 mg/kg, s.c.). EOPs inhibited the edema formation induced by carrageenan and dextran in rats. In mice, EOPs inhibited the edema formation by croton oil as well as the leukocyte and neutrophil migration, the rolling and the adhesion of leukocytes.
Conclusions: These data show for the first time that EOPs has a significant and peripheral antinociceptive effect that seems unrelated to interaction with the opioid system. EOPs also displays a significant anti-inflammatory effect in acute inflammation models. This effect seems to be related to components which inhibit the production of several inflammatory mediators. These results support the widespread use of *Peperomia serpens* in popular medicine to treat inflammation and pain.

1. Introduction

The genus *Peperomia*, belonging to Piperaceae, comprises an estimated 1500–1700 species (Mathieu et al., 2008). *Peperomia serpens* (Sw.) Loud. [syn. *Acrocarpidium guildingianum* Miq., *Acrocarpidium scandens* (Ruiz et Pav.) Miq., *Peperomia praecox* Trel., *Peperomia repens* Kunth, *Peperomia scandens* Ruiz et Pav., *Piper scandens* (Ruiz et Pav.) Vahl, *Verhuellia serpens* Miq., among other] (Missouri Botanical Garden, 2011) is an epiphyte herbaceous liana, with petiolate, cordate and succulent leaves, known as "carrapatinho" or "carapitinha," grown wild on different host trees in the Amazon rainforest. The decoction of its leaves is recommended for their anti-inflammatory and analgesic properties, particularly against flu, asthma, cough, earache and irritation provoked by ant bites (Grenand et al., 1987; Schultes and Raffauf, 1990).

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The essential oil composition of *Peperomia subespatulata* (Diaz and Cardoso, 1988), *Peperomia rotundifolia*, *Peperomia macrostachya* (Lira et al., 2009), *Peperomia pellucida*, *Peperomia circinnata* (Silva et al., 1999), *Peperomia alata*, *Peperomia obtusifolia* (Moreira et al., 1999) and *Peperomia emarginella* (Abreu et al., 2005) has been reported. The essential oil of *Peperomia pellucida* showed antibacterial, anti-inflammatory and analgesic activities (Khan and Omoloso, 2002; Arrigoni-Blank et al., 2004). Other oils of *Peperomia* contains mono- and sesquiterpenes, as the case of *Peperomia serpens*, whose main constituents were α-humulene, (*E*)-caryophyllene, (*E*)-nerolidol and (*Z*)-nerolidol acetate (Silva et al., 2006), except the oil of *Peperomia pellucida* rich in dillapiole, a poly-oxygenated phenylpropanoid (Silva et al., 1999). The anti-inflammatory activities of α-humulene and (*E*)-caryophyllene have been reported previously (Fernandes et al., 2007).

Despite the widespread use of *Peperomia serpens* in popular medicine to treat inflammation and pain, surprisingly no studies have been carried out to examine its basic pharmacological properties. Therefore, the aim of the present study was to investigate the effects of essential oil of *Peperomia serpens* (EOPs) in standard rodent models of pain and inflammation. Additionally, the chemical composition of EOPs has been reviewed.

2. Materials and methods

2.1. Plant material

The epiphytic *Peperomia serpens* was collected in July 2009 in the locality of Flechal, municipally of Santa Izabel, Pará State, Brazil. Its identification was confirmed by comparison with an authentic voucher of *Peperomia serpens* (MG 157255), that is deposited in the herbarium of Emilio Goeldi Museum, in the city of Belém, Pará State, Brazil.

2.2. Essential oil extraction and analysis

The whole plant (1.6 kg) was air-dried at room temperature for 4 days and submitted to steam distillation (980 g, 3 h) using a Clevenger-type apparatus. Essential oil of *Peperomia serpens* (EOPs) was dried over anhydrous sodium sulfate and its percentage content was calculated on basis of the plant dry weight, resulting in an oil yield of 0.4%. The moisture content was calculated after the phase separation in a Dean–Stark trap (5 g, 30 min) using toluene.

The analysis of the oil was carried out on a Thermo DSQ II GC–MS instrument, under the following conditions: DB-5 ms (30 m × 0.25 mm; 0.25 μm film thickness) fused-silica capillary column; programmed temperature: 60–240 °C (3 °C/min); injector temperature: 250 °C; carrier gas: helium, adjusted to a linear velocity of 32 cm/s (measured at 100 °C); injection type: splitless (2 μL of a 1:1000 hexane solution); split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 mL/min; EIMS: electron energy, 70 eV; temperature of ion source and connection parts: 200 °C. The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a Focus GC/FID operated under conditions similar to those in GC–MS, except for the carrier gas, which was nitrogen. The retention index was calculated for all the volatiles constituents using an *n*-alkane homologous series.

Individual components were identified by a comparison of both mass spectra and GC-retention data with authentic compounds previously analyzed and stored in the data system. Other identifications were carried out by comparison of the mass spectra with those existing in the data system libraries and cited in the literature (NIST, 2005; Adams, 2007).

