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Anti-inflammatory and antinociceptive effects of the hydroethanolic extract of the flowers of *Pyrostegia venusta* in mice

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Abstract: *Pyrostegia venusta* (Ker Gawl.) Miers, Bignoniaceae, is native to the Brazilian Cerrado and popularly known as "cipó-de-são-joão". In Brazilian folk medicine, the flowers of *P. venusta* are used as a general tonic and a treatment for diarrhea, vitiligo, cough, and common infections and inflammatory diseases of the respiratory system. Nevertheless, there are still no studies on its possible anti-inflammatory and antinociceptive effects. The *P. venusta* hydroethanolic extract (PvHE) was used to evaluate the anti-inflammatory and analgesic effects in carrageenan-induced paw edema, peritonitis induced by lipopolysaccharide, acetic acid-induced writhing, and formalin-induced paw-licking tests in Swiss male mice. PvHE at doses of 30-300 mg/kg *p.o.* demonstrated anti-inflammatory effect. PvHE reduced paw edema induced by carrageenan and inhibited leukocyte recruitment into the peritoneal cavity. The extracts showed antinociceptive activity in acetic acid-induced writhing and formalin tests. Our results showed that the PvHE demonstrated anti-inflammatory and antinociceptive action in mice. All the anti-inflammatory actions obtained are also suggested to due the presence of acacetin-7-*O*-β-glucopyranoside.

Introduction

Many species belonging to the Bignoniaceae family, such as *Pyrostegia venusta* (Ker Gawl.) Miers are known to be of medicinal value (Emmanuel et al., 2010). *P. venusta* is popularly known in Brazil as "cipó-de-são-joão", "cipó-caititu", "cipó-tingá" and "dedo-de-moça" (Pool, 2008) and is widely distributed in the cerrado, which is noted as being a typical ecosystem of central and southeastern Brazil (Blatt et al., 1998). In folk medicine, the aerial parts of *P. venusta* are mainly used as an infusion or decoction. They are administered orally as a general tonic for the treatment of diarrhea, vitiligo, cough and common infections and inflammatory diseases of the respiratory system, such as bronchitis, flu and the common cold (Ferreira et al., 2000; Scalon et al., 2008; Cardozo et al., 2009). Previous studies demonstrated that the hydroethanolic extract of flowers of *P. venusta* attenuated the sickness behavior induced by lipopolysaccharide (LPS) in the forced swim and open field test, suggesting that the extract inhibits immune and inflammatory responses, including cytokine and prostaglandin production, supporting the popular use of *P. venusta* as a general tonic as well as a treatment for the general symptoms of flu and cold (Veloso et al., 2010).

The literature records the phytochemical study of the flowers of *P. venusta*, from which the compounds β-sitosterol, *n*-hentriacontane, acacetin-7-*O*-β-glucopyranoside and meso-inositol have been isolated (Dubey & Misra, 1976; Veloso et al., 2010). Other studies have indicated the presence of carotenoids in the flowers (Harborne, 1967) and rutin in the leaves (Blatt et al., 1998). It has been demonstrated that the compounds acacetin-7-*O*-β-glucopyranoside and β-sitosterol showed anti-inflammatory activity (Gupta et al., 1980; Shen et al., 2010).

Due to the large variety of compounds found in the flowers of this species and a need to effectively identify anti-inflammatory and analgesic therapies, the objective of this study was to evaluate the anti-inflammatory and analgesic effects of the hydroethanolic extract of *P. venusta* flowers in animal models.

Material and Methods

Plant material

Pyrostegia venusta (Ker Gawl.) Miers, Bignoniaceae, were collected in Alfenas, Minas Gerais, Brazil. The plant was identified by Dr. G. Alves-da-Silva from the Department of Pharmacy at the Federal University of Alfenas, and the voucher specimen (699) has been deposited at the Herbarium of the Federal University of Alfenas-MG.

Preparation of the plant extracts and reference drugs

The flowers of *P. venusta* were dried in an oven at 40 °C and powdered. The *P. venusta* hydroethanolic extract (PvHE) was obtained by maceration in a 50% hydro-alcoholic solution for 48 h at room temperature, and this procedure was repeated twice. The PvHE was concentrated on a rotary evaporator and then dried with a spray dryer (Büchi Mini Spray Dryer B-290). The yield of the PvHE was 6.0%.

