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Anti-inflammatory and analgesic potential of *Caesalpinia ferrea*

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Abstract: *Caesalpinia ferrea* Mart. belongs to the family Fabaceae. Known as pau-ferro and jucá, it is used in folk medicine to treat diabetes, as antipyretic and antirheumatic. This study aimed to evaluate the anti-inflammatory and antinociceptive activities of the ethanol extract of the fruits of *C. ferrea* (EECf). In the evaluation of anti-inflammatory activity, EECf (50 mg/kg) produced significantly inhibition of ear edema by 66.6% compared to control. Indomethacin (10 mg/kg) showed inhibition of 83.9% compared to control. EECf (50 mg/kg) inhibited of vascular permeability induced by acetic acid and was also able to reduce of cell migration to the peritoneal cavity induced by thioglycolate. In the writhing test induced by acid acetic, EECf (12.5, 25 and 50 mg/kg) significantly reduced the number of contortions by 24.9, 46.9 and 74.2%, respectively. In the formalin test, EECf presented effects only in the second phase. The results provided experimental evidence for the effectiveness of the traditional use of *C. ferrea* in treating various diseases associated with inflammation and pain.

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Introduction

Caesalpinia ferrea Mart. is a tree that belongs to the family Fabaceae. It is commonly known as "pau-ferro" or "jucá", which grows throughout Brazil and is widely distributed in the North and Northeast, especially in Pernambuco and Ceará (Pio Correia, 1984).

Some therapeutic properties of *C. ferrea* were described and include antiulcerogenic (Bacchie et al., 1995), anti-inflammatory and analgesic properties (Thomas et al., 1998). In addition, it has been shown to have cellulase, amylase and anticoagulant activities and to kill *Aedes aegypti* larvae (Cavalheiro et al., 2009). The fruits have also been used to treat diabetes (Balbach, 1972) and to prevent cancer. Treatment with the fruits significantly decreased the average number of papillomas per mouse in experiments of the promoting effects of 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) on skin tumor formation in mice initiated with 7,12-dimethylbenz[α]anthracene (DMBA) (Nakamura et al., 2002). *C. ferrea* roots are used as antipyretics and antidiarrheals, and the decoction of the wood is healing and antisecretory (Lewis, 1987). The stem bark is used as a decongestant, for treatment of enterocolitis, an antidiarrheal (Balbach, 1972), and for the treatment of rheumatism and may be beneficial to the cardiovascular system (Menezes et al., 2007). A preliminary phytochemical study of the hydroalcoholic extracts of stem, bark and leaves showed the presence of flavonoids, saponins, tannins, coumarins, sterols and phenolic compounds (Gonzalez et al., 2004). However, despite indications of the popular use of this plant for inflammatory processes, the literature reports few studies with the ethanol extract of the fruits, and there are no comprehensive studies on its possible mechanisms of action. Therefore, this study aimed to evaluate the anti-inflammatory and antinociceptive activities of the ethanol extract of the fruits of *Caesalpinia ferrea* Mart.

Material and Methods

Plant material

The pods (peels and seeds) of *Caesalpinia ferrea* Mart., Fabaceae, were collected at Barbalha-CE, Brazil (latitude 7° 18' 40", longitude 7° 18' 40"), in June 2007. They were dried at 40 °C in an incubator with circulating air for 48 h and ground in a grinder. The botanical material was identified by researchers at

the Instituto Agronômico de Pernambuco. A voucher specimen was prepared and stored at the Herbarium IPA - Dárdano de Andrade Lima, under the number 83.566.

Reagents

Carrageenan and Evans blue were purchased from Sigma (St. Louis, MO), sodium thioglycolate, xylene and formalin were obtained from Vetec (Rio de Janeiro, Brasil). Indomethacin was purchased from Merk Sharp & Dome and Fentanyl was obtained from Janssen Cilag Farmaceutica.

Preparation of extract

The ethanol extract of *C. ferrea* (EECf) was obtained by repeated soaking under agitation in 95% ethanol P.A. at a ratio of 1:5, until the depletion of substances extractable by ethyl alcohol. To concentrate the extract, ethanol was removed from the filtrate using a rotary evaporator at 40 °C under reduced pressure.

