From popular use to pharmacological validation: A study of the anti-inflammatory, anti-nociceptive and healing effects of *Chenopodium ambrosioides* extract

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**A B S T R A C T**

*Ethno-pharmacological relevance:* *Chenopodium ambrosioides* (Amaranthaceae) is an annual or perennial plant popularly known as ‘erva de Santa Maria’, ‘mastruço’ and ‘erva-do-formigueiro’. This herb is used in folk medicine in the form of teas, poultices and infusions for inflammatory problems, contusions and lung infections, and as an anthelmintic and anti-fungal.

**Aim of the study:** The aim of the present study was to further the understanding of the anti-nociceptive, anti-inflammatory and wound healing effects of ethanol extract (EE) obtained from the leaves and stems of *Chenopodium ambrosioides* in animal models of acute pain, inflammation and wound healing, thus supporting its medicinal use for the treatment of pain and inflammatory conditions.

**Materials and methods:** The anti-nociceptive activity of EE (15–500 mg/kg) was evaluated using the nociception induced by formalin (2.5%), prostaglandin-E2 (PGE2; 3 nmol/paw), capsaicin (CAP, 1.6 mg/paw) and bradykinin (BK, 10 nmol/paw). The anti-inflammatory activity of EE (150–500 mg/kg) was evaluated in carrageenan- (Cg, 300 mg/paw), PGE2- (3 nmol/paw), substance P- (SP, 20 nmol/paw) and BK-(3 nmol/paw) induced paw oedema. The topical anti-inflammatory activity of EE (1%, 3% and 5%) was evaluated in arachidonic acid- (AA, 2 mg/ear), oil croton- (1 mg/ear) and CAP- (250 mg/ear) induced ear oedema. The effect of this extract in the inhibition of the influx of neutrophils, myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities and nitric oxide (NO) and TNF-α levels was also determined using the mouse of pleurisy induced by Cg. The excision wound model in rats was used to evaluate the wound healing efficacy of EE (1%, 3% and 5%). To exclude the possible non-specific muscle relaxant or sedative effects of EE, mice motor performance was also evaluated with the rota-rod test.

**Results:** EE (5% per ear) was effective in reducing ear oedema induced by croton oil by 78.09%, CAP by 70.85% and AA by 77.02%. EE (500 mg/kg; p.o.) also significantly inhibited paw oedema induced by Cg by 40%, PGE2 by 51%, SP by 56% and BK by 57%. EE (500 mg/kg; p.o.) inhibited the cell influx of leucocytes by 78% and neutrophils by 53%, MPO activity by 62.22% and ADA activity by 23.07%, as well as NO by 77.77% and TNF-α levels by 50% in the fluid leakage due to the carrageenan-induced pleurisy. EE also inhibited the formalin-induced nociceptive in both phases of pain (neurogenic and inflammatory) at a dose of 500 mg/kg, resulting in inhibitions of 77.39% and 95.60%, respectively. EE (500 mg/kg; p.o.) was also effective in inhibiting the nociception induced by PGE2 (68%), CAP (53%) and BK (32%). Topical application of EE (5%) on excision wounds caused a significant reduction in wound area when compared with the untreated controls. Finally, treatment with EE (150–500 mg/kg) did not show any significant alterations in motor performance or body temperature compared with the control group.

**Conclusions:** The results, including the inhibition of mediators (BK, NO, SP, PGE2 and TNF-α) and enzyme (MPO and ADA) activity, validate the use of the plant under study for therapeutic treatment of anti-inflammatory, painful and wound healing processes.

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1. Introduction

In Brazil, many medicinal plants provide relief comparable to that of conventional medicinal agents in the treatment of different diseases, and they contain new bioactive molecules. The biodiversity present in different regions of the country is a potential source of bioactive compounds (Kingston, 2011). Recently, the Brazilian government launched a programme known as the National List of Plants of Interest to the (RENISUS), which lists 71 plants used with emphasis on Brazilian folk medicine. The programme aims to support phytotherapy as an alternative treatment of diseases and also encourage Brazilian researchers to validate the pharmacological properties of these plants, including their efficacy and safety (Brasil, 2008). It is important to note that Chenopodium ambrosioides (L) (Amaranthaceae) belongs to this list.

Chenopodium ambrosioides (L) is an annual or perennial plant with a strong aromatic odour and can grow up to 1 m tall. It is native to Central and South America that adapts to diverse conditions and is considered invasive. In Brazil, this species has a wide distribution, occurring in almost every territory. It is popularly known as ‘erva do Santa Maria’, ‘mastrucho’ and ‘erva-dos-formigueiro’ (Lorenczi and Matos, 2002). In Brazilian folk medicine, the plant is crushed and served in the form of teas, infusions or poultices for several purposes: it acts as a diuretic and an anthelmintic, and it is used to treat wounds, respiratory problems, inflammatory and painful processes, bronchitis, tuberculosis and rheumatism (Kumar et al., 2007).