The PvHE were administered in 30, 100, and 300 mg/kg doses after being suspended in a vehicle (1% sodium carboxymethylcellulose suspension in distilled water). Dexamethasone (1 mg/kg) and morphine sulphate (10 mg/kg) was diluted in sterile saline (0.9% NaCl). Indomethacin (10 mg/kg) was diluted in Tris buffer (pH 8.5). The animals in the control group received the same experimental handling as those in the test groups, with the exception that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Dexamethasone, indomethacin and morphine sulphate were used as reference drugs. All test drugs were intraperitoneally administered, except for indomethacin, which was orally administered.

Determination of the total phenolics and flavonoids in the PvHE

The total phenolic content in the PvHE was determined using the Folin-Ciocalteu methods, and the total flavonoid content was determined according to previous described (Singleton et al., 1999).

Pharmacological procedures

Animals

Adult male Swiss mice (22-28 g) were obtained from the Central Animal Facility of the Federal University of Alfenas and housed under controlled light (12:12 h light:dark cycle; lights on at 6:00 am) and temperature conditions (23±1 ºC) with access to water and food *ad libitum*. The animals were allowed to habituate to the housing facilities for at least one week before the experiments were started. All experiments were conducted according to Brazilian Regulations for animal experimentation (COBEA), after approval by the Ethical Commission of Animal Experimentation at the Federal University of Alfenas (#269/2009). The doses used in the present study were selected based on previous studies (de Paiva et al., 2010; Vilela et al., 2010).

Evaluation of anti-inflammatory activity in mice

Carrageenan-induced mice paw edema

Paw edema was measured with a plethysmometer (Model 7140, Ugo Basile, Italy). The basal volume of the right hind paw was determined before the administration of any drug. After determination of the basal volume, the animals (n=8 per group) were divided into the experimental groups in such a way that the mean volumes of the different groups were similar. The vehicle, PvHE or indomethacin was orally administered 1 h before *i.pl.* injection of carrageenan (1 mg/paw, 20 µL). The paw volume was measured at 1, 2, 3 and 4 h after injection of the inflammatory stimulus. The results are presented as the paw volume (mL) variation in relation to the basal values.

Peritonitis induced by lipopolysaccharide

To assess the possible effect of the PvHE on leukocyte recruitment into the peritoneal cavity, the animals (n=8 per group) were orally pre-treated with the vehicle and PvHE and intraperitoneally administered with dexamethasone. Thirty minutes later, lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (100 µg/kg *i.p.*) dissolved in pyrogen-free sterile saline was administered. Four hours after the injection of LPS, the mice were killed by an inhalatory overdose of halothane, and the cells from the peritoneal cavities were harvested by injecting 5.0 mL of PBS containing 0.5% of sodium citrate. The abdomens were gently massaged and the blood-free cell suspension was carefully aspirated with a syringe. Abdominal washings were placed into plastic tubes, and total cell counts were performed in a Neubauer chamber (Cunha et al., 1989; Vilela et al., 2010).

Evaluation of antinociceptive activity in mice

Acetic acid-induced writhing in mice

Acetic acid (0.6% v/v, 10 mL/kg) was injected into the peritoneal cavities of mice, which were placed in a large glass cylinder, and the intensity of the nociceptive behavior was quantified by counting the

total number of writhes occurring between 0 and 20 min after the stimulus injection, as described earlier by Koster et al., (1959). Oral treatments with the vehicle, indomethacin or the PvHE, were given 1 h prior to the acetic acid injection (n=6 per group). The writhing response consists of a contraction of the abdominal muscle together with a stretching of the hind limbs. The antinociceptive activity was expressed as writhing scores over a period of 20 min.

Formalin-induced nociception

A formalin solution (5% in 0.9% saline; 20 µL/paw) was injected into the hind paw plantar surface (*i.pl.*), and the animals were individually placed in transparent observation chambers, as previously described (Santos & Calixto, 1997). Oral treatments (*p.o.*) with the vehicle, indomethacin or the PvHE were given 1 h prior to formalin injection (n=8 per group). Morphine was administrated (*i.p.*) 30 min before the test. The time spent in licking the injected paw was recorded and expressed as the total licking time in the early phase (0-5 min) and late phase (20-30 min) after formalin injection.

