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Antinociceptive and anti-inflammatory effects of extract of *Celtis iguanaea* (Jacq.) Sargent leaves in mice

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Abstract: The antinociceptive and anti-inflammatory activities of crude ethanolic extract of *Celtis iguanaea* leaves and their active fractions are reported. The oral treatment with crude ethanolic extract (CEE; 100, 300 or 1000 mg/Kg) inhibited the number of writhings in a dose-dependent manner. The intermediate dose also inhibited formalin-induced nociception in both phases. The oral treatment with dichloromethane fraction (DF; 9 mg/Kg) produced antinociceptive effect in both phases of formalin test; however, the treatment with ethyl acetate fraction (EAF; 16 mg/Kg) reduced pain only in the second phase of this test. The oral treatments with CEE (300 mg/Kg) or DF (9 mg/Kg) reduced the nociception induced by capsaicin and pre-treatment with naloxone did not change these effects. The oral administration of CEE (300 mg/Kg), DF (9 mg/Kg) or ethyl EAF (16 mg/Kg) reduced ear edema, leukocytes migration and myeloperoxidase activity. Furthermore, the oral treatment with CEE (300 mg/Kg) or EAF (16 mg/Kg) reduced the level of Tumor Necrosis Factor - Alpha (TNF- α) in the pleurisy test. In conclusion, the DF showed antinociceptive activity that involves the vanilloid system as well as anti-inflammatory effect and the EAF showed anti-inflammatory activity involving the reduction of TNF- α cytokine.

Key words: Esporão-de-galo, herbal medicine, preclinical evaluation, pain management, TNF- α , vanilloid receptors.

INTRODUCTION

Pain and inflammation diseases are among the main problems that significantly influence the lifestyle of millions of people (Nathan & Ding 2010, Manjiani et al. 2014). However, existing therapies are not always effective and can cause several adverse effects related mainly to the gastrointestinal tract (Harirforoosh et al. 2013, Ungprasert et al. 2012), which brings the challenge of seeking for drugs with enhanced therapeutic effects and minor adverse effects.

In this sense, medicinal plants, a source of a wide variety of pharmacologically active molecules, appear as an important tool in the search for new safer and more effective drugs (Balunas & Kinghorn 2005, García-Rodríguez et al. 2014).

Celtis iguanaea (Jacq.) Sargent, a member of the Cannabaceae family, is popularly known as “esporão-de-galo, taleira, taleira-unha-de-gato, gurrupiá or grão-de-galo” in midwest Brazil. The leaves of this plant are traditionally used for the treatment of body aches, colic, asthma, urinary infections and digestive disorders (Carneiro

2012, Paula et al. 2010, Silva & Proença 2008, Piliackas et al. 2007, Tene et al. 2007).

Phytochemical screening of the leaves and stem of *C. iguanaea* showed the presence of flavonoids, coumarins and mucilage (Paula et al. 2010). Moreover, the leaves aqueous extracts presented low toxicity with median lethal dose (LD_{50}) higher than 2000 mg/Kg and less than 5000 mg/Kg (Gonçalves et al. 2015).

A preclinical survey showed that the crude hexane extract of *C. iguanaea* possess gastroprotective effect, as well as hexane fraction obtained from the partitioning of crude ethanolic extract (CEE) of leaves of this plant (Sousa et al. 2013, Martins et al. 2014). Herein, our aims were to evaluate the antinociceptive and anti-inflammatory activities of CEE of *C. iguanaea* leaves and its fractions, hexane (HF), dichloromethane (DF), ethyl acetate (EAF), and aqueous (AF), to better elucidate the therapeutic effects of this plant and point out its value in traditional Brazilian medicine.

MATERIALS AND METHODS

Plant material

Leaves of *C. iguanaea* were collected in Hidrolândia, Goiás, Brazil (161 53059,4" S 491 130 29,4' W) with an altitude of 786 m. The sample was authenticated by Prof. Dr. José Realino de Paula, and a voucher specimen was deposited at the Herbarium of the Federal University of Goiás (40.110/UFG).

Preparation and fractionation of the crude ethanolic extract

The CEE was obtained by static maceration. Briefly, 450 g of dried and powdered *C. iguanaea* leaves were immersed in 900 mL of ethanol (96°GL), for 48–72 h, with occasional stirring. Thereafter, the extract was vacuum filtered; concentrated in a rotary evaporator at reduced

pressure and temperature lower than 50°C; lyophilized; and stored sheltered from the light at 4°C. The extraction yield was 6% (w/w). The CEE was dissolved in water and subsequently partitioned with solvents of increasing polarity i.e., hexane, dichloromethane, and ethyl acetate.

The remaining aqueous portion and the several organic portions were evaporated to dryness under reduced pressure to obtain the AF (yield of 60% w/w) HF (yield of 30% w/w), DF (yield of 1.5% w/w), and EAF (yield of 2.6%w/w) (Sousa et al. 2013). Fractions obtained were tested in the same experimental models as the CEE to identify a possible separation of biological activities. For this, the fraction doses were calculated based on their yields to ensure that the same quantity of phytochemicals present in the CEE were maintained in the fractions. In this pursuit, it was used a correction factor of 2-folds the yields of the fractions in relation to the crude extract. Therefore, the greater the yield of the fraction in relation to the CEE, the greater the dose to be used, thus reaching the dose of the CEE.