Animals

Females Swiss albino mice (*Mus musculus*) (n=3) were used for acute toxicity evaluation and males (n=7) for evaluation of anti-inflammatory and anti-nociceptive activities. The animals were approximately 50 days old and weighed 25±5 g. Mice were obtained from the animal house of the Antibiotics Department of the Federal University of Pernambuco (UFPE), which is registered with the Brazilian College of Animal Experimentation (COBEA) under nº 18. The animals were divided into groups and kept in plastic boxes at room temperature (22±3 °C) with a light:dark cycle of 12:12 h. They received standard chow (Purina) and water *ad libitum*. All animals used for determination of anti-inflammatory and antinociceptive activities were fasted for 4 h before the experiment.

The protocol was approved by the Animal Ethics (CEEA) of UFPE (number 40/06) and is in accordance with the Brazilian legislation dealing with the use of animals for scientific purposes.

Acute toxicity single dose

The acute toxicity study of *C. ferrea* followed the guidelines of the Organization for Economic Cooperation and Development, which authorizes the use of three animals per dose according to Guideline 423 and the guidelines of the Ordinance of the Ministry of Health 116/96 for evaluation of chemical substances and toxicity studies of single doses of new drugs (OECD, 2001). The study also followed the requirements of the RDC 17 (24/02/2000) from Anvisa/MS. The technique involved administration of sequentially lower doses from the maximum of 2000 mg/kg to groups of three animals sequentially lower doses from the maximum of 2000 mg/kg if they note the deaths of more than one animal. This yielded an estimate of the LD50 according to the standards of the GHS (Globally Harmonized System of Classification). The treated animals received the EECf (2000 mg/kg) orally, while the negative control animals received saline:propylene glycol (1:1). After the administration of EECf, the mice were observed for 24 h, and mortality was noted. The test was repeated once.

Evaluation of anti-inflammatory activity

Thioglycolate induced peritonitis

The mice were treated orally with the vehicle, EECf (12.5, 25 and 50 mg/kg) or indomethacin (10 mg/kg). The doses were chosen according to the results of a pilot study conducted in our laboratory and also based on studies of the literature (Carvalho et al., 1996). One hour after treatment, the animals received an intraperitoneal injection of 0.5 mL of sodium thioglycolate (3%). After 4 h, the animals were euthanized in a CO_2 chamber, and 2 mL of saline containing EDTA was injected into the peritoneal cavity. The total leukocytes that migrated into the peritoneal cavity in the recovered samples were counted in a Neubauer chamber (Bonhomme-Faivre et al., 2000). This test was used for initial screening to select the best dose (50 mg/kg) for the other tests of anti-inflammatory activity. Indomethacin was chosen as a positive control because it is a very effective anti-inflammatory nonsteroidal drug that is widely used in the literature (Gupta et al., 2003; Tunalier et al., 2007).

Xylene-induced ear edema

Sixty minutes after oral administration of the EECf (50 mg/kg), 20 µL of xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was utilized as a control. Two hours after xylene application, the mice were killed and both ears were removed. Circular sections were taken using a cork borer with a diameter of 7 mm, and the sections were weighed. The increase in weight caused by the irritant was measured by subtracting the weight of the untreated left ear section from that of the treated right ear section. The formula to calculate the percentage of inhibition was the average of the edema in the control group (normal saline) minus the average of the edema in the drug group divided by the average of the edema in the control group x 100%. Individuals in the control

groups received vehicle, and indomethacin (10 mg/kg) was used as a reference; both were given orally (Akindele & Adeyemi, 2006).

Vascular permeability induced by acetic acid in mice

The test was performed using the technique described by Whittle (1964). The mice were treated orally with vehicle, EECf (50 mg/kg) or indomethacin (10 mg/kg). One hour after treatment, Evans blue dye (1%) was injected into the retro-orbital plexus (0.2 mL/animal) under associative anesthesia with ketamine/xylazine. Next, 0.5 mL of acetic acid (1%) was administered intraperitoneally. After 30 min, the mice were euthanized in a CO_2 chamber and intraperitoneally injected with 2 mL of saline. The exudate was collected and centrifuged at 200 × *g* for 10 min. The absorbance of the supernatant was read at 610 nm with ELISA (thermo plate).

