Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
21(6): 1077-1083, Nov./Dec. 2011

Antinociceptive and anti-inflammatory effects of *Caesalpinia pyramidalis* in rodents

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Abstract: *Caesalpinia pyramidalis* Tul., Fabaceae, is a plant with an anti-inflammatory activity that is used in folk medicine. To evaluate the mechanism of action of this plant, studies were performed on its antinociceptive and anti-inflammatory properties using an ethanol extract (EE) made from the inner bark. Oral treatment of mice with the EE (100, 200, and 400 mg/kg) decreased their acetic acid-induced abdominal writhes (p<0.001) and their formalin-induced paw licking in both the first and second phases (p<0.001). This treatment increased the reaction time of mice on the hot-plate test (400 mg/kg, p<0.05); however, it did not alter their performance on the Rotarod performance test. The carrageenan-induced paw edema in the rats and the leukocyte migration into the peritoneal cavity of the mice were also reduced by the EE given at a dose of 400 mg/kg (p<0.05). In addition, the EE (100-400 mg/kg, *v.o.*) did not alter the arterial pressure of non-anesthetized rats. In conclusion, the EE of *C. pyramidalis* shows antinociceptive and anti-inflammatory activities in rodents, supporting the usage of this plant to treat various inflammatory diseases for which it has traditionally been used.

Article

Received 4 Aug 2010
Accepted 16 Feb 2011
Available online 30 Sep 2011

Keywords:
Anti-inflammatory
antinociceptive
Caesalpinia pyramidalis
Fabaceae

ISSN 0102-695X
http://dx.doi.org/10.1590/S0102-695X2011005000179

Introduction

Inflammation is one of the most important processes involved in the defense of an organism, however, it often progresses to painful and sometimes chronic diseases needing pharmacological treatment. Unfortunately, the therapies currently available to treat inflammation and pain are associated with unwanted side effects and low efficacy. There has been a resurgence of interest in herbal medicines in Western countries (Phillipson & Anderson, 1989) as alternative sources of drugs for often intractable diseases.

Previous studies of species of the genus *Caesalpinia,* Fabaceae, report remarkable biological activities, such as antimicrobial (Saeed & Sabir, 2001), antidiabetic (Sharma et al., 1997) (*C. bonducella*), antimalarial (Deharo et al., 2001; Kuria et al., 2001) (*C. volkensii, C. pluviosa*), and anti-inflammatory (Hikino et al., 1977; Carvalho et al., 1996) (*C. sappan, C. ferrea*) activities.

Caesalpinia pyramidalis Tul. is an endemic tree of northeastern region of Brazil and is one of the predominant species in the "caatinga" vegetation. The plant *C. pyramidalis*, known as "catingueira," is a plant species used in folk medicine to treat cough, bronchitis, respiratory infection, influenza, asthma, gastritis, colic, fever, heartburn, flatulence, diarrhea, collision, injury, diabetes, and stomach ache and is used as an aphrodisiac and expectorant (Albuquerque et al., 2007).

In this study, we evaluate the antinociceptive and anti-inflammatory effects of the ethanol extract (EE) made from *C. pyramidalis* inner bark.

Materials and Methods

Plant material and extraction of Caesalpinia pyramidalis *inner bark*

The inner bark of *Caesalpinia pyramidalis* Tul., Fabaceae, was collected in the municipality of Canindé de São Francisco-SE, Brazil, in September 2008 (09º66'00"S, 37º78'94"W). The plant was authenticated by Professor Ana Paula Prata, Department of Biology, Federal University of Sergipe, and a voucher specimen was deposited in the Federal University of Sergipe

Herbarium (number ASE 13,164). The inner bark was dried at 40 ºC in a forced air oven for two days and subsequently powdered (2.840 g) and extracted by maceration at room temperature with 90% ethanol for five days. The extract was filtered in vacuum, and the solvent was removed using a rotary evaporator (45 °C). The percentage of yield of the EE was 2.6% (73.8 g).

Phytochemical screening

The methods of Matos (1997) were used to screen the EE of *C. pyramidalis* inner bark used in this study for its chemical constituents.

Animals

Young adults Wistar rats (120-180 g) and Swiss mice (20-30 g) of both sexes were obtained from the Central Biotery of the Federal University of Sergipe (São Cristóvão, Brazil). Animals were maintained at controlled room temperature (21±2 °C) with free access to food (Purina®) and water, under a 12 h light/dark cycle. All the experimental procedures were carried out during the light period of the day (8:00 a.m. to 5:00 p.m.) and complied with the guidelines on animal care of the Federal University of Sergipe Ethics Committee for Animal Use in Research (CEPA/UFS 05/09).