Animals

Experiments were performed using female Swiss albino mice (25–30 g) from the Central Animal House of the Federal University of Goiás. The animals were kept in plastic cages at 22 ± 2 °C, with free access to pellet food and water and under a 12 h light/dark cycle, in compliance with the International Guiding Principles for Biomedical Research Involving Animals (National Research Council (US) Institute for Laboratory Animal Research 2004). The animals were acclimatized for 7 days before the beginning of the experiments. All experimental protocols were developed and approved according to the principles of ethics and animal welfare designated by the Ethics Committee on Animal

Use (CEUA) of the Federal University of Goiás (number: 106/08).

Drugs and chemicals

The chemicals used in this study were: acetic acid (Merck, San Louis, Missouri, USA); acetone (Synth, Diadema, SP, Brazil); capsaicin (Sigma Chemical, San Louis, Missouri, USA); capsazepine (Sigma Chemical, San Louis, Missouri, USA); carrageenan (Sigma Chemical, San Louis, Missouri, USA); croton oil (Sigma Aldrich, San Louis, Missouri, USA); dexamethasone (Prodome, Campinas, SP, Brazil); 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB - Sigma Chemical, San Louis, Missouri, USA); formaldehyde (Synth, Diadema, SP, Brazil); heparin (Hipolabor, Belo Horizonte, MG, Brazil); indomethacin (Merck Sharp & Dohme Farmacêutica Ltda, São Paulo, SP, Brazil); morphine hydrochloride (Dimorf®, Cristália, SP, Brazil); and Türk solution (New Prov, Pinhais, PR, Brazil).

Antinociceptive activity

Acetic acid-induced abdominal writhing test

Groups of mice (n=8) were treated by gavage (p.o.) with vehicle (distilled water – 10 mL/Kg), CEE (100, 300 or 1000 mg/Kg), or indomethacin (10 mg/Kg - positive control for antinociceptive activity) 60 min before the application of acetic acid solution (1.2% v/v; 10 mL/Kg, i.p.). The number of abdominal writhing was counted for each animal over a period of 30 min after acetic acid injection as described by Silva et al. (2018a). The results are expressed as the means \pm S.E.M. of number of writhings.

Formalin test

The mice (n=8) were treated by gavage (p.o.) with vehicle (distilled water – 10 mL/Kg); CEE (300 mg/Kg); AF (360 mg/Kg); EAF (16 mg/Kg); DF (9 mg/Kg); HF (180 mg/Kg); indomethacin (10 mg/Kg,

p.o. - positive control for antinociceptive activity in the second phase); or morphine (5 mg/Kg, s.c. - positive control for antinociceptive activity in the first and second phases). Sixty minutes after the oral treatments or thirty minutes after the subcutaneous treatment, 20 μ L of formalin (3%) were injected into the plantar surface of the right hind paw and the licking time of the paw was timed from 0-5 min and from 15-30 min after formalin injection, as described by Florentino et al. (2016). These results were expressed as the means \pm S.E.M of licking time, in seconds (s).

Involvement of vanilloid receptor in the antinociceptive effect of CEE or DF

To investigate the role of the vanilloid TRPV1 receptor in the modulation of CEE antinociceptive action, capsaicin (TRPV1 agonist) was used to induce nociception (Sakurada et al. 1992, Oliveira et al. 2012). The mice (n=8) were pre-treated orally with vehicle (distilled water – 10 mL/Kg), CEE (300 mg/Kg), DF (9 mg/Kg) or intraperitoneally with capsazepine (10 mg/Kg – Vanilloid receptor antagonist) or subcutaneously with morphine (5 mg/Kg). Sixty minutes after the oral treatments or thirty minutes after intraperitoneally or subcutaneous treatments, 20 μ L of capsaicin (1.6 μ g/paw) were injected into the plantar surface of the right hind paw of the animals and the time the animals spent licking the paw was recorded, during a 7 min period. These results were expressed as the means \pm S.E.M of licking time, in seconds (s).

Involvement of opioid receptors in effect of CEE or DF in the capsaicin test

To evaluate the involvement of opioid receptors in the antinociceptive effect of CEE and DF in the capsaicin test, the mice (n = 8) were pre-treated with saline (10 mL/Kg s.c.) or naloxone (3 mg/Kg s.c. – non-selective opioid antagonist) 15 min before the treatment with vehicle (10

mL/Kg, p.o.), CEE (300 mg/Kg, p.o.), DF (9 mg/Kg p.o.), or morphine (5 mg/Kg s.c.). Sixty minutes after treatment by gavage (p.o.) or 30 min by subcutaneous administration (s.c.) the animals received 20 μ L of capsaicin (1.6 μ g/paw) by injection into the plantar surface of the right hind paw and the experiment proceeded as discussed in the previous section (Costa et al. 2013).

Anti-inflammatory activity

Croton oil-induced ear edema test

The experimental groups were treated with vehicle (distilled water – 10 mL/Kg, p.o.), CEE (300 mg/Kg), EAF (16 mg/Kg), DF (9 mg/Kg), or dexamethasone (1 mg/Kg p.o. – positive control for anti-inflammatory activity). Posteriorly, 20 μ L of croton oil in acetone (2.5%) were administered topically in the right ear of each animal. The same volume of acetone was applied to the left ear for control. Four hours after, animals were sacrificed by decapitation and a 6 mm diameter disk was removed from each ear lobe. The difference between the right and left ear disk weights was taken as a measure of edema (Emim et al. 2000). The results are expressed as means \pm S.E.M of difference between the weights of ears, in mg.