Evaluation of anti-nociceptive activity

Writhing test

One hour after the oral administration of the EECf (12.5, 25 and 50 mg/kg), the mice were injected intraperitoneally with 1% v/v acetic acid solution (volume of injection 0.1 mL/10 g). The mice were placed individually into glass beakers for an initial ten-minute period. The number of writhes produced in these animals was counted for the next 20 min, according to the methodology described with minor modifications (Koster et al., 1959). The control group received vehicle, and ibuprofen (300 mg/kg) was used as a reference drug (Sun et al., 2008; Jones et al., 2007). This test was used as an initial screening to select the best dose (50 mg/kg) for the remaining tests of the antinociceptive activity.

Formalin test

The formalin test was performed according to the methodology described by Hunskaar & Hole (1987). The animals were injected with 20 µL of formalin 2.5% (0.92% formaldehyde diluted in saline) in the sub-plantar area of the right hind paw. The duration of paw licking was measured for 1-5 min (first phase) and 15-30 min (second phase) after the formalin injection. The amount of time spent licking the injected paw was considered to be the nociceptive response. Animals received vehicle and EECf (50 mg/kg) orally 30 min prior to the injection of formalin. Fentanyl (200 µg/kg, *s.c.*) was administered 15 min prior to the injection of formalin. Fentanyl was used as a standard drug based on literature (Rocha et al., 2008; Romero et al., 2010).

Statistical analysis

The results were expressed as mean±SD for each experimental group. Statistical comparison of groups was performed using one-way analysis of variance (ANOVA), followed by a Bonferroni´s test. Statistical significance was considered to be indicated by a p value <0.05 in all cases, using the software Graph Pad prism. 5.0.

Results

Acute toxicity

The method proposed by Litchfield & Wilcoxon (1949) recommended determining the LD50 when the approximate lethal dose is less than 2 g/kg. In this study, we showed that a dose of 2 g/kg did not cause death in animals, so we discarded the determination of an LD50. The treated group exhibited ptosis and drowsiness immediately following gavage; however, no animals died.

Evaluation of anti-inflammatory activity

Thioglycolate-induced peritonitis

According to statistical analysis, the EECf was effective in inhibiting the inflammatory response (Figure 1). The effect was most pronounced at the doses of 25 and 50 mg/kg, which inhibited cell migration of 68.4% (4.14±0.5 x 10^6) and 71.8% (3.69±0.5 x 10^6), respectively, compared to control.

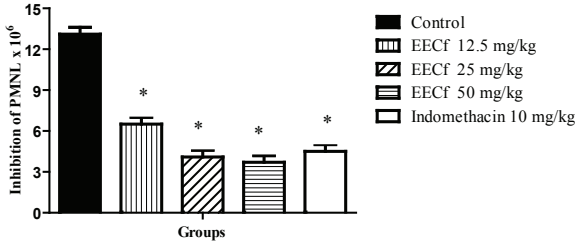

Figure 1. Inhibition of cell migration by EECf (12.5, 25 and 50 mg/kg, *p.o.*) in thioglycollate-induced peritonitis. Results are expressed as mean±SD for each experimental group (n=7). Significant after analysis of variance (ANOVA) followed by Bonferroni´s test with confidence interval of 95% compared to the control group. *p<0.05 compared to control.

Xylene-induced ear edema

The effect of the extract on the xylene-induced ear edema in mice is shown in Figure 2. The administration of EECf (50 mg/kg) reduced the ear

edema by 66.6% (5.5±1.2) compared to the control group (16.4±1.7). Indomethacin (10 mg/kg) produced an inhibition of 83.9% compared to control.

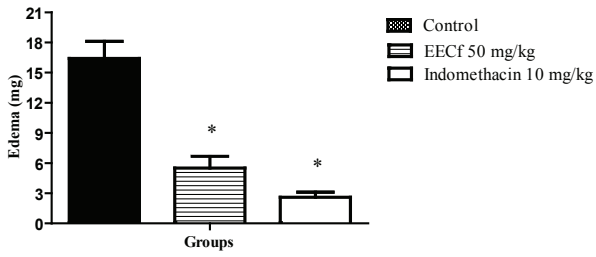

Figure 2. Effect of EECf in the ear edema induced by xylene. Results are expressed as mean±SD for each experimental group (n=7). Significant after analysis of variance (ANOVA) followed by Bonferroni´s test with confidence interval of 95% compared to the control group. *p<0.05 compared to control group.