Open-field test

To discard the possible nonspecific muscle relaxants or the sedative effects of the extract, the motor performance of the mice was evaluated on the open-field apparatus (Archer, 1973). Groups of mice (n=10) were treated with the vehicle or PvHE one hour before the test. Each animal was placed in the center of the open-field arena and allowed to have free ambulation for 5 min for the observation of the locomotion frequency (number of floor units the animal entered on all its limbs).

Evaluation of acute toxicity of the Pyrostegia venusta *extract*

The PvHE (0.5-5 g/kg) was orally administered to a group of mice, both male and female. The behavior parameters observed after administration were convulsion, hyperactivity, sedation, grooming, and increased or decreased respiration during a period of seven days. Food and water were provided *ad libitum*.

Statistical analysis

The data obtained were analyzed using the GraphPad software program v.4.0 and expressed as mean±SEM Statistically significant differences between the groups were calculated by the application of an analysis of variance (ANOVA), followed by the Newman-Keuls test ($p<0.05$ were considered significant).

Results

Total phenols and flavonoids contents

The analysis showed that the level of polyphenolics compounds in the PvHE was 998 mg/g extract. The content of total flavonoids was 543 mg quercetin equivalent/g extract. Our previous reports showed that a principal constituent was identified as the flavonoid acacetin-7-*O*-glycopyranoside by HPLC/DAD analysis (Veloso et al., 2010).

Carrageenan-induced mice paw edema

Figure 1 shows that the PvHE significantly inhibited ($F_{4,35}=6.12$; $p=0.0009$) the carrageenan-induced mice paw edema at doses of 30-300 mg/kg at 3 h post carrageenan, with inhibitions of 56.5%, 74.78% and 89.13%, for 30, 100 and 300 mg/kg, respectively. Indomethacin (10 mg/kg) gave a percentage inhibition of 73.48%.

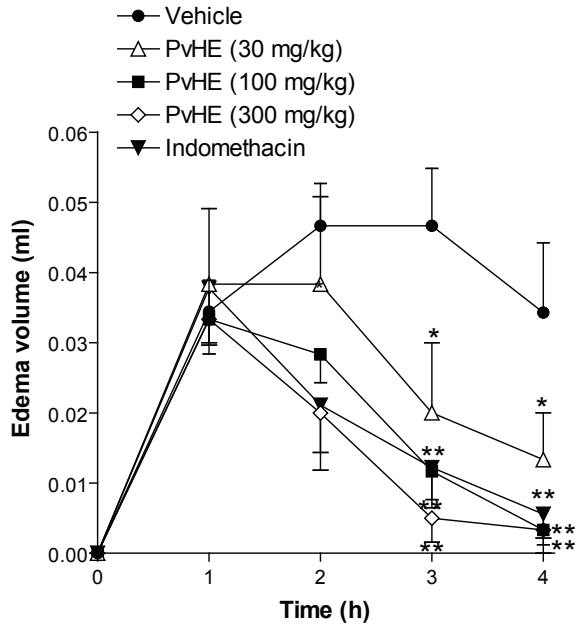

Figure 1. Effects of the administration of the hydroethanolic extract of *Pyrostegia venusta* flowers (PvHE; 30, 100 and 300 mg/kg, *p.o.*) or indomethacin (10 mg/kg, *p.o.*) on mice paw edema induced by intraplantar (*i.pl.*) carrageenan injection (1 mg/paw). Each point represents the mean±SEM of eight animals. The asterisks denote the significance levels when compared with the control group: *$p<0.05$, **$p<0.01$.