2.3. Drugs and chemicals

The following drugs and chemicals were used: acetic acid and formaldehyde used was of analytical grade (Vetec Química Fina Ltda, Rio de Janeiro, Brazil); indomethacin, carrageenan, dextran, dexamethasone, croton oil and tribromoethanol (Sigma Chemical Co., St. Louis, MO, USA); pizotifen (Novartis, Rio de Janeiro, Brazil); ketamin hydrochloride (Dopalen, Paulinia, São Paulo, Brazil), xylazine hydrochloride (Rompun Bayer®, Rio de Janeiro, Brazil), morphine sulfate and naloxone hydrochloride (Cristália, Rio de Janeiro, Brazil). The EOPs was dissolved in Tween 80 (1%), brought to the chosen volume with sterile isotonic saline and sonicated just before use. Oral treatment with EOPs or drugs was performed in a volume of 0.1 mL/10 g for mice and 0.1 mL/100 g for rats. All drugs were dissolved in 0.9% saline.

2.4. Animals

Experiments were conducted using male Swiss mice (20–30 g) and male adult Wistar rats (160–220 g) obtained from the Central Housing Facility of the Evandro Chagas Institute, Belém, State of Pará, Brazil. Animals were housed at 23 ± 1 °C under a 12 h light/dark cycle and with access to water and food *ad libitum*. On the day of experiment, animals which have been starved overnight with water *ad libitum*, were acclimatized to the laboratory for at least 2 h before performing any test and were used only once throughout the study. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals (1996). All procedures described here were reviewed by and had prior approval from the local animal Ethics committee (Federal University of Pará, process number FAR001-10).

2.5. Antinociceptive activity

2.5.1. Acetic acid-induced writhing in mice

The writhing test induced by intraperitoneal (i.p.) injection of acetic acid (0.6%) was carried out according to the procedure described previously by Koster et al. (1959). Briefly, mice were randomly divided in 7 groups (*n* = 10 per group). An aqueous acetic acid solution (0.6%, v/v) was injected by intraperitoneal (i.p., 0.1 mL/10 g body weight) route to mice pre-treated 60 min earlier by gavage (p.o.) with vehicle (0.9% saline plus 1% Tween 80 to 0.1 mL/10 g, control group), the standard drug indomethacin (10 mg/kg), or EOPs (31.25, 62.5, 125, 250 and 500 mg/kg). After the challenge, the mice were individually placed in a glass cylinder of 22 cm diameter. The total numbers of abdominal contractions (writhes), which consist in the contraction of the flank muscles associated with inward movements of the hindlimb or with whole body stretching, were counted cumulatively after 10 min of stimulus over a period of 20 min. The antinociceptive activity was determined as the difference in number of writhes between control group and each treated group.

2.5.2. Formalin test

Formalin-induced nociception was induced in mice according to a previously described procedure (Hunskaar et al., 1985). Animals were randomly divided in 6 groups (*n* = 10 per group). A volume of 20 μL of a 1% formalin solution (0.92% formaldehyde) in saline was injected intraplantarly (i.pl.) in the plantar surface of the right hind paw to mice pretreated 60 min earlier with vehicle (0.9% saline plus 1% Tween 80 to 0.1 mL/10 g, control group) or EOPs (188.8 mg/kg, p.o.), or pretreated 30 min earlier with morphine (4 mg/kg, subcutaneously). After formalin injection, the mice were individually placed in a glass cylinder of 22 cm diameter and were observed from 0 to 5 min (neurogenic phase) and 15 to 30 min (inflammatory phase). The time spent licking the injected paw was recorded with

a chronometer for both phases and considered as indicative of nociception. In order to verify the possible involvement of the opioid system in antinociceptive effect of EOPs, three more groups of mice were pretreated with the nonselective opioid antagonist naloxone (0.4 mg/kg, s.c.) 15 min before receiving EOPs (188.8 mg/kg, p.o.), morphine (4 mg/kg, s.c.) or vehicle.

2.5.3. Hot plate test in mice

The hot plate test was used to measure response latency according to a previously described method (MacDonald et al., 1946). Mice ($n = 10$) were placed in a hot plate (Ugo Basile, model 35100, Varese, Italy) kept at a temperature of $50 \pm 1\,^{\circ}C$. Animals with baseline latencies of more than 20 s were eliminated from the study and the cut-off time of 40 s was fixed to avoid damage to the paws. Mice were pre-treated with vehicle (0.9% saline plus 1% Tween 80 to 0.1 mL/10 g, control group), the positive reference drug morphine (10 mg/kg, s.c.) or EOPs (188.8 mg/kg, p.o.), and the response latency (in seconds) of the first noxious behavior of the animal (licking of paw, jumping or shaking) was recorded using a stopwatch at 0, 30, 60, 90 and 120 min after administration.

2.6. Anti-inflammatory activity

2.6.1. Paw edema induced by carrageenan

The paw edema was induced according to Winter et al. (1962). Rats were randomly divided in 3 groups ($n = 8$–11 per group). They were pretreated orally with the vehicle (0.9% saline plus 1% Tween 80 to 0.1 mL/100 g, control group), EOPs (188.8 mg/kg) or the reference anti-inflammatory indomethacin (5 mg/kg, p.o). After 60 min, edema was induced with the injection of 0.1 mL of carrageenan (100 μg/paw) in saline into the right hind paw. Left hind paw, used as control, received the vehicle saline (100 μL). The inflammation was quantified by measuring the volume (mL) displaced by the paw using a plethysmometer (Ugo Basile, model 7140, Varese, Italy) at 0, 1, 2, 3, 4 and 5 h after carrageenan injection. Results were expressed as variation in volume (mL) between the right and left paws at each time.