Carrageenan-induced pleurisy

The experimental groups (n=8) were treated with vehicle (distilled water – 10 mL/Kg, p.o.), CEE (300 mg/Kg), EAF (16 mg/Kg), DF (9 mg/Kg), or dexamethasone (1 mg/Kg p.o.) 1 h before injection of 100 μ L of 1 % carrageenan into the pleural cavity. Four hours after carrageenan administration, the animals were euthanized, and the pleural exudate was collected with 1 mL of heparinized PBS as described by Silva et al. (2018b). One aliquot of pleural exudate was used to count the number of total leukocytes

using Turk solution in a Neubauer chamber and another aliquot was used to determine activity of the myeloperoxidase enzyme (MPO) and to quantify the Tumor Necrosis Factor -Alpha (TNF- α) levels.

Activity of MPO

To measure the MPO activity 40 μ L of pleural lavage of mice treated with vehicle (10 mL/Kg, p.o.), CEE (300 mg/Kg), EAF (16 mg/Kg), DF (9 mg/Kg), or dexamethasone (1 mg/Kg p.o.) were added to 360 μ L of phosphate buffer pH 6.0 containing 0.167 mg/mL of o-dianisidine, 2 HCl and 0.0005% H₂O₂. The enzymatic reaction was stopped after 15 min by addition of 20 μ L of 1% (w/v) sodium azide. The samples were subsequently centrifuged for 5 min at 300 x g. The supernatant (200 μ L) was transferred to a microplate well, and the absorbance was monitored at a wavelength of 450 nm (Silva et al. 2018b). The results are expressed as means \pm S.E.M of enzymatic activity in mU/mL.

Assay of TNF- α

The pleural exudates of mice treated with vehicle (10 mL/Kg, p.o.), CEE (300 mg/Kg), EAF (16 mg/Kg), DF (9 mg/Kg), or dexamethasone (1 mg/Kg p.o.) also were used to determine the concentrations of TNF- α using an immunosorbent assay kit (ELISA) (Ebioscience – Thermo Fisher Scientific – Waltham, Massachusetts, USA). Initially the plate was incubated overnight at 2-8 $^{\circ}$ C with 100 μ L capture antibody. Subsequently, the plate was washed with specific wash buffer and the wells were blocked with 200 μ L of the ELISA / ELISPOT diluent.

The plate was incubated for 1 hour at room temperature. After this time, the plate was washed again, 100 μ L of samples or the standard was added to the wells and the plate was incubated again for 2 hours at room temperature. After, the plate was washed, 100 μ L of the detection

antibody was added and the plate was incubated for 1 hour at room temperature. After this time, the wells were washed, 100 µl of avidin-HRP was added and the plate was incubated at room temperature for 30 minutes.

Once again, the wells were washed and 100 µl of TMB solution was added to the wells. The plate was incubated for 15 minutes and after this time, 50 µl of stop solution was added for reading the plate at 450 nm. Subsequently, standard curve calculations were performed, and the results were expressed as means ± S.E.M in pg/mL.

Statistical analysis

The data obtained in this study were analyzed statistically by one-way ANOVA followed by the Newman-Keuls test as post-hoc (Sokal & Rohlf 1981). All statistical analysis was carried out using GraphPad Prism version 5.0. Values of $P \leq 0.05$ were considered significant.

RESULTS

Antinociceptive activity

Acetic acid-induced abdominal writhing test

In the acetic acid-induced abdominal writhing test CEE decreased the number of writhings in a dose-dependent manner. The treatment with CEE at doses of 300 or 1000 mg/Kg decreased the number of abdominal writhings by 19 % ($P < 0.05$) and 29 % ($P < 0.001$), respectively, when compared to the control group (vehicle 10 mL/Kg). However, the lowest dose of the extract was not able to reduce the number of abdominal writhings. The positive control, Indomethacin (10 mg/Kg), reduced abdominal writhings by 24 % ($P < 0.001$), as shown in Table I.

Formalin test

Table I. Antinociceptive activity of crude ethanolic extract (CEE) of *Celtis iguanaea* leaves in acetic acid-induced abdominal writhing in mice.

	Number of writhes	
	(Mean ± S.E.M)	(%) Inhibition
Vehicle 10 mL/Kg	115.7 ± 5.4	-
CEE 100 mg/Kg	108 ± 5.2	7
CEE 300 mg/Kg	93.2 ± 7.5*	19
CEE 1000 mg/Kg	82.3 ± 3.8***	29
Indomethacin 10 mg/Kg	87.6 ± 4.0***	24

Values are expressed as mean ± S.E.M of cumulated writhings in 30 min for each experimental group. * $P < 0.05$ and *** $P < 0.001$ compared to vehicle, according to ANOVA followed by Student-Newman-Keuls' test.