Vascular permeability induced by acetic acid

In order to evaluate the action of EECf in the release of vasoactive amines (histamine, bradykinin and serotonin) and edema formation, we evaluated its effect on vascular permeability induced by acetic acid (Figure 3). EECf (50 mg/kg) significantly inhibited vascular permeability in 66.1% compared to control.

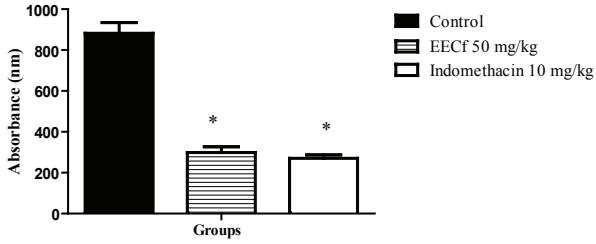

Figure 3. Effect of EECf in the vascular permeability induced by acetic acid measured as Evans blue dye extravasation in swiss albino mice males (n=7). Significant after analysis of variance (ANOVA) followed by Bonferroni´s test with confidence interval of 95% compared to the control group. *p<0.05 compared to control.

Evaluation of anti-nociceptive activity

Writhing test

Figure 4 shows that EECf (12.5, 25 and 50 mg/kg) significantly inhibited writhing induced by acetic acid in 24.9, 46.9 and 74.2%, respectively, when compared to control. This response was dose-dependent. Ibuprofen (300 mg/kg) inhibited the writhing response by 78%. Ibuprofen was used as positive control for its potent analgesic properties, as described in the literature (Sun et al., 2008; Jones et al., 2007).

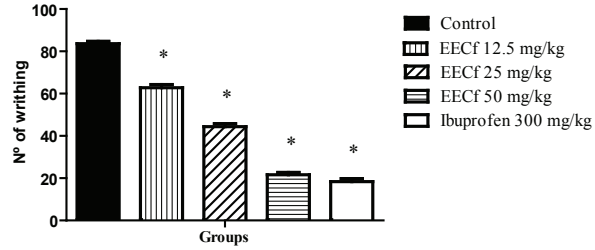

Figure 4. Response observed in the writhing test induced by acetic acid with EECf in the doses of 12.5, 25 and 50 mg/kg in swiss albino mice (*Mus musculus*) males (n=7). Results are expressed as mean±SD for each experimental group. Significant after analysis of variance (ANOVA) followed by Bonferroni´s test with confidence interval of 95% compared to the control group. *p<0.05 compared to control group.

Formalin test

Table 1 shows that in the first phase of formalin-induced nociception, the oral pre-treatment with EECf (50 mg/kg) significantly modified the licking time of the animals when compared to control group. The second phase of the test extract showed 34.0% inhibition of nociception compared to control. The fentanyl inhibited the licking time in 92.9 and 96.7% in the first and second phases, respectively. The fentanyl was used as reference drug in accordance with the studies of Rocha et al. (2008).

Table 1. Effect of EECf in the nociception induced by formalin.

Group	Dose (mg/kg)	Paw licking (s) (mean±SEM)		Inhibition (%)	
		0-5 min	20-30 min	0-5 min	20-30 min
Control	___	57.8±4.1	177.9±13.9	____	____
EECf	50	47.6±3.5*	116.7±7.9*	17.6	74.3
Fentanyl	200	4.1±0.4*	5.8±0.5*	93.0	96.7

*p<0.05 compared to the control. Significant after analysis of variance (ANOVA) followed by Bonferroni´s test with confidence interval of 95% compared to the control group; n=7 animals for group.

Discussion

Our results indicated that the ethanol extract of *C. ferrea* has low oral acute toxicity. The 2 g/kg dose did not cause death in animals, so we discarded the determination of an LD50 (Litchfield & Wilcoxon, 1949).