Peritonitis induced by lipopolysaccharide

In agreement with previous studies, LPS-induced peritonitis was followed by a significant increase in the number of leukocytes in the peritoneal cavity of mice when compared to the control group treated only with the vehicle (Vilela et al., 2010). The PvHE (30-300 mg/kg) significantly inhibited ($F_{5,35}$=8.29; p<0.001; Figure 2) leukocyte recruitment induced by LPS (100 µg/kg) into the peritoneal cavity in mice at doses of 30-300 mg/kg. The inhibition of leukocyte recruitment at 4 h post-LPS were 51.7%, 87.3% and 95.2% for 30, 100 and 300 mg/kg, respectively. Dexamethasone (1 mg/kg) showed an inhibition (p<0.001) of 98.9%.

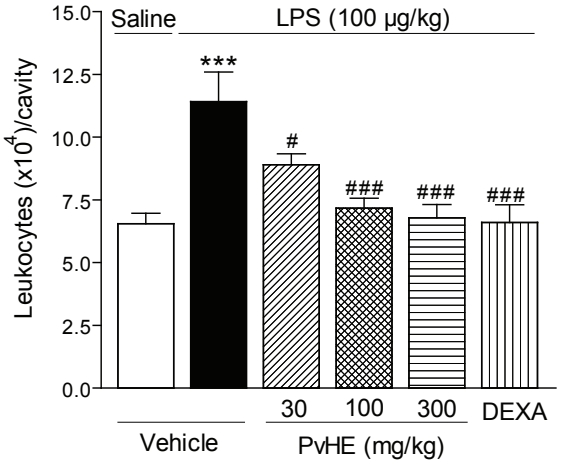

Figure 2. Effects of the administration of the hydroethanolic extract of *Pyrostegia venusta* flowers (PvHE; 30, 100 and 300 mg/kg, *p.o.*) or dexamethasone (DEXA, 1 mg/kg, *i.p.*) on the lipopolysaccharide-induced recruitment of leukocytes to the peritoneal cavity of mice. Each column represents the mean±SEM of six animals. ***p<0.001 compared with the saline+vehicle group. #p< 0.05; ###p<0.01 compared with LPS+vehicle group.

Acetic acid-induced writhing in mice

Figure 3 shows the results of the acetic acid writhing test. The PvHE at doses of 100 and 300 mg/kg significantly reduced ($F_{4,42}$=7.67; p<0.001) the number of writhes by 43.20% and 69.10%, respectively. Indomethacin at a dose of 10 mg/kg exhibited a significant percentage reduction (p<0.001) in acetic acid-induced writhing of 65.32%.

Formalin test in mice

The PvHE at doses of 30-300 mg/kg *p.o.* produced a significant antinociceptive activity compared to the control, reducing formalin-induced nociceptive responses during the first ($F_{5,57}$=6.14; p<0.001; Figure 4A) and second ($F_{5,57}$=7.90; p<0.001; Figure 4B) phases. The reference drug indomethacin suppressed only the second phase of the formalin test, whereas morphine inhibited both phases of the pain stimulus.

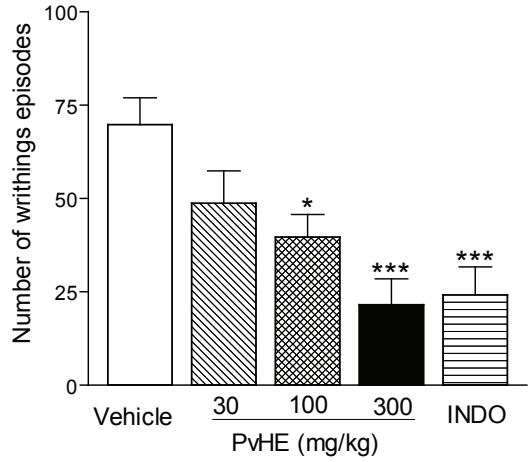

Figure 3. Effects of the hydroethanolic extract from *Pyrostegia venusta* flowers (PvHE) administered orally against acetic acid-induced writhing movements in mice. Animals were pretreated orally with the vehicle, PvHE (30, 100, and 300 mg/kg), and indomethacin (INDO, 10 mg/kg) prior to the acetic acid (0.6%, *i.p.*). Each column represents the mean with SEM for eight mice. The asterisks denote the significance levels when compared with the control group: *p<0.05; ***p<0.001.

Open-field test

Mice treated with the PvHE at 30-300 mg/kg did not display a reduction in the numbers of crossings and rearings when compared to the control group in the open-field test (data not shown).