The treatment with CEE (300 mg/Kg, p.o.) decreased the licking time in both phases of formalin-induced pain; in neurogenic (first phase) by 42 % ($P < 0.001$) and in inflammatory (second phase) by 39 % ($P < 0.001$), when compared to the control group. The treatment with DF (9 mg/Kg, p.o.) significantly decreased the licking time of both phases, in neurogenic by 26 % ($P < 0.05$) and in inflammatory by 22 % ($P < 0.05$). The treatment with EAF (16 mg/Kg, p.o.) or indomethacin (10 mg/Kg, p.o.) only significantly decreased the licking time in the inflammatory phase by 55 % ($P < 0.001$) and 51 % ($P < 0.001$), respectively. However, with AF (360 mg/Kg) or HF (180 mg/Kg) the licking time was not reduced in any phase. Morphine (5 mg/Kg, s.c.) however decreased significantly both phases of this test by 98 % and 100 % (both $P < 0.001$), respectively (Table II).

Involvement of vanilloid receptors in the antinociceptive effect of CEE and DF

From the results obtained, CEE at all doses caused a significant inhibition of the capsaicin-induced nociception (Figure 1). The treatment with CEE (300 mg/Kg, p.o.) decreased the licking time in

Table II. Antinociceptive activity of crude ethanolic extract (CEE) of *Celtis iguanaea* leaves, aqueous fraction (AF), ethyl acetate fraction (EAF) and dichloromethane fraction (DF) and hexane fraction (HF) in pain induced by formalin test in mice.

	Licking time (s) (Mean \pm S.E.M and % Inhibition)			
	Neurogenic pain (0–5 min)		Inflammatory pain (15–30 min)	
Vehicle 10 mL/Kg	64.2 \pm 3.0	-	159.6 \pm 6.8	-
CEE 300 mg/Kg	37.2 \pm 2.2***	42	97.7 \pm 9.2***	39
AF 360 mg/Kg	66 \pm 0.7	-	176.2 \pm 15.5	-
EAF 16 mg/Kg	54.8 \pm 2.2	-	72 \pm 11.6***	55
DF 9 mg/Kg	47.2 \pm 2.4*	26	124 \pm 11.6*	22
HF 180 mg/Kg	69 \pm 6	-	152 \pm 13.8	-
Morphine 5 mg/Kg	1.3 \pm 1.0***	98	0.33 \pm 0.3***	100
Indomethacin 10 mg/Kg	56.8 \pm 3.6	-	77.6 \pm 5.9***	51

Values are expressed as mean \pm S.E.M of reaction time pain, in neurogenic pain (0–5 min) and inflammatory pain (15–30 min) in seconds. * $P < 0.05$ and *** $P < 0.001$ compared to vehicle, according to ANOVA followed by Student-Newman-Keuls' test.

neurogenic nociception, by 47 % ($P < 0.001$) when compared to the control group. Administration of DF (9 mg/Kg, p.o.) or capsazepine (10 mg/Kg, i.p.) significantly reduced the licking time by 46 % ($P < 0.001$) and 51 % ($P < 0.001$) respectively when compared to the control group. Morphine (5 mg/Kg, s.c.) significantly decreased the licking time by 84 % in neurogenic nociception, when compared to control group.

Involvement of opioid receptors in effect of CEE and DF in the capsaicin test

Administration of the non-selective opioid receptor antagonist naloxone (3 mg/Kg, s.c.) 15 min prior to the test, did not change the antinociceptive activity produced by CEE (300 mg/Kg, p.o.) or DF (9 mg/Kg, p.o.) in the pain induced by capsaicin. On the other hand, pre-treatment with naloxone reversed the antinociceptive effect produced by morphine (5 mg/Kg, s.c.). The administration of naloxone alone, in the

dose tested, did not affect capsaicin-induced nociception (Figure 2).

Anti-inflammatory activity

Croton oil-induced ear edema test

In the croton oil-induced ear edema test, CEE decreased the formation of the edema. The treatment with CEE (300 mg/Kg, p.o.) decreased the ear edema by 22 % ($P < 0.05$) when compared to the control group (vehicle 10 mL/Kg, p.o.). The treatment with EAF (16 mg/Kg, p.o.), DF (9 mg/Kg, p.o.), or dexamethasone (1 mg/Kg, p.o.) also reduced the edema by 37 % ($P < 0.001$), 20 % ($P < 0.01$) and 78 % ($P < 0.001$), respectively, when compared to the control group, as shown in Table III.

Carrageenan-induced pleurisy

In the carrageenan-induced pleurisy test, the treatment with CEE (300 mg/Kg, p.o.) reduced the total number of leukocytes by 33% ($P < 0.01$)