2.6.2. Paw edema induced by dextran

Anti-inflammatory activity was evaluated by inhibition of the dextran-induced paw edema in rats (Carvalho et al., 1999). Rats were randomly divided in 3 groups ($n = 5$ per group). A volume of 0.1 mL of a 1% dextran solution was injected i.pl. in the plantar surface of the right hind paw to rats pretreated 60 min earlier with vehicle (0.9% saline plus 1% Tween 80 to 0.1 mL/100 g, control group), EOPs (188.8 mg/kg, p.o.), or the reference drug pizotifen (0.5 mg/kg, p.o.). The inflammation was quantified by measuring the volume (mL) displaced by the paw using a plethysmometer (Ugo Basile, model 7140, Varese, Italy) at 0, 30, 60, 90 and 120 min after dextran injection. Results were expressed as variation in volume (mL) between the right and left paws at each time.

2.6.3. Ear edema induced by croton oil

Ear edema was induced according to a previously described procedure (Tubaro et al., 1985). Three groups of 10 mice each were used. Under anesthesia with ketamine and xylazine (3:1, i.p.), cutaneous inflammation was induced by application of 20 μL of an acetone solution containing the irritant agent (2.5% of croton oil) in the inner surface of the right ear of each mouse (surface: about 1 cm^2). Left ear, used as control, received the vehicle acetone (20 μL). Sixty min before the application of the irritant agent, the animals were pretreated with vehicle (10 mL/kg, p.o.) (negative control), EOPs (188.8 mg/kg, p.o.) or the reference steroidal anti-inflammatory drug dexamethasone (10 mg/kg, p.o.) (positive control). After 6 h, animals were sacrificed and a plug (6 mm) was removed from both ears with irritant and with acetone. The edematous response was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as a percentage of the edema reduction in pretreated mice with EOPs or dexamethasone compared to the negative control group.

2.6.4. Leukocyte and neutrophil migration into the peritoneal cavity in mice

The determination of leukocyte and neutrophil migration to peritoneal cavity was performed as previously described by Souza and Ferreira (1985). Mice ($n = 5$ per group) were pretreated with the EOPs (188.8 mg/kg, p.o.) or vehicle (0.1 mL/10 g, p.o.) 60 min before injection of carrageenan (500 μg/cavity, i.p., 0.5 mL) or sterile saline (0.5 mL) into the peritoneal cavity. Dexamethasone (10 mg/kg, p.o.) has been used as reference anti-inflammatory drug. The animals were then sacrificed by cervical displacement 3 h after carrageen injection. Immediately after, a volume of 3 mL of phosphate buffered saline (PBS) containing ethylenediamine tetracetic acid (EDTA, 1 mM) was injected into the peritoneal cavity and the counting number of both total (leukocytes) and differential (neutrophils) cells was achieved.

To perform the total count, the peritoneal fluids (20 μL) were diluted in Turk solution (0.4 mL). The counting was done in a Newbauer camera and the results expressed in number of leukocytes per millilitre in the peritoneal washing. Then, part of peritoneal fluid was centrifuged at 78.4 g (10 min) and the supernatant was re-suspended to determine the neutrophils count. The cells were stained with hematoxylin–eosin and counted under a light microscope, using oil immersion objective. The number of differentiated cells was calculated by the percentage found in the total number of cells (100 cells in total).

2.6.5. Real time microscopic analysis of the neutrophils rolling and adhesion in mesenteric microcirculation of mice

The experiment was conducted according Fortes et al. (1991). The microscopic analysis was performed after the induction of leukocytes migration by injection of carrageenan (500 μg/cavity) in sterile saline solution. Three hours after the stimulus injection the mice were anesthetized with 2.5% tribromoethanol (250 mg/kg, i.p.) injection. A lateral incision in the abdominal wall allowed the exposure of the mesentery for observation of microcirculation *in situ*. The animals were kept on a heated plate (37 °C) adapted to the chariot of an optical microscopic with TV camera and video monitor for projection and image recording. The preparation was kept moist and warmed by irrigation with saline 0.9%. The images were viewed with long-distance objective lenses (40×) and numerical aperture (0.65). The vessels are the post-capillary venues with diameters from 10 to 18 μm. The rolling leukocytes were defined as white blood cells that moved on at a slower when compared to other cells on the same vessel and it was determined 5 min after the carrageenan injection. It was expressed as rolling 'leukocytes/min'. For leukocytes adherence were considered white blood cells that remained static in the endothelium and were determined by area variation between 350 and 450 μm^2. The results were expressed as the number of adherent leukocytes/100 μm^2 of venue area. For measurement, the following relationship was considered: 10 μm tissue corresponds to 3.4 cm on the monitor screen. Three groups of mice were used, one treated orally with EOPs (188.8 mg/kg, p.o), other with the vehicle 0.1 mL/10 g (control group), 1 h before injection of carrageenan (500 μg/cavity, i.p.; 0.1 mL). A third group of mice was treated only with sterile saline (0.5 mL) into the peritoneal cavity.

2.7. Statistical analysis

The results were presented as the mean ± standard error of the mean (SEM). The ED_{50} value, defined as the dose of EOPs at which 50% of the maximal response was achieved, was determined using linear regression GraphPad (GraphPad software, San Diego, CA, USA). Groups of data were analyzed using unpaired Student's *t*-test, one-way or two-way analysis of variance (ANOVA) test followed by Bonferroni's test multiple comparison tests when appropriate. *P*-Values less than 0.05 ($P < 0.05$) were considered to be indicative of significance.

3. Results

The chemical analysis of the sample of EOPs used in the present investigation allowed the identification of twenty-four compounds, representing 89.6% of total oil (Table 1). (*E*)-Nerolidol (38.0%), ledol (27.1%), α-humulene (11.5%), (*E*)-caryophyllene (4.0%) and α-eudesmol (2.7%) were found to be the major constituents of the oil (Table 1).