Acetic acid-induced abdominal writhes

Abdominal writhes were induced by intraperitoneal (*i.p.*) injection of acetic acid (0.6%, 0.1 mL/10 g) in mice (Koster et al., 1959). Animals were pre-treated orally (*p.o.*) with *C. pyramidalis* EE (100-400 mg/kg), vehicle (0.2% Tween 80, 0.1 mL/10 g), or acetylsalicylic acid (ASA, 300 mg/kg) 60 min before initiating the algesic stimulation (n=6/group). The abdominal writhes were observed for a period of 20 min and began 5 min after the injection of the nociceptive agent.

Formalin test

The formalin test was conducted according to the method of Hunskaar & Hole (1987). Mice were pre-treated with the *C. pyramidalis* EE (100-400 mg/kg, *p.o.* 60 min before the start of the experiment), vehicle (0.2% Tween 80, *p.o.*, 60 min before the start of the experiment), morphine (10 mg/kg, *i.p.*, 30 min before the start of the experiment), or ASA (300 mg/kg, *p.o.*, 60 min before the start of the experiment). An intraplantar injection of 2% formalin solution (20 µL) was given to the right hindpaw of the animal (n=6/group). The time that the animal spent licking or biting its paw was measured during the first-phase (0-5 min) and the second-phase (15-30 min) of the test.

Hot-plate test

Mice were pre-treated with *C. pyramidalis* EE (100-400 mg/kg, *p.o.*), vehicle (0.2% Tween 80, *p.o.*), or morphine (3 mg/kg, *i.p.*), and after 30 min, they were placed on a metallic plate warmed to 55.0±0.5 °C (n=6/group). In another set of experiments, naloxone (5 mg/kg, *i.p.*) was injected 30 min prior of the EE (400 mg/kg) or morphine (3 mg/kg) treatment (n=6/group). The time that elapsed between the start of the experiment and the appearance of reactions (latency, in seconds) to the thermal stimulus, such as lifting or licking the paws, was recorded as an index of nociception (Woolfe & Macdonald, 1944). Measurements were performed 0, 30, 60, 90, and 120 min after the first thermal stimulus. To avoid damage to the animals, the maximal time standing on the plate was limited to 30 s.

Motor function assay: rotarod

To evaluate the possible non-specific muscle-relaxant or sedative effects of EE, mice were submitted to the Rotarod Performance Assay (Duham & Miya, 1957). The rotarod apparatus (AVS, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into five compartments. Animals were treated with EE (100-400 mg/kg, *v.o.*), vehicle (0.2% Tween 80, *p.o.*), or diazepam (1.5 mg/kg, *i.p.*), and after 60 min, the animals were placed on the rotating rod (7 rpm, n=6/group). The latency to falling was measured for up to 180 s. The results are expressed as the average time (s) that the animals remained on the rotarod.

Measurement of edema and myeloperoxidase (MPO) activity in rat paws

The anti-inflammatory activity of the EE was studied using the paw edema induced by carrageenan (1%, 0.1 mL), which was administered into the subplantar region of the right hindpaw of the rat (Winter et al., 1962). *C. pyramidalis* EE (100-400 mg/kg, *p.o.*), dexamethasone (2 mg/kg, *s.c.*), or vehicle (0.2% Tween 80, *p.o.*) were administrated 1 h before the edematogenic agent was injected (n=6/group). The paw edema was measured plethysmographically (model 7150, Ugo Basile, Varese, Italy) at 1, 2, 3, and 4 h after the carrageenan was administered. The data obtained were expressed in mL. The percentage inhibition was calculated based on the area under the time-course curves (AUC_{0-4h}).

Myeloperoxidase activity was measured in paw tissue samples obtained from animals after the end (4 h) of edema measurement. These samples were homogenized in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl-trimethylammonium bromide. These

homogenates were incubated for 2 h at 60 °C to inactivate endogenous catalases. The supernatants were mixed to a solution of o-dianisidine dihydrochloride (0.167 mg/mL, in 50 mM phosphate buffer) containing 0.005% of H_2O_2. The changes of absorbance at 460 nm were measured with a microplate reader (Labsystem Multiskan, Helsinki, Finland). The results were expressed as units of MPO (UMPO)/mg tissue, where one UMPO is defined as the amount of enzyme that degrades 1 µmol of H_2O_2/min (Bradley et al., 1982).