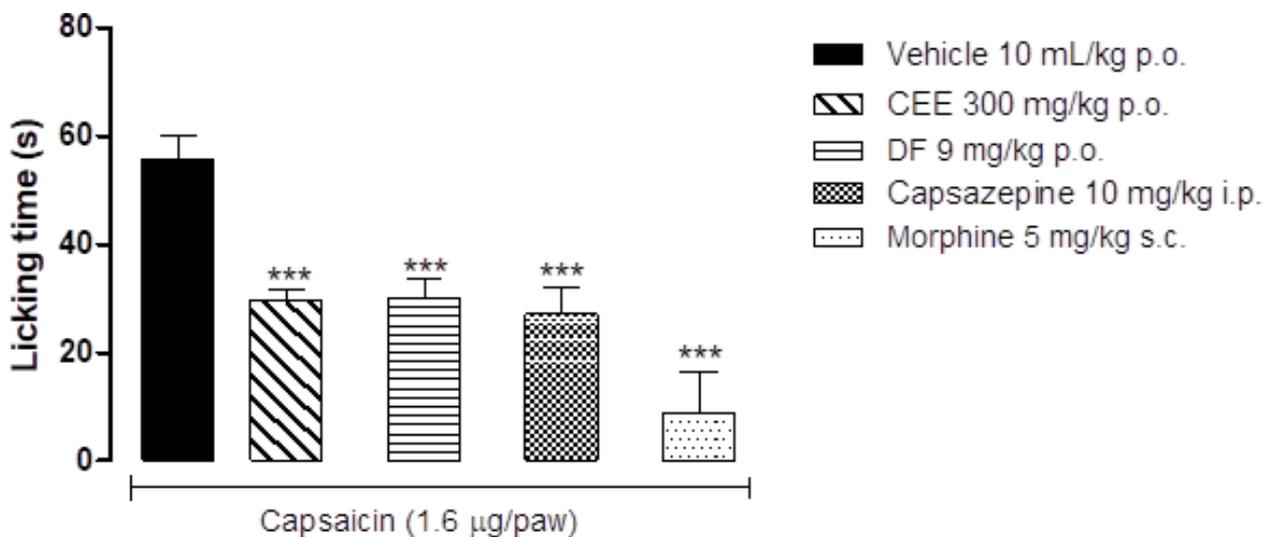


Figure 1. Antinociceptive effect of CEE (300 mg/Kg, p.o.) or DF (9 mg/Kg, p.o.) in the neurogenic phase (0 - 7 min) of pain induced by capsaicin test in mice (n = 8). Capsazepine (10 mg/Kg, i.p., antagonist of TRPV-1 receptor) and morphine (5 mg/Kg, s.c., agonist of opioids receptors) were used as positive controls, in the test. Vertical bars represent mean \pm S.E.M. of pain reaction time, in seconds. *** $P \leq 0.001$ compared to vehicle, according to ANOVA followed by Newman-Keuls' post-hoc test.

compared to the control group. As shown in Table IV, the groups treated with EAF (16 mg/Kg, p.o.), DF (9 mg/Kg, p.o.), or dexamethasone (1 mg/Kg, p.o.) also reduced the total number of leukocytes by 57 % ($P < 0.001$), 35 % ($P < 0.01$) and 51 % ($P < 0.001$) respectively, compared to the control group.

Activity of MPO

Treatment with CEE (300 mg/Kg, p.o.) reduced the MPO enzyme activity by 27 % ($P < 0.01$) compared to the control group. The groups treated with EAF (16 mg/Kg, p.o.), DF (9 mg/Kg, p.o.), or dexamethasone (1 mg/Kg, p.o.) reduced MPO activity by 64 % ($P < 0.001$), 43 % ($P < 0.01$) and 72 % ($P < 0.001$) respectively, compared to the control group, as shown in Table IV.

Assay of TNF- α levels

The treatment with CEE (300 mg/Kg, p.o.) reduced the levels of TNF- α by 62 % ($P < 0.001$) compared to the control group. The groups treated with EAF (16 mg/Kg, p.o.) or dexamethasone (1 mg/Kg, p.o.) also reduced the levels of TNF- α by

32 % ($P < 0.001$) and 81 % ($P < 0.001$), respectively compared to the control group. As shown in Table IV, the treatment with DF (9 mg/Kg, p.o.) did not reduce the levels of TNF- α .

DISCUSSION

C. iguanaea has been one of the species most frequently mentioned in folk medicine in Goiás State, Brazil, due to several therapeutic effects observed from its use (Borges et al. 2013, Martins et al. 2015). The current study investigated the antinociceptive and anti-inflammatory activity of the CEE obtained from leaves of *C. iguanaea*, as well as its active fractions.

The acetic acid-induced abdominal writhing test was the first test used in this study to evaluate possible anti-nociceptive activity of CEE. The administration of acetic acid into the abdominal cavity induces an acute inflammatory process, with pain as the main signal, and the behavioral response appears as abdomen contraction with extension followed by the animal rotating one or both hind legs (Koster et al. 1959, Ribeiro et