3.1. Effects of EOPs on acetic acid-induced abdominal writhes in mice

Fig. 1 shows that the oral pretreatment with EOPs (61.25–500 mg/kg) 1 h before testing evoked a dose-dependent ($P < 0.05$, one-way ANOVA) inhibition of acetic acid-induced abdominal writhes in mice when compared to control group, with an ED_{50} value of 188.8 mg/kg. The standard drug reference indomethacin resulted in a significant ($P < 0.001$, Fig. 1) reduction (73.3%) of the control writhes.

3.2. Effects of EOPs on hot plate test in mice

The pretreatment of animals with the standard drug morphine (10 mg/kg, s.c.) significantly ($P < 0.05$, two-way ANOVA) increased the response latency of the first noxious behavior at 30, 60, 90 and 120 min after administration (28.1 ± 3.65, 27.5 ± 3.87, 25 ± 4.28 and 16.7 ± 3.02 s, respectively) when compared to the control group (9.74 ± 0.55, 8.39 ± 0.42, 9.70 ± 1.20 and 9.22 ± 1.06 s, respectively). At the dose studied (188.8 mg/kg, p.o.), EOPs did not show any antinociceptive effect at all analyzed periods.

Table 1
Chemical composition and retention indices of the essential oil of *Peperomia serpens* (data bank of the Department of Chemical Engineering of the Federal University of Pará).

Constituents	Retention indices	Composition (%) of total oil)
α-Copaene	1372	0.2
(*E*)-Caryophyllene	1415	4.0
β-Gurjunene	1430	0.1
α-Humulene	1450	11.5
Trans-cadina-1(6),4-diene	1474	0.1
γ-Muurolene	1476	0.1
α-Amorfene	1481	0.2
β-Selinene	1488	0.2
α-Zingiberene	1492	0.1
Viridiflorene	1496	0.9
α-Muurulene	1499	0.4
β-Bisabolene	1503	0.6
δ-Amorfene	1510	1.0
(*Z*)-γ-Bisabolene	1513	0.3
Trans-calamenene	1519	0.2
(*E*)-Nerolidol	1560	38.0
Caryophyllene oxide	1580	0.2
Ledol	1601	27.1
Humulene II epoxide	1607	0.1
1-*Epi*-cubenol	1625	0.1
Eremoligenol	1628	0.5
α-Muurolol	1642	0.7
α-Eudesmol	1650	2.7
(2*Z*,6*Z*)-Farnesol	1697	0.3
Total identified		89.6

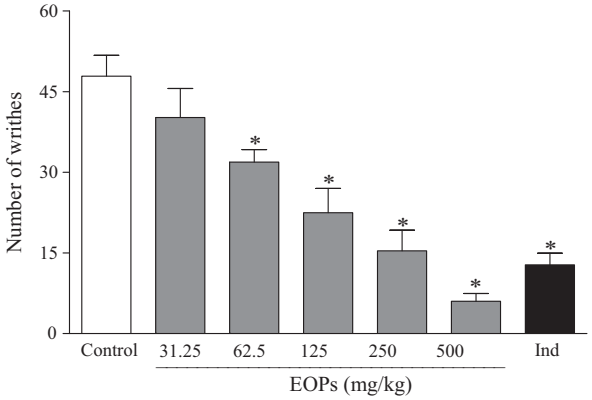

Fig. 1. Effects of oral pretreatment with the essential oil of *Peperomia serpens* (EOPs; 31.25–500 mg/kg, grey columns) or indomethacin (Ind; 10 mg/kg, black column) on acetic acid-induced writhing in mice. Each value represents mean ± SEM ($n = 10$ per group). *$P < 0.05$ by one-way ANOVA followed by post hoc Bonferroni's test compared to control group (pretreated orally by 0.9% saline plus 1% Tween 80, white column).

3.3. Effects of EOPs on formalin-induced nociception in mice

Fig. 2 shows that both first (neurogenic pain) and second (inflammatory pain) phases of formalin-induced nociception were significantly ($P < 0.05$, one-way ANOVA and Bonferroni's test compared to control group) inhibited in mice pretreated orally with EOPs (188.8 mg/kg) with an inhibition of 48.1 and 93.8%, respectively. Such an effect of EOPs was not significantly altered by naloxone (0.4 mg/kg, s.c.) pretreatment (Fig. 2; $P > 0.05$, unpaired Student's *t*-test). Likewise, pretreatment of mice with morphine (4 mg/kg, s.c.) caused significant inhibition of both first and second phases of formalin-induced nociception by 66.1 and 88.9%, respectively, an effect that was abolished by naloxone (Fig. 2; $P < 0.05$, unpaired Student's *t*-test).