Leukocyte migration into the peritoneal cavity of mice

The leukocyte migration was induced by injection of carrageenan (1%, 250 µL, *i.p.*) into the peritoneal cavity of mice (n=6/group) 1 h after the administration of the EE (100-400 mg/kg, *p.o.*), vehicle (0.2% Tween 80, *p.o.*), or dexamethasone (2 mg/kg, *s.c.*) as previously described by Mendes et al. (2010). The mice were euthanized 4 h after the carrageenan injection, and 3.0 mL of saline containing EDTA (1.0 mM) was injected into the peritoneal cavity. The peritoneal lavages were collected and centrifuged at 1000 x *g*, and the cell pellets were resuspended in 1 mL of saline. The total number of cells was counted in a Neubauer chamber, and cytospin preparations were stained with May-Grunwald-Giemsa for the differential leukocyte counts. The results were expressed as the number of leukocytes/mL.

Measurement of blood pressure in non-anesthetized rats

To measure the mean arterial pressure (MAP), the rats were anesthetized with sodium thiopental (50 mg/kg, *i.p.*). A polyethylene catheter was inserted into the abdominal aorta via the left femoral artery for pressure recordings. The catheter was filled with heparinized saline and placed under skin, exiting between the scapulae. Twenty-four hours after the surgery, the rats were placed in large individual cages, and the experiments were performed in non-anesthetized rats. The arterial catheter was connected to a pre-calibrated pressure transducer (Edwards Lifescience, Irvine, CA, USA), and pressure outputs were recorded in an amplifier-recorder (BioData, Model BD-01, PB, Brazil) connected to a personal computer equipped with an analog-to-digital converter board (DI148U, DATAQ Instruments, OH, EUA) (Menezes et al., 2007). After the hemodynamics parameters had stabilized, the MAP was recorded before (baseline values) and 1, 2, 3, and 4 h after oral administration of *C. pyramidalis* EE (100, 200, and 400 mg/kg, *p.o.*).

Statistical analysis

The results are presented as the means±SEM of n animals per group. Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. *p* values lower than 0.05 were considered significant.

Results

Phytochemical screening

Phytochemical screening showed that the EE of *C. pyramidalis* inner bark contains flavonoids, phenols, saponins, steroids, tannins, and triterpenes.

Acetic acid-induced writhing in mice

The writhes evoked by injection of acetic acid in the abdominal cavity were markedly reduced by the pre-treatment with *C. pyramidalis* EE at 100, 200, and 400 mg/kg ($p<0.001$, Table 1). ASA (300 mg/kg) also significant inhibited ($p<0.001$) the writhes induced by acetic acid (Table 1).

Table 1. The antinociceptive effect of *Caesalpinia pyramidalis* EE on acetic acid-induced writhing.

Treatment	Dose (mg/kg)	Number of writhes	Inhibition (%)
Vehicle	--	31.2±0.9	--
EE	100	24.7±0.7[a]	20.9
	200	18.2±0.3[a]	41.7
	400	9.5±0.7[a]	69.5
ASA	300	7.5±0.5[a]	75.9

[a]$p<0.001$ *vs*. vehicle (n=6/group).

Formalin reaction time in mice

The intraplantar injection of the formalin solution produced nociceptive behavior in both the first and second phases (105±3 and 122±6 s, respectively, Figure 1). *C. pyramidalis* EE produced marked inhibition of formalin-induced neurogenic (45.5, 31.9, and 42.0% at 100, 200, and 400 mg/kg, respectively, $p<0.001$) and inflammatory (92.8, 81.0, and 93.0% at 100, 200, and 400 mg/kg, respectively, $p<0.001$) phases (Figure 1). Similarly, morphine (10 mg/kg) caused significant inhibition of 75.9% and 96.3% of formalin-induced nociceptive behavior in the first and second phases, respectively ($p<0.001$, Figure 1). ASA (300 mg/kg) caused inhibition of 90.6% in the second phase of formalin-induced nociception ($p<0.001$, Figure 1).