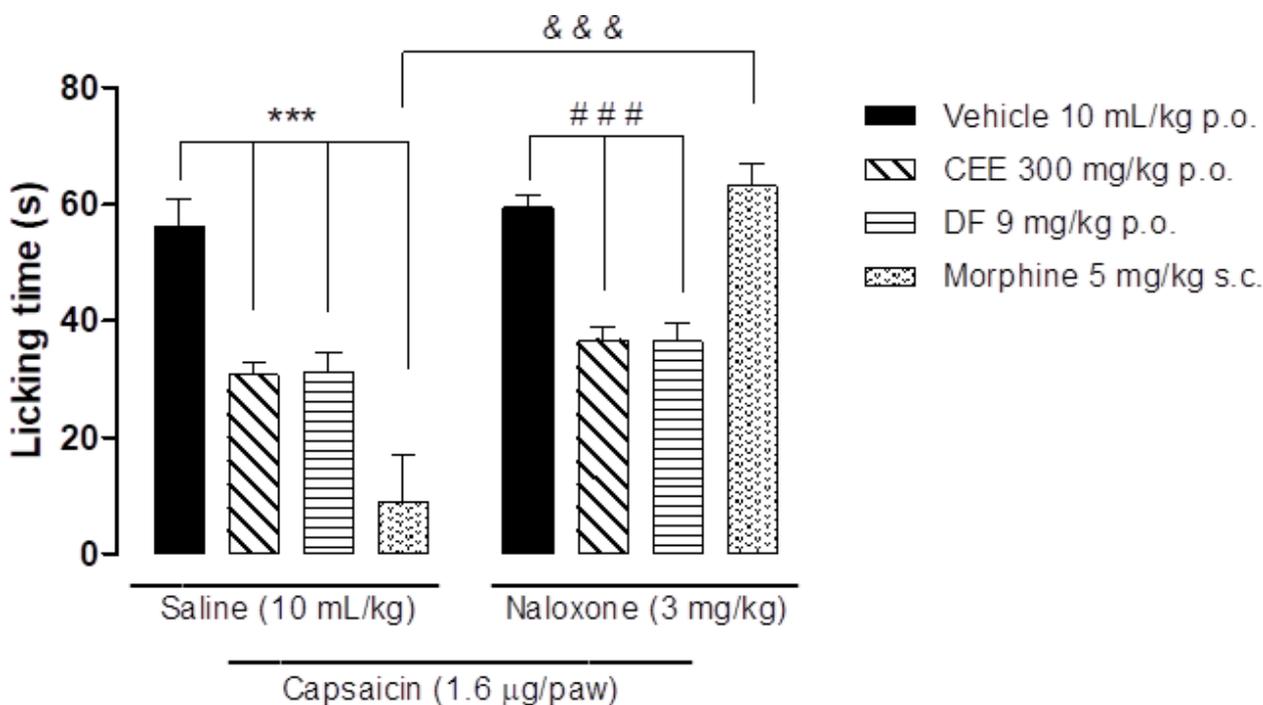


Figure 2. Effect of pre-treatment with saline (10 mL/Kg, s.c.) or naloxone (3 mg/Kg, s.c., opioid antagonist) on the antinociceptive effect of CEE (300 mg/Kg, p.o.) or DF (9 mg/Kg, p.o.) in the neurogenic phase (0 - 7 min) of pain induced by capsaicin in mice (n = 8). Morphine (5 mg/Kg, s.c., agonist of opioid receptors) was used as positive control in the test. Vertical bars represent mean \pm S.E.M of pain reaction time, in seconds. *** $P \leq 0.001$ compared to control group pre-treated with saline; ### $P \leq 0.001$ compared to control group pre-treated with naloxone; &&& $P \leq 0.001$ compared to group treated with morphine and pre-treated with saline according to ANOVA followed by Newman-Keuls' post-hoc test.

al. 2000). In this test, it was possible to observe that the oral treatments with CEE decreased in a dose-dependent manner the number of writhings induced by acetic acid, suggesting antinociceptive activity for the extract. Thus, in the subsequent tests only the intermediate dose of CEE was used, with the perspective of reducing the number of animals as recommended by the CEUA.

Since the antinociceptive effect of *C. iguanaea* was suggested, we were confronted with the challenge of determining the effects of the active fraction(s) of CEE. Using organic solvents with increasing polarities, the HF, DF, EAF and AF were obtained. The antinociceptive effect of the CEE and its fractions was tested in the formalin-induced pain test, which evaluates neurogenic pain (first phase) that is associated

with direct activation of nociceptors by formalin, and preforms mediators released in the injured tissue; and the inflammatory pain (second phase) that occurs due to the action of pro-inflammatory mediators (Hunskar & Hole 1987, Shibata et al. 1989, Barrot 2012).

The results obtained in this study showed that treatment of animals with CEE (300 mg/Kg, p.o.) as well as DF (9 mg/Kg, p.o.) significantly reduced the reactivity to pain time in both phases of the test, not permitting the differentiation of the antinociceptive effect of CEE or DF from possible anti-inflammatory activity. On the other hand, EAF (16 mg/Kg, p.o.) significantly reduced the reactivity to pain time only in the second phase of the test, suggesting that the antinociceptive effect of this fraction is dependent on anti-inflammatory activity. The HF

Table III. Anti-inflammatory activity of crude ethanolic extract (CEE) of *Celtis iguanaea* leaves, ethyl acetate fraction (EAF) and dichloromethane fraction (DF) in croton oil-induced ear edema test in mice.

	Δ weight between the ears (mg)	
	(Mean \pm S.E.M)	% Inhibition
Vehicle 10 mL/Kg	17.8 \pm 0.8	-
CEE 300 mg/Kg	13.9 \pm 0.3**	22
EAF 16 mg/Kg	11.3 \pm 0.4***	37
DF 9 mg/Kg	14.3 \pm 0.4**	20
Dexamethasone 1 mg/Kg	4.0 \pm 1.3***	78

Values are expressed as mean \pm S.E.M of difference of weight between the ears, in milligrams. ** $P < 0.01$ and *** $P < 0.001$ compared to vehicle, according to ANOVA followed by Student-Newman-Keuls' test.

and AF were not able to significantly reduce the reactivity to pain time in any phase of the test.