Acute toxicity

The PvHE given to mice at a dose of 0.5-5 g/kg *p.o.* had no affect on their behavioral responses during the observation period of seven days after administration. No mortality was observed up to seven days of monitoring. The estimated LD50 value of these extracts in mice was therefore more than 5 g/kg *p.o.* Because the effective dose used in the present study (100 mg/kg *p.o.*) was 5-fold less than the dose used in the acute toxicity test, we assume that the doses of 30, 100, and 300 mg/kg *p.o.* given to mice in this study were safe.

Discussion

The flowers of *Pyrostegia venusta* (Ker Gawl.) Miers, Bignoniaceae, are commonly used in

traditional Brazilian medicine for the treatment of various inflammatory diseases. Our previous study demonstrated that the PvHE attenuated the sickness behavior in mice, supporting the popular use of flowers of *Pyrostegia venusta* as treatment for the general symptoms of flu and cold (Veloso et al., 2010). However, its pharmacological actions have not been completely investigated to date. The present study demonstrated that the hydroethanolic extract of the flowers of *Pyrostegia venusta* display antinociceptive and anti-inflammatory properties. Because the extract did not produce any mortality in mice even at a dose of 5 g/kg, it may be considered relatively safe.

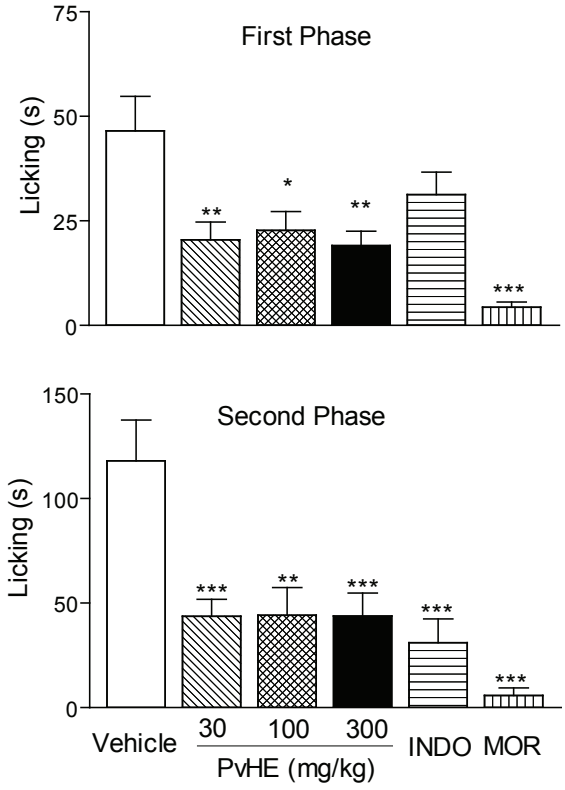

Figure 4. Effects of the hydroethanolic extract from *Pyrostegia venusta* flowers (PvHE) given by the oral route on the formalin test in mice. Animals were pretreated orally with the vehicle, PvHE (30, 100, and 300 mg/kg), indomethacin (INDO; 10 mg/kg) or morphine (MOR; 1 mg/kg) prior to formalin. The total time spent licking the hindpaw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with S.E.M. for eight mice. The asterisks denote the significance levels when compared with the control group: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

In this study, the anti-inflammatory activity of the hydroethanolic extract from the flowers of *Pyrostegia venusta* were evaluated using the carrageenan-induced mice paw edema and peritonitis induced by LPS. In mice, the inflammatory response induced by carrageenan is characterized by a biphasic response (Vinegar et al., 1969). Marked edema formation resulting from a rapid production of several inflammatory mediators, such as histamine, serotonin and bradykinin, is observed in the first-phase. The second-phase is characterized by the release of prostaglandins and nitric oxide with a peak at 3 h, produced by inducible isoforms of COX (COX-2) and nitric oxide synthase (iNOS), respectively (Seibert et al., 1994). Oral administration of the PvHE suppressed the edematous response in a dose-dependent manner 3 h after the carrageenan injection. The inhibitory effect of the PvHE on the carrageenan-induced inflammation in mice may be due to the inhibition of cyclooxygenase (Morris, 2003) because its effect can be compared to that caused by indomethacin. In another model of acute inflammation (peritonitis induced by lipopolysaccharide), the PvHE significantly reduced the leukocyte migration to the peritoneal cavity induced by lipopolysaccharides.