Hot-plate reaction time in mice

The EE of *C. pyramidalis* (400 mg/kg) caused a significant increase in the pain latency in the hot-plate

test (55 °C) at all time points analyzed (p<0.05, Table 2). Similarly, morphine (3 mg/kg) caused a significant and marked increase in the reaction time of mice (p<0.05, Table 2). Naloxone (5 mg/kg) significantly prevented the antinociceptive effect caused by both *C. pyramidalis* EE (400 mg/kg) and morphine (3 mg/kg) at all time points observed (p<0.05, Table 2).

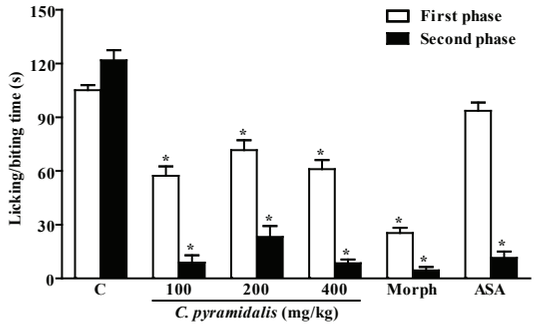

Figure 1. The effect of *Caesalpinia pyramidalis* EE on formalin-induced nociception. Mice were pre-treated with vehicle (C), morphine (Morph, 10 mg/kg), acetylsalicylic acid (ASA, 300 mg/kg), or EE (100-400 mg/kg) before a formalin injection. *p<0.001 *vs*. the respective control group (n=6/group).

Motor performance

In the rotarod test, EE-treated mice did not show any significant motor performance alterations with the 100, 200, and 400 mg/kg dose (180±0, 180±0, and 180±0 s, respectively) compared to control mice (180±0 s). As expected, the injection of the diazepam (1.5 mg/kg) reduced the time the mice were on the rotarod after the treatment (31±3 s, p<0.001).

Carrageenan-induced edema and MPO activity in rat paws

As observed in Figure 2, the single oral treatment of *C. pyramidalis* EE at the 400 mg/kg dose was capable of reducing (p<0.05) the edema induced by carrageenan at 2, 3 and 4 h after the injection of the phlogistic agent. Likewise, dexamethasone (2 mg/kg) inhibited (p<0.01) the edematogenic response evoked by carrageenan in rats at 2, 3 and 4 h (Figure 2).

Based on $AUC_{0\text{-}4h}$ values, the EE at the 400 mg/kg dose caused a 41.2% (p<0.05) inhibition of the edema response compared to carrageenan-treated group (4.6±0.7 mL x hour). Dexamethasone (2 mg/kg) caused an inhibition of 54.4% (p<0.001).

C. pyramidalis EE (400 mg/kg) also produced a marked inhibition (p<0.05) of carrageenan-induced MPO activity in the paws of rats compared to vehicle-treated controls (4.5±0.5 and 7.1±0.9 UMPO/mg tissue, respectively). Similarly, dexamethasone (2 mg/kg) caused significant inhibition of carrageenan-induced MPO activity (2.6±0.3 UMPO/mg tissue, p<0.001).

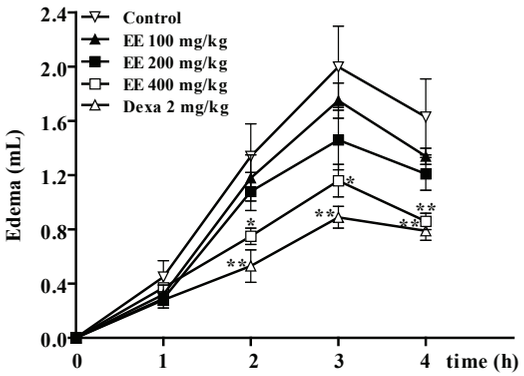

Figure 2. The effect of *Caesalpinia pyramidal*s EE on rat paw edema. Animals were pre-treated with vehicle (control), dexamethasone (Dexa, 2 mg/kg), or EE (100-400 mg/kg) before a carrageenan injection. *p<0.05 and **p<0.01 *vs*. the control group (n=6/group).

Carrageenan-induced peritonitis in mice

The carrageenan injection in control animals induced leukocyte migration into the peritoneal cavity after 4 h (7.22±0.99 x 10^6 leukocytes/mL). *C. pyramidalis* EE (400 mg/kg) significantly inhibited this response (2.63±0.23 x 10^6 leukocytes/mL, p<0.01), but 200 or 100 mg/kg EE did not (4.87±0.69 x 10^6 and 7.09±1.03 x 10^6 leukocytes/mL, respectively). The dexamethasone injection also (2 mg/kg) inhibited (0.68±0.40 x 10^6 leukocytes/mL, p<0.001) the carrageenan-induced leukocyte migration.