Considering the effect of CEE and DF in the first phase of the formalin test, the next step of this study was to investigate the mechanism of action involved in the antinociceptive effect of the extract and this specific fraction. Therefore, the possible participation of vanilloid receptors, also known as transient receptor potential cation channel subfamily V member 1 (TRPV1), in the antinociceptive effect of CEE and DF was initially investigated. The intra-plantar administration of capsaicin, an active ingredient in hot chili peppers, which acts selectively as an agonist of TRPV1 receptors, activates neurons within the peripheral and central nervous systems inducing acute nociception (Sakurada et al. 1992, Szolcsányi 1993, Cui et al. 2006, Trevisan et al. 2012).

This preclinical model has been relevant for the discovery of new compounds capable of modulating the TRPV1 receptor or vanilloid system. The findings in the present study

suggests that antinociceptive effect of CEE and DF involves a block of vanilloid system based on their ability to inhibit nociceptive transmission in capsaicin-induced pain test.

The pre-treatment with naloxone (3 mg/Kg, s.c.), a non-selective opioid antagonist, did not reverse the antinociceptive effect of CEE (300 mg/Kg, p.o.) or DF (9 mg/Kg, p.o.) in the capsaicin-induced pain test, confirming that the antinociceptive effect of *C. iguanaea* involved the participation of vanilloid system. These results agree with several studies which show that vanilloid system blockers are important for the antinociceptive activity of different plants (Zakaria et al. 2016, Li et al. 2017, Prá et al. 2017) and analgesic drugs designed or extracted from plant extracts (e.g., eriodictyol and α -spinasterol) (Rossato et al. 2011, Trevisan et al. 2012).

Additionally, the involvement of TRPV receptors in the inflammatory process has been described in the literature. Once activated, TRPV1 receptors stimulate the release of pro-inflammatory cytokines (Veronesi et al. 1999, Zhang et al. 2007) which, together with other inflammatory mediators, sensitize TRPV1 receptors, contributing to hyperalgesia (Obreja et al. 2002, Tang et al. 2004).

Another important function of TRPV1 receptors is the stimulation of cell migration via the release of pro-inflammatory cytokines and increased calcium influx, which is important for the regulation of components of cell migration machinery (Pettit & Fay 1998, Waning et al. 2007). Therefore, a blockade of the vanilloid system may also be important for reducing inflammation.

Some studies have shown that other species in the Cannabaceae family such as *Celtis sinensis* and *Humulus lupulus* L. are known for the anti-inflammatory effect (Kim et al. 2005, Hougee et al. 2006, Hall et al. 2008, Akazawa et al. 2012). Therefore, another part of this study was directed to the evaluation of anti-inflammatory

Table IV. Anti-inflammatory activity of crude ethanolic extract (CEE) of *Celtis iguanaea* leaves, ethyl acetate fraction (EAF) and dichloromethane fraction (DF) in carrageenan-induced pleurisy test in mice.

	Carrageenan-induced pleurisy (Mean \pm S.E.M and % Inhibition)					
	Nº of leukocytes $\times 10^6$ /mL		MPO activity (mU/mL)		TNF- α (pg/mL)	
Vehicle 10 mL/Kg	4.9 \pm 0.4	-	212 \pm 39.2	-	58.4 \pm 3.6	-
CEE 300 mg/Kg	3.3 \pm 0.5**	33 %	154.3 \pm 18.7 *	27%	21.9 \pm 3.0***	62 %
EAF 16 mg/Kg	2.1 \pm 0.1***	57 %	75.6 \pm 3.2***	64 %	39.6 \pm 1.3***	32 %
DF 9 mg/ kg	3.2 \pm 0.7**	35 %	121 \pm 26.5**	43 %	50.4 \pm 4.2	-
Dexamethasone 1 mg/Kg	2.4 \pm 0.2***	51 %	59.2 \pm 33.7***	72 %	11 \pm 1.3***	81 %

Values are expressed as mean \pm S.E.M of number of migrated leukocytes $\times 10^6$ /mL, of the activity of MPO enzyme (mU/mL) and of the levels of TNF- α (pg/mL) in the pleural exudate after carrageenan injection. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to vehicle, according to ANOVA followed by Student-Newman-Keuls' test.

activity of CEE, DF and EAF, which showed effect in the second phase of the formalin test.

To evaluate the anti-inflammatory activity of CEE, DF, and EAF, croton oil-induced ear edema and carrageenan-induced pleurisy tests were used. The main irritant present in croton oil is 12-O-Tetradecanoylphorbol-13-acetate (Stanley et al. 1991), which induces an acute inflammatory process with consequent edema formation from the action of multiple inflammatory mediators, for example histamine, serotonin, and prostaglandins (Saraiva et al. 2011). The treatments with CEE (300 mg/Kg, p.o.), EAF (16 mg/Kg) or DF (9 mg/Kg) significantly reduced the edema formation, suggesting an anti-inflammatory effect.