3.4. Effects of EOPs on carrageenan-induced paw edema in rats

Carrageenan (100 μg/paw) induced significant (Fig. 3; $P < 0.05$, two-way ANOVA) and intense paw edema in rats, an effect that

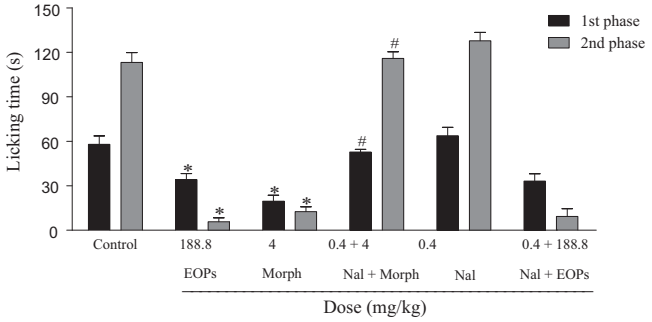

Fig. 2. Influence of naloxone (Nal, s.c.) pretreatment on the antinociceptive action of morphine (Morph, s.c.) and the essential oil of *Peperomia serpens* (EOPs, p.o.) on both first (neurogenic pain, 0–5 min) and second (inflammatory pain, 15–30 min) phases of the formalin-induced nociception. Each value represents mean ± SEM ($n = 10$ per group). *$P < 0.05$ by one-way ANOVA followed by post hoc Bonferroni's test compared to control group (pretreated orally by 0.9% saline plus 1% Tween 80). #$P < 0.05$ by unpaired Student's *t*-test compared to group pretreated with Morph (s.c).

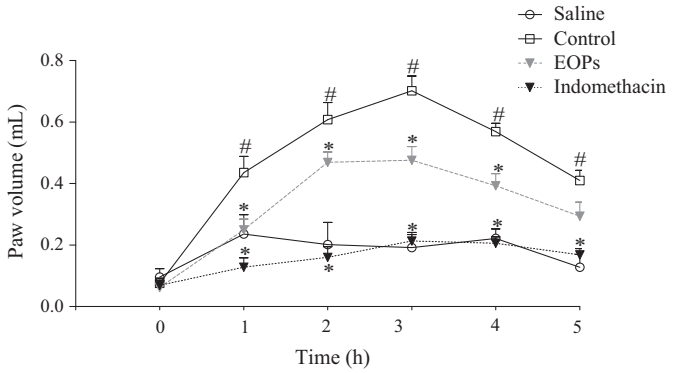

Fig. 3. Effects of oral pretreatment with the essential oil of *Peperomia serpens* (EOPs, 188.8 mg/kg) or indomethacin (5 mg/kg) on carrageenan-induced paw edema in rats. Each value represents mean ± SEM (n = 8–11 per group). $^{*}P < 0.05$ by two-way ANOVA followed by post hoc Bonferroni's test compared to control group (pretreated orally by 0.9% saline plus 1% Tween 80) and $^{\#}P < 0.05$ by two-way ANOVA followed by post hoc Bonferroni's test compared to saline group (received only saline into the hind paw).

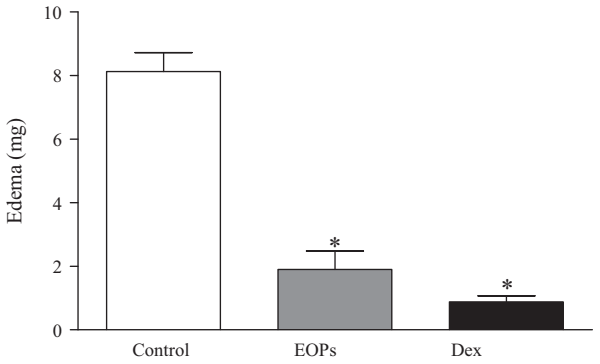

Fig. 5. Effects of oral pretreatment with the essential oil of *Peperomia serpens* (EOPs, 188.8 mg/kg, grey column) or dexamethasone (Dex, 10 mg/kg, black column) on the ear edema induced by croton oil in mice. Each value represents mean ± SEM (n = 10 per group). $^{*}P < 0.05$ by one-way ANOVA followed by post hoc Bonferroni's test compared to control group (pretreated orally by 0.9% saline plus 1% Tween 80, white column).

reached a maximum level at 3 h (0.70 ± 0.04 mL) after administration. Treatment of animals with EOPs (188.8 mg/kg, p.o.) 1 h before injection of carrageenan significantly (Fig. 3; $P < 0.05$, two-way ANOVA) inhibited the edema formation at 1, 2, 3 and 4 h when compared to control group. Indomethacin (5 mg/kg, p.o.) also produced a significant inhibition of the carrageenan-induced paw edema at 1, 2, 3, 4 and 5 h when compared to control (Fig. 3; $P < 0.05$, two-way ANOVA).

3.5. Effects of EOPs on dextran-induced paw edema in rats

Dextran 1% induced intense paw edema in rats, an effect that reached a maximum level at 1 h (0.94 ± 0.04 mL) after administration and decreased over the subsequent hours. Treatment of animals with EOPs (188.8 mg/kg, p.o.) 1 h before injection of dextran significantly (Fig. 4; $P < 0.05$, two-way ANOVA) inhibited the edema formation at 60 and 90 min when compared to control group. The reference drug pizotifen (0.5 mg/kg, p.o.) produced a significant ($P < 0.05$, two-way ANOVA) inhibition of dextran-induced paw edema at 30, 60, 90 and 120 min after administration when compared to control (Fig. 4).