The PMN migration evoked by carrageenan was reduced by 200 and 400 mg/kg EE by 42.6% and 80.2% (p<0.001), respectively (Figure 3). Dexamethasone (2 mg/kg) exhibited significant inhibition (95.7%, p<0.001) of the PMN migration in the control group (Figure 3).

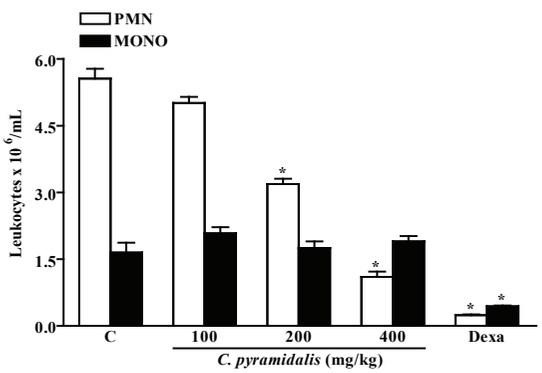

Figure 3. The effect of *Caesalpinia pyramidalis* EE on leukocyte migration. Mice were pre-treated with vehicle (C), dexamethasone (Dexa, 2 mg/kg), or EE (100-400 mg/kg) before carrageenan-induced peritonitis. *p<0.001 *vs*. the respective control group (n=6/group).

Table 2. The antinociceptive effect of *Caesalpinia pyramidalis* EE using the hot-plate test.

		Reaction time after first stimulus (s)				
Treatment	Dose (mg/kg)	0 min	30 min	60 min	90 min	120 min
Vehicle	--	8.2±0.9	10.2±0.5	8.5±0.6	9.8±0.5	8.7±0.8
EE	100	12.3±0.8	13.8±2.2	14.7±1.7	11.7±0.6	10.3±1.1
	200	13.2±1.4	14.0±2.0	13.8±1.4	11.5±1.0	11.2±0.7
	400	12.5±1.0	17.5±2.0[a]	18.0±2.4[a]	18.2±1.8[a]	14.8±0.4[a]
Morphine	3	30.0±0.0[a]	30.0±0.0[a]	29.0±0.7[a]	28.7±0.6[a]	30.0±0.0[a]
Naloxone+morphine	5+3	11.4±4.4[b]	11.6±5.8[b]	10.5±4.9[b]	9.7±6.2[b]	8.5±2.9[b]
Naloxone+EE	5+400	6.7±0.5[c]	10.7±0.8[c]	8.7±1.4[c]	11.3±1.6[c]	9.0±1.5[c]

$p<0.05$ vs. [a]vehicle, [b]morphine, and [c]EE (400 mg/kg) (n=6/group).

MAP in non-anesthetized rats

In non-anesthetized normotensive rats, the oral administration of *C. pyramidalis* EE (100, 200, and 400 mg/kg) did not show any significant MAP alterations at any of the time points analyzed (data not shown).

Discussion

This study evaluated the effects of *Caesalpinia pyramidalis* Tul., Fabaceae, EE using several acute models of nociception and inflammation in rodents. Our data revealed that *C. pyramidalis* EE significantly diminished the nociceptive and inflammatory responses induced by various agents.

In the abdominal constriction assay, acetic acid acts indirectly causing the release of nociceptive endogenous mediators, such as bradykinin, serotonin (5-HT), histamine, sympathomimetic amines, prostaglandins (PG), and pro-inflammatory cytokines (Ribeiro et al., 2000; Ikeda et al., 2001). Acetic acid can also directly activate non-selective cation channels located at primary afferent pathways (Julius & Basbaum, 2001). This nociceptive effect can be prevented by non-steroidal anti-inflammatory drugs, opioids, analgesics with central actions, sympatholytic agents, and substances that reduce intestinal motility (Duarte et al., 1988; Reichert et al., 2001). Our results demonstrated that the EE of *C. pyramidalis* was able to significantly diminish the abdominal writhing induced by acetic acid, likely by interfering with the generation or mechanisms of action of the inflammatory mediators.