Subsequently, carrageenan-induced pleurisy test was performed to confirm and characterize the anti-inflammatory effect of CEE, DF, and EAF. This experimental model is characterized by the accumulation of exudate and cell migration, as well as an increase of pro-inflammatory mediators in the pleural cavity (Saleh et al. 1999, Murai et al. 2003, Di Paula et al. 2004) permitting the evaluation and quantification of several inflammatory parameters such as cell migration,

MPO activity, and cytokines levels (Vinegar et al. 1973, Higgs et al. 1980, Mikami & Miyasaka 1983).

In this model, the treatment with CEE, EAF, or DF at the same doses tested in the ear edema, showed a reduction in the number of leukocytes migrated as well as in MPO activity. MPO is predominantly present in active neutrophils, therefore, the activity of this enzyme is directly proportional to the polymorphonuclear leukocytes concentration in inflamed tissue (Rosen & Michel 1997, Lanza 1998). Thereby, the reduction in MPO activity by treatment with CEE, EAF, or DF can be due to the reduction in the number of migrated neutrophils.

Furthermore, the treatment with CEE or EAF also reduced the levels of TNF- α , which is involved mainly in cellular chemotaxis and can induce the expression of cyclooxygenase 2, amplifying the formation of inflammatory mediators (Dinarello 2009). Therefore, the ability to reduce TNF- α levels by treatment with CEE or EAF may in part explain the anti-inflammatory effect observed throughout this study.

In contrast, the treatment with DF at dose of 9 mg/Kg was not able to reduce the levels of TNF- α . This result suggests that the mechanism of action by which DF promotes

an anti-inflammatory effect does not involve reduction of this cytokine. Furthermore, the anti-inflammatory effect of this fraction, at the dose used, has been less than the anti-inflammatory effect promoted by CEE or EAF, probably because the EAF fraction retained most of the metabolites responsible for the anti-inflammatory effect of CEE. However, it is noteworthy that DF showed improved results in the pain tests (acid-induced abdominal writhing and formalin-induced pain) as compared to EAF. These findings suggest that the DF fraction retained the main active metabolites responsible for the antinociceptive effect, irrespective of the anti-inflammatory effect.

Altogether, these findings make it possible to assert that fractions with average polarity i.e., DF and EAF, gather phytochemicals with prospects to be used in the treatment of painful conditions and inflammatory diseases. Typically, EAF and DF enable the recovery of low polarity free aglycones from herbal material i.e., flavones, flavonols, flavanones, di-hydroflavonols, isoflavones and other aglycones with great methylation degree (Stalikas 2007).

Over the last few years, there has been a significant advance towards the full characterization of the phytochemical profile of *C. iguanaea* extracts. By using HPLC-PDA, da Silva et al. (2016) found appreciable amounts of phenolic acids (i.e., gallic, chlorogenic and ellagic acids) and to a greater degree flavonoids (i.e., rutin and quercetin) in a crude extract obtained from the leaves of *Celtis iguanaea* (maceration with 70 % ethanol at 1:20 w/v, 5 days).

Moreover, Zanchet et al. (2018) used HPLC-ESI-IT-MSⁿ and ESI-MSⁿ analyses in the identification of phytochemicals in a dichloromethane extract from the leaves of *Celtis iguanaea* (maceration at 1:20 w/v, 5 days). Such a hyphenated technique enabled the identification of several flavonoids in the

sample, namely 2-O-pentosyl-8-C-hexosyl-apigenin, luteolin-4'-O-rhamnosyl (1→2) glycoside, orientin, genistin, rutin, vitexin, and tetrahydroxyisoflavone-O-hexoside. This research also led to the identification of orientin and (9S,10E,12Z,15Z)-9-hydroxy-10,12,15-octadecatrienoic acid in the hydroalcoholic extract of the leaves obtained with 70 % ethanol under the same maceration conditions.

Due to the similarities in the extraction procedures, it is reasonable to consider that some of these phytochemicals might also be identified in our samples and thus related to the pharmacological activities showcased. Further investigations will be performed aiming to identify the active metabolites present in *C. iguanaea* and the probable synergic or additive effect between them to characterize the mechanism(s) responsible for the antinociceptive and anti-inflammatory actions of these fractions.

CONCLUSIONS

We have provided convincing evidence of the antinociceptive and anti-inflammatory effects of CEE of *Celtis iguanaea* leaves and its average polarity active fractions. Our results showed that CEE and its DF reduced the pain in different experimental models and suggest that this antinociceptive effect involves the participation of vanilloid system. Furthermore, the CEE, DF, and EAF were able to reduce the edema, leukocytes migration, and the activity of myeloperoxidase. The TNF- α levels in pleural exudate were also reduced by CEE and EAF. Therefore, these results suggest that *Celtis iguanaea* may hold great promise for treating painful conditions and inflammatory diseases, supporting its popular use in the Brazilian folk medicine.

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Author contributions

Weuller F. Moraes, Daiany P. B. Silva and E. A. Costa (Head) conceived the study, designed pharmacological assays and drafted the manuscript. Daiany P. B. Silva, Iziara F. Florentino, Dionys de S. Almeida, Lorrane K. S. Moreira, Marcus V. M. Nascimento and Pablinny M. G. de Carvalho designed and carried out the pharmacological assays. Renê O. Couto and José R. de Paula (Head) conceived the study, performed the collection and identification of vegetable material and preparation of the extract and fractions. All authors critically revised the manuscript for important intellectual content and approved its final version.