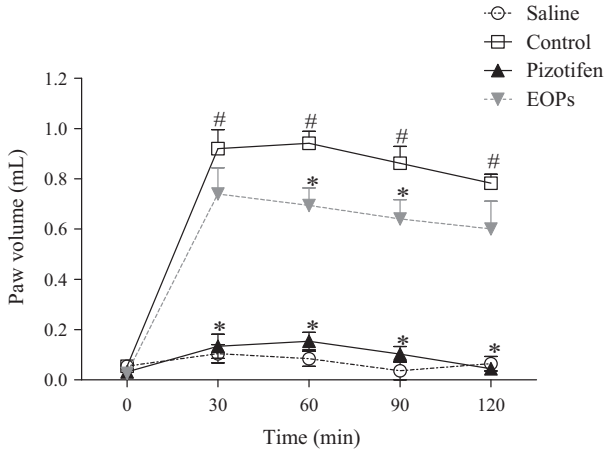

Fig. 4. Effects of oral pretreatment with the essential oil of *Peperomia serpens* (EOPs, 188.8 mg/kg) or pizotifen (0.5 mg/kg) on dextran-induced paw edema in rats. Each value represents mean ± SEM (n = 5 per group). $^{*}P < 0.05$ by two-way ANOVA followed by post hoc Bonferroni's test compared to control group (pretreated orally by 0.9% saline plus 1% Tween 80) and $^{\#}P < 0.05$ by two-way ANOVA followed by post hoc Bonferroni's test compared to saline group (received only saline into the hind paw).

3.6. Effects of EOPs on ear edema in mice

Pretreatment with EOPs (188.8 mg/kg, p.o.) inhibited in 76.63% the ear edema formation induced by croton oil injection (Fig. 5; $P < 0.05$, unpaired Student's t-test when compared to control group). Likewise, pretreatment with the reference steroidal anti-inflammatory drug dexamethasone (10 mg/kg, p.o.) (positive control) inhibited the edema formation by 89.18% (Fig. 5; $P < 0.05$, unpaired Student's t-test), when compared to control group (negative control).

3.7. Effects of EOPs on carrageenan-induced leukocyte and neutrophil migration into the peritoneal cavity in mice

Carrageenan (500 μg/cavity, i.p., 0.5 mL) induced a significant leukocyte (5.9 ± 0.35 leukocytes × 10^6 mL^{-1}) and neutrophil (4.12 ± 0.55 neutrophils × 10^6 mL^{-1}) migration in mice. Pretreatment with vehicle had no effect on carrageenan-induced response. However, pretreatment with EOPs (188.8 mg/kg; p.o., 1 h beforehand) significantly inhibited by 58.5% (2.45 ± 0.25 leukocytes × 10^6 mL^{-1}) and 63.1% (1.52 ± 0.3 neutrophils × 10^6 mL^{-1}) the leukocyte and neutrophil migration caused by carrageenan, respectively, when compared to control group (Fig. 6A and B; $P < 0.05$, unpaired Student's t-test). The reference anti-inflammatory drug dexamethasone (10 mg/kg, p.o.) promoted significant (Fig. 6A and B; $P < 0.05$, unpaired Student's t-test) reduction in leukocyte (2.55 ± 0.27 leukocytes × 10^6 mL^{-1}) and neutrophil (1.36 ± 0.45 neutrophils × $10^6/mL^{-1}$) recruitment into peritoneal cavities of mice.

3.8. Effects of EOPs on rolling and adhesion of leukocytes in mice

Carrageenan (500 μg/cavity; i.p., 0.5 mL) caused a significant (Fig. 7; $P < 0.05$, unpaired Student's t-test) increase in leukocyte rolling and adhesion on endothelium 3 h after stimuli, when compared to mice pretreated only with i.p. saline. Pretreatment of mice with EOPs (188.8 mg/kg, p.o.) significantly (Fig. 7A and B; $P < 0.05$, unpaired Student's t-test) decreased leukocyte rolling and adhesion when compared to mice treated orally with only vehicle (control group).

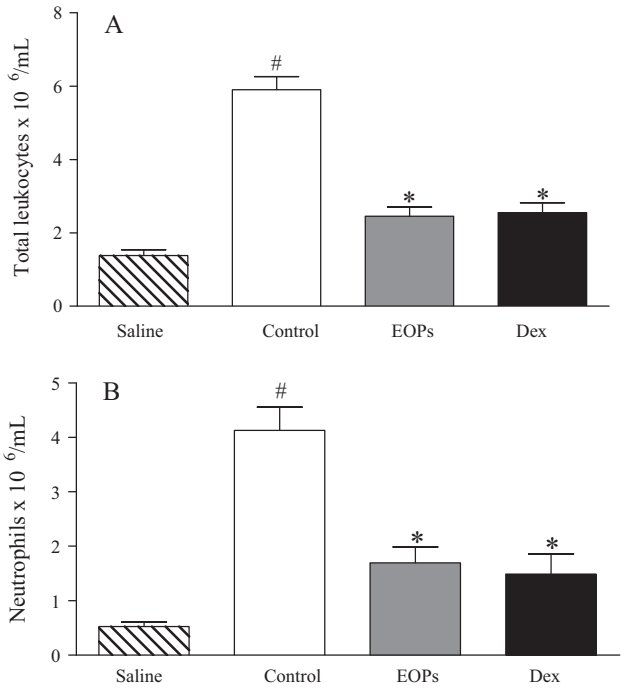

Fig. 6. Effects of oral pretreatment of the essential oil of *Peperomia serpens* (EOPs, 188.8 mg/kg, grey column) or dexamethasone (Dex, 10 mg/kg, black column) on carrageenan-induced leukocytes (A) and neutrophils (B) migration into the peritoneal cavity in mice. Each value represents mean ± SEM ($n = 5$ per group). $^{*}P < 0.05$ by one-way ANOVA followed by post Bonferroni's hoc test compared to control group (pretreated orally by 0.9% saline plus 1% Tween 80, white column). $^{\#}P < 0.05$ by unpaired Student's *t*-test compared to saline group (received only saline into the peritoneal cavity without carrageenan injection, hatched column).