This work shows that the PvHE p.o. produces significant antinociception according to the assessment of the abdominal writhes elicited by acetic acid and the formalin test in mice. The PvHE was shown to possess antinociceptive activity to the abdominal writhes elicited by acetic acid. Pretreatment with the PvHE (100 and 300 mg/kg) reduced the number of acetic acid-induced writhes in mice. This model, which is a visceral pain model, releases arachidonic acid via cyclo-oxygenase (COX); prostaglandins biosynthesis plays a notably important role in the nociceptive mechanism (Duarte et al., 1988). The results of the present study indicate that the analgesic effect of the PvHE may possibly be triggered by the inhibition of the synthesis or action of prostaglandin. The PvHE was found to be effective in both phases of formalin response. The early phase, named the non-inflammatory pain, is a result of the direct stimulation of nociceptors and reflects centrally-mediated pain; the late phase, named the inflammatory pain, is caused by local inflammation with a release of inflammatory and hyperalgesic mediators (Hunskaar & Hole, 1987). In this study, administration of the PvHE induced antinociceptive activities in both the early and late phases of the formalin test. The first phase is sensitive to drugs that interact with opioid system and the second phase is inhibited by nonsteroidal anti-inflammatory drugs. Drugs that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the second phase (Rosland et al., 1990). In the present study, PvHE produced antinociception in both the early and late phases of formalin test. Considering the inhibitory property of PvHE on the first phase of formalin, is

presumed that its possible antinociceptive activity is due a possible central action since its effect could be compared to that caused by morphine. Moreover, the antinociceptive effect of the PvHE in the second phase is due to its possible anti-inflammatory action, inhibiting the release of the inflammatory mediators. The possible nonspecific action of the muscle relaxants or the sedative effects was discarded, as tested on the open-field apparatus. This result corroborates the anti-inflammatory and antinociceptive effect of the PvHE suggested by the nociceptive tests.

In previous studies, β-sitosterol, *n*-hentriacontane, acacetin-7-*O*-β-glucopyranoside and meso-inositol were identified (Dubey & Misra, 1976). The anti-inflammatory activities of β-sitosterol and acacetin were demonstrated (Gupta et al., 1980; Shen et al., 2010). The anti-inflammatory activities of β-sitosterol isolated from the *Cyperus rotundus*, have shown carrageenan-induced edema in rats. β-sitosterol is found to possess potent anti-inflammatory activities, similar to that of hydrocortisone, when administered intraperitoneally (Gupta et al., 1980). Others studies have demonstrated that acacetin inhibits the induction of nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2) in macrophages that are activated with LPS by inhibiting the transcriptional activation (Pan et al., 2006; Shen et al., 2010). LPS produces pro-inflammatory cytokines which provokes a number of neuropsychological symptoms collectively referred to sickness behavior (Dantzer 2009; de Paiva et al., 2010). In addition, Veloso et al. (2010) observed that the hydroethanolic extract from flowers of *Pyrostegia venusta* attenuated the depressive-like and exploratory behaviors induced by LPS, suggesting that the PvHE inhibited the imune and inflammatory responses.

The precise mechanisms that are involved in the production of the anti-nociceptive and anti-inflammatory responses of the *Pyrostegia venusta* extract are not completely understood, but they may be caused by the presence of flavonoid acacetin-7-*O*-glycopyranoside identified by Veloso et al. (2010). The presence of flavonoids and phenolic compounds has been associated with various degrees of anti-inflammatory and analgesic activities (Garcia-Leme, 2008).

Finally, the obtained results point out the potential of *Pyrostegia venusta* extract for the pharmacological control of pain and inflammatory processes. This study has shown that the hydroethanolic extract of the flowers of *Pyrostegia venusta* possess significant antinociceptive and anti-inflammatory effects in laboratory animals. Moreover, the results support the traditional use of this plant in some inflammatory conditions.

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