In addition, the formalin-induced paw licking assay was employed to evaluate the antinociceptive effect of the EE of *C. pyramidalis*. This test allowed the evaluation of two distinct phases: the first (also called neurogenic) and second (also called inflammatory) phases. The former occurs during the first 5 min after the formalin injection and is characterized by the direct stimulation of nociceptors presents on afferent C and in part by Aδ fibers (glutamate and substance P release). The latter occurs between the 15th and 30th min after formalin injection and is putatively caused by the release of pro-inflammatory mediators such as adenosine, bradykinin, histamine, PG, and 5-HT (Reeve & Dickenson, 1995). The treatment with the EE was capable of diminishing the nociceptive response in both the neurogenic and inflammatory phases. This result suggests that the EE of *C. pyramidali*s might possess anti-inflammatory activity. However, the inhibition presented in the first phase suggests a disruption of either the production or the release of some central neurotransmitters.

Another interesting result of the current study was the fact that EE of *C. pyramidalis* produced a significant antinociceptive effect after the mice were exposed to a thermal stimulus. The hot-plate test, which is performed at a constant temperature, produces two kinds of behavioral responses: paw licking and jumping. Both of these are considered to be supraspinally integrated responses (Chapman et al., 1985). The present results lead us to the conclusion that the opioid system is involved because the pre-treatment with naloxone, a non-selective opioid receptor antagonist, reversed the antinociceptive effect caused by EE of *C. pyramidalis*. Although the hot-plate test is commonly used to assess the effect of narcotic analgesics, some sedatives, muscle relaxants, and psychotomimetics have also shown activity in this test (Eddy & Leimbach, 1953). This could indicate that this EE may have non-specific central actions, but this hypothesis was proven unlikely by the observation that EE did not influence the performance of mice in the rotarod test, indicating that EE actions may not be due to a motor impairment.

To complement the results obtained in the second phase of the formalin-induced licking response, the EE of *C. pyramidalis* was tested on models of inflammation (paw edema and peritonitis) induced by carrageenan.

In this model, carrageenan-induced rat paw edema occurs as a non-immune reaction and is used to evaluate the effects of anti-inflammatory drugs. The edema formed is a multi-mediated phenomenon divided in two phases. The first phase (which last up to 2h after carrageenan

injection) is due to liberation of histamine, 5-HT, and bradykinin in paw tissue, while the second phase (3 and 4 h after carrageenan treatment) is sustained by liberation of PG (Di Rosa, 1972). In this study, EE of *C. pyramidalis* effectively reduced the edematogenic responses evoked by carrageenan between 2 and 4 h after the injection. These effects may be related to a reduction in the release or actions of histamine, 5-HT, bradykinin, or PG on local tissue.

The EE of *C. pyramidalis* also significantly decreased the elevated paw MPO activity, which is currently used as an indicator of neutrophil presence in inflamed tissues, suggesting that the inhibition of neutrophil infiltration may be another characteristic of the anti-inflammatory actions of this EE. This was further investigated using carrageenan as a stimulus to produce an acute inflammatory response in the peritoneal cavity of mice, which is a widely accepted model for induction of a massive influx of leukocytes (mainly neutrophils) to this cavity. The EE of *C. pyramidalis* inhibited both the total migration and the PMN migration induced by carrageenan at the same dose that inhibited the paw edema formation. The mechanism of action of carrageenan on peritonitis involves synergistic action between PG, leukotriene B_4, and other chemotactic agents, which promote an increase of the vasodilatation, exudation, and recruitment of leukocytes (Foster et al., 1986). As these anti-edematogenic and anti-chemotatic actions of EE could be strongly influenced by the possible vasoconstrictor effects of this extract, we conducted experiments to evaluate this possibility by measuring the mean arterial pressure of non-anesthetized rats after the administration of EE. We found that the doses of EE of *C. pyramidalis* used did not alter the mean arterial pressure, indicating that the anti-inflammatory activity of EE is not linked to alterations in the blood supply to the local of injury and might instead be related to the interference of the generation or mechanisms of action of inflammatory mediators. Furthermore, this hypothesis is supported by the antinociceptive effects of this extract, such as those observed in the second phase of the formalin-induced nociceptive test and the acetic acid-induced writhing test.

In summary, the data reported in this work confirmed the anti-inflammatory indications of *C. pyramidalis,* which have been observed in traditional medicinal practices. Additionally, this study suggests, for the first time, that *C. pyramidalis* has relevant antinociceptive properties in acute pain-like behavioral animal models. The mechanisms by which the EE of *C. pyramidalis* exerts its actions require further studies.

Acknowledgements

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico. CA Santos and AMPR Passos received grants from CNPq.

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