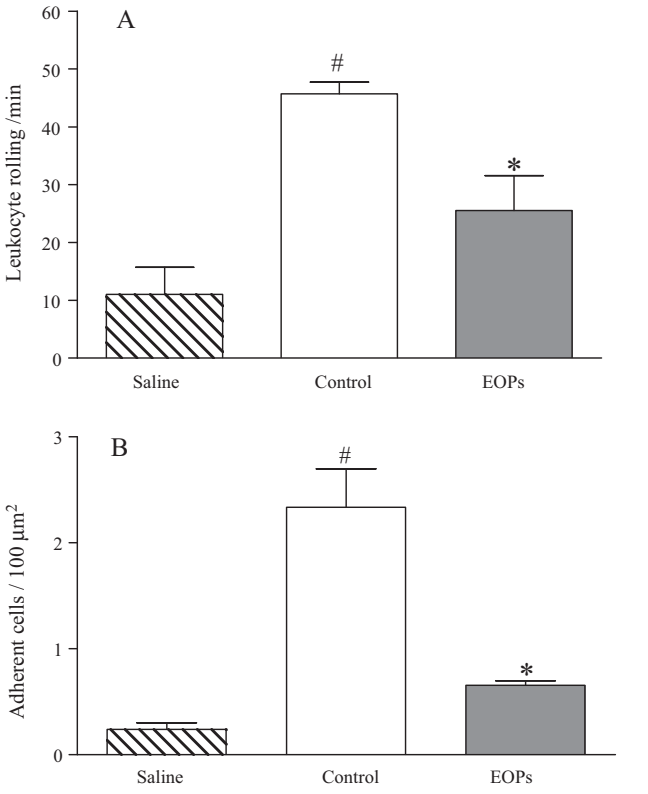

Fig. 7. Effects of oral pretreatment of the essential oil of *Peperomia serpens* (EOPs, 188.8 mg/kg, grey column) on carrageenan-induced leukocyte rolling (A) and adhesion (B) in mesenteric microcirculation of mice. Each value represents mean ± SEM ($n = 5$ per group). $^{*}P < 0.05$; $^{\#}P < 0.05$ by unpaired Student's *t*-test with respect to control group (pretreated orally by 0.9% saline plus 1% Tween 80, white column) and saline group (received only saline into the peritoneal cavity without carrageenan injection, hatched column), respectively.

4. Discussion and conclusion

The present study demonstrates for the first time that the EOPs induces anti-inflammatory and antinociceptive effects in several model of nociception (acetic acid-induced abdominal writhing, hot plate and formalin) and inflammation (ear edema, paw edema, peritonitis and intravital microscopy). Acute study from our laboratory showed that neither mortality nor sign of toxicity was detected during the behavioral observations, indicating a lack of toxicity for the EOPs (data not shown). Therefore, it is highly unlikely that the anti-inflammatory and antinociceptive effects observed herein could be related to a putative toxic effect of this essential oil.

The writhing test induced by acetic acid in mice is described as a typical model of study of inflammatory pain, being used as a screening for evaluation of analgesics or anti-inflammatory drugs (Vinegar et al., 1979). The local irritation provoked by intraperitoneal injection of acetic acid triggers the liberation a variety of mediators such bradykinin, substance P, and prostaglandins, especially PGI_2, as well as some cytokines such as IL-1β, TNF-α and IL-8 (Correa and Calixto, 1996; Ribeiro et al., 2000; Ikeda et al., 2001). Such as mediators activate chemosensitive nociceptors that contribute to the development of this type of inflammatory pain, which is known to be sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, diclofenac and indomethacin. Like indomethacin, EOPs was able to reduce dose-dependently the acetic acid-induced writhing response, suggesting a mechanism resulting on peripheral antinociceptive effect. Despite EOPs displayed a significant antinociception response on the acetic acid-induced pain, it was ineffective on hot plate test which indicates a non-participation on thermal stimulation associated with central neurotransmission in what the heat activates nociceptors (Aδ and C fibers) by driving the momentum of the dorsal horn of the spinal cord and subsequently to cortical centers.

We also used the formalin test which involves two phases: the first phase (0–5 min) is characterized by neurogenic pain caused by a direct chemical stimulation of nociceptors. The second phase (15–30 min) is characterized by inflammatory pain triggered by a combination of stimuli, including inflammation of the peripheral tissues and mechanisms of central sensitization (Tjolsen et al., 1992). Substance P is involved in the first phase whereas histamine, serotonin, prostaglandins and bradykinin are involved in the second one (Murray et al., 1988; Tjolsen et al., 1992). Formalin test is a very useful method for not only assessing the antinociceptive drugs but also helping in the elucidation of the action mechanism. Centrally acting drugs such as narcotics inhibited both phases equally. Peripheral acting drugs such as NSAIDs and corticoids inhibited mainly the second phase (Shibata et al., 1989). In the present study, oral pretreatment with EOPs significantly inhibited the first (neurogenic pain) and second (inflammatory nociception) phases of formalin-induced licking in mice, an effect that remained significantly unaffected by pretreatment with the opioid receptor antagonist naloxone. This may indicate that EOPs induces its antinociceptive action by direct action on nociceptive afferent fibers without interaction with the opioid system. In addition, EOPs was effective in the second phase of the formalin test indicating an anti-inflammatory activity. These results may suggest that the antinociceptive action of EOPs is more related to a peripheral mechanism rather than a central one.