In order to evaluate the effect of EECf on cell migration to the peritoneal cavity, we tested its effect on the induction of peritonitis by thioglycolate. The levels of inhibition of cellular migration by 25 and 50 mg/kg of EECf were not statistically different and

were comparable to the effects produced by 10 mg/kg indomethacin. In the model of peritonitis induced by thioglycolate, both mast cells and macrophages act to increase vascular permeability (Kolaczkowska et al, 2002). Based on the response to EECf treatment, we hypothesize that substances present in the extracts are inhibiting the activation of mast cells and macrophages.

Xylene is an irritant that causes increased vascular permeability and edema due to the release of mediators and cellular migration (Hosseinzadeh et al., 2003). EECf significantly inhibited the production of edema compared to the vehicle treatment, probably due to a reduction in vascular permeability. The technique developed by Henriques et al., (1987) is a classic model to evaluate specific acute inflammation in mice that involves various types of chemical mediators of inflammation, such as histamine, serotonin, bradykinin and prostaglandins.

To confirm the reduction of edema mediated by EECf in the xylene-induced ear edema assay, we tested the extract in the model of vascular permeability induced by acetic acid. The results showed that EECf reduced permeability and was effective in the first phase of the inflammatory response, which is characterized by the action of vasoactive amines. The effect of treatment with 50 mg/kg EECf was comparable to that of treatment with 10 mg/kg indomethacin, which is a promising level of activity. Acetic acid can cause an increase of chemical mediators such as prostaglandin E2 (PGE2), histamine and serotonin in peritoneal fluids, leading to an increase in vascular permeability (Choi et al., 2006). The ethanol extract of the *C. ferrea* significantly inhibited the acetic-acid-induced vascular permeability in mice. These results suggested that *C. ferrea* exerted an anti-inflammatory action similar to that of indomethacin (a nonselective COX inhibitor) and inhibited the mediators of the acute phase of inflammation.

The analgesic activities of EECf were evaluated using two animal models. EECf was effective in the writhing test (Figure 4), which is used to screen for both peripherally and centrally acting agents (Le Bars et al., 2001). Induction of writhing by acetic acid allows the evaluation of antinociceptive responses to different types of drugs (*i.e.,* anticholinergics and antihistamines). It triggers the activation of various events that determine the nociception, such as the release of mediators such as histamine, bradykinin and PGE2. This model is sensitive to analgesic substances that have either central or peripheral mechanisms of action (Koster et al., 1959). EECf exhibited a dose-dependent effect in the writhing test, and a dose of 50 mg/kg had antinociceptive effects comparable to those of ibuprofen.

These findings indicated, therefore, the antinociceptive activity of EECf may result from direct or indirect inhibition of the release of pro-inflammatory mediators induced by acetic acid, such as prostaglandins and cytokines. Acetic acid acts indirectly to cause the release of endogenous substances involved in the modulation of nociception, including bradykinin, serotonin, histamine and prostaglandins (Witkin et al., 1961). Recently, Ribeiro et al. (2000) showed that the nociception induced by this stimulus also depends on the release of cytokines such as IL-1β, TNF, IL-8 from macrophages and basophils residents in the abdominal cavity. Together with the other mediators mentioned previously, these factors could induce the nociception observed in this study. These data, together with the data obtained on the effect of EECf on vascular permeability, lead us to hypothesize that that the activity of the extract may involve the participation of resident macrophages.

The formalin test has an advantage over other frequently used tests as it involves a biphasic response with an early and a late phase representing, respectively, neurogenic and inflammatory pain (Hunskaar & Hole, 1987). This is of interest considering that both phases are sensitive to centrally acting drugs, such as opioids (Shibata et al., 1989). However, the second phase is also sensitive to NSAID (non-steroidal anti-inflammatory drugs) and corticosteroids (Hunskaar & Hole, 1987). In this study, EECf (50 mg/kg) significantly inhibited (p<0.05) the second phase and weakly affected the first phase, suggesting that the main mechanism of action occurs at the peripheral level.

In our study, the ethanol extract of *C. ferrea* was capable of inhibiting inflammatory reactions and pain. The results provided experimental evidence for its traditional use in treating various diseases associated with inflammation and pain. The mechanism involved was not elucidated in the present study. Further studies currently in progress will enable us to understand the precise mechanisms involved.

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