The anti-inflammatory activity of EOPs was evaluated in carrageenan-induced paw edema, a largely used test for screening

both steroidal anti-inflammatory drugs and NSAIDs, since it involves several mediators. This kind of test induces an inflammatory reaction characterized by two different phases. The initial phase, which occurs between 0 and 2.5 h after injection of the phlogistic agent, has been attributed to the action of histamine, serotonin, and bradykinin on vascular permeability (Vinger et al., 1987). The edema volume reaches its maximum approximately 3 h post-treatment and then begins to decline. The second phase, which is also a complement-dependent reaction has been shown to be a result of a overproduction of prostaglandins in tissues (Di Rosa, 1974). The oral pretreatment with EOPs inhibited the edema formation in both phases after administration of carrageenan. This allowed us to suggest that the antiedematogenic response of EOPs is related to interference in the production of histamine, serotonin, kinins (bradykinin) and arachidonic acid metabolites (prostaglandins). Dextran induces mast cell degranulation releasing histamine and serotonin which contribute to increased vascular permeability and leakage of fluid (Ankier and Neat, 1972; Van Wauwe and Goossens, 1989). The finding that EOPs was effective in dextran-induced paw edema corroborates the putative involvement of histamine and serotonin in the mediation of its anti-inflammatory activity.

The topical application of croton oil in the mice ear induces vascular irritation, leukocyte migration and consequently the edema formation, an effect that is attributed to the action of its irritant principle the 4β-12-O-tetradecanoylphorbol-13-acetate (TPA) (Garg et al., 2008). TPA can activate protein kinase C, a calcium-dependent enzyme, and induced arachidonic acid formation through activation of phospholipase A_2. Phospholipase A_2 inhibitors, cyclooxygenase inhibitors, and lipoxygenase inhibitors as well as corticoids are effective at suppressing ear edema after topical application of TPA at high concentration (Young and De Young, 1989). Oral pretreatment with EOPs was able to reduce the edema formation 6 h after croton oil application. It is possible that this inhibitory activity of EOPs is related to its ability to inhibit the calcium intracellular stores by inhibiting the protein kinase C and therefore the liberation of inflammatory mediators. However, further experiments are needed to corroborate this hypothesis.To evaluate the anti-inflammatory activity of EOPs on migration of inflammatory cells, carrageenan-induced peritonitis test was performed in mice. During peritonitis induced by carrageenan, several events occur such as neutrophil and leukocyte migration from mesenteric venules to peritoneal cavity, vasodilatation and increased vascular permeability, and the liberation of several mediators of the inflammation such as TNF-α, IL-1β, IL-8, prostanoids, LTB_4 and component of the complement C5a (Hall and Heel, 1998). The step of rolling is mediated by E- and P-selectins (on endothelial cells) and L-selectin (on leukocytes) interacting with their respective carbohydrate ligand. Thereafter, adhesion and transmigration are mediated by CD11/CD18 complex (β2-integrins) on leukocyte, which interacts with its ligand immunoglobulins, such as intercellular adhesion molecule-1 (ICAM-1), present mostly on endothelial cells (Kasama et al., 2005). In addition, pro-inflammatory cytokines as TNF-α, IL-1β and IL-8 have the ability to induce the expression of selectins (rolling), ICAM-1 and vascular adhesion molecule-1 (VCAM-1) (Shigeta et al., 2008). EOPs were able to reduce the neutrophil and leukocyte migration into the peritoneal cavity after carrageenan injection. This result was corroborated by intravital microscopy test which evaluate the mesenteric microcirculation by means of video surveillance *in vivo*, interactions between leukocytes and the wall of blood vessels in the inflammation site (Granger and Kubes, 1994) in which the EOPs was able to reduce rolling and adhesion of leucocytes in mesenteric venules. This suggests that EOPs may inhibit adhesion molecules that are expressed by pro-inflammatory cytokines. This hypothesis is reinforced by the finding that α-humulene and (*E*)-caryophyllene inhibited TNF-α and IL-1β in rats (Fernandes et al., 2007). However, further experiments are needed to confirm of this hypothesis.

Chemical composition of the EOPs was analyzed by gas GC/MS and identified the presence of twenty-four compounds, representing 89.6% of total oil. The main components were the oxygenated sesquiterpenes (*E*)-nerolidol (38.0%) and ledol (27.1%), and the hydrocarbon sesquiterpenes α-humulene (11.5%), (*E*)-caryophyllene (4.0%) and α-eudesmol (2.7%). The antinociceptive and anti-inflammatory effects of EOPs could be attributed to the single or synergic action of these main components or even other minor constituents present in the oil. It noteworthy that (*E*)-caryophyllene and α-humulene were reported to reduce the edema formation induced by carrageenan, histamine, bradikinin, PGE_2 and platelet activating factor (Fernandes et al., 2007). Furthermore, α-eudesmol is also reported as responsible for the inhibition of neurogenic inflammation in models of the electrical stimulation of trigeminal ganglion (Asakura et al., 2000).

These data show for the first time that EOPs has a significant and peripheral antinociceptive effect that is unrelated to interaction with the opioid system. EOPs also display a significant anti-inflammatory effect in acute inflammation models. This effect seems be related to components which inhibit the production of several inflammatory mediators. These results support the widespread use of *Peperomia serpens* in popular medicine to treat inflammation and pain. Further experiments are needed to be carried out to identify the EOPs's constituents responsible for these effects.

Conflict of interest

The authors have declared that there is no conflict of interest.

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