Phytochemical investigations revealed that the plant contains terpenes, sterols and phenols (Kokanova-Nedialkova et al., 2009). The monoterpene ascaridole is the main component of essential oil. This compound has sedative and anti-inflammatory effects (Olajide et al., 1997; Effert et al., 2002). Iboronke and Ajiboye (2007) demonstrated in a preliminary study that methanol extract of the leaves of Chenopodium ambrosioides (L) has anti-inflammatory and anti-ociceptive properties. Considering the importance of Chenopodium ambrosioides (L) to Brazilian folk medicine, the aim of this study was to investigate in more depth the anti-inflammatory, anti-nociceptive and healing properties of the ethanol extract (EE) of the leaves and stems of this specimen in animal models.

2. Materials and methods

2.1. Collection and authentication of plant material

The aerial parts (stems and leaves) of Chenopodium ambrosioides (L) were collected in March 2010 in Cáceres, a city in the state of Mato Grosso, Brazil. The plant was dried at room temperature and protected from light and sun. Taxonomic identification of the collected material was done by comparison with the proved samples that was analysed by botanist M.Sc. Oscar Benignolza and deposited in the Herbarium Barbosa Rodrigues (HBR), Itajai, SC (number 52802).

2.2. Preparation of extract and purification of active compounds

The dried vegetal material (leaves, 2.22 kg and stems, 1.24 kg) of Chenopodium ambrosioides (L) was crushed and macerated with 100% ethanol (12 L) at room temperature for 7 days. Solvent removal was carried out under reduced pressure at temperatures below 45 °C until the desired concentrations were achieved with dried leaves (48.44 g) and stems (24.42 g). The extracts were combined due to similar chromatographic profiles evaluated through thin layer chromatography (TLC).

A portion of the ethanol extract (EE) of the leaves and stems (9.37 g) was subjected to column chromatography (CC) using silica gel 60 as the stationary phase and eluted with hexane (Hex) and increasing the polarity with ethyl acetate (EtOAc) and ending with methanol. In total, 122 fractions (20 ml each) were collected and analysed by TLC. The fraction eluted with Hex:EtOAc (95:5) designate 1 was submitted to ¹H and ¹³C nuclear magnetic resonance (NMR) analysis.

The fraction 131–154 (811.67 mg) eluted with Hex: EtOAc (60:40) was subjected to column chromatography packed with silica gel. Fraction 15 eluted with Hex:EtOAc 70:30 designate 2 as submitted to NMR analysis.

2.3. Animals

Experiments were carried out using male Swiss mice (20–30 g), with an exception for wound-healing experiments in which Wistar rats were used (200–250 g). The animals were kept at controlled room temperature (22 ± 3 °C) under a 12 h:12 h light-dark cycle (lights on at 0600 h), with access to water and food ad libitum. The animals were acclimatised to the laboratory 1 h before testing and were used once throughout the experiments. All procedures used in the present study were approved by the Institutional Ethics Committee of UNIVALI (037/2009CEP/UNIVALI) and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental inflammation and pain in conscious animals (Zimmermann, 1983). The number of animals (N=6–8) and the intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the herb treatment. The doses used in the study were lower than those that produce toxic effects.

2.4. Drugs and reagents

The following substances were used: croton oil, arachidonic acid (AA), capsaicin (CAP), bradykinin (BK), prostaglandin (PGE₂), grade IV lambda carrageenan (Cg), substance P (SP), captopril, indomethacin, myeloperoxidase, sodium azide, adenosine phosphate-buffered solution, adenosine, phenol, sodium nitroprussiate, the alkaline buffer NaOCl, naphthyl ethylenediamide dihydrochloride, sulphamidamide, phosphoric acid, vanadium chloride and dexamethasone; all substances were obtained from Sigma Chemical Co. (St Louis, MO, USA). Formaldehyde was obtained from Chemical Ind. e Com. (SP, Brazil), Heparin™ (Roche, Brazil), Evans blue and Türk solution (Merck, Rio de Janeiro, Brazil), May–Grunwald (Newprov, Pinhais PR, Brazil), and Giemsa (Laborclín, Pinhais, PR, Brazil) were also used. The enzyme-linked immunosorbent assay (ELISA) for quantitative determination of mouse TNF-α was from BD Biosciences Pharmingen (San Diego, CA, USA). The drugs and extracts were dissolved in saline (vehicle), except capsaicin, which was dissolved in absolute ethanol. All doses and routes of administration of the Chenopodium ambrosioides (L) materials were chosen in accordance with previously established protocol based on our pilot experiments (unpublished data).

2.5. Determination of anti-inflammatory effect of Chenopodium ambrosioides (L)

2.5.1. Effect of Chenopodium ambrosioides (L) in ear oedema induced by different phlogistic agents

The oedema was expressed as an increase in ear thickness due to inflammation. Ear thickness was measured before and after induction of the inflammatory response using a micrometre (Mitutoyo Series 293). The micrometre was applied near the tip...
of the ear just distal to the cartilaginous ridges, and the thickness was recorded in millimetre. To minimise variation in technique, a single investigator performed the measurements throughout each experiment (Zanini et al., 1992).

The animals received the cream with EE of the leaves and stems of *Chenopodium ambrosioides* (L) at different concentrations (1%, 3% and 5%) in the inner surface of the right ear. Another group of animals received dexamethasone cream (1%, positive control), and another received only the base (cream without active compounds, negative control). After 30 min of treatment, each animal received 20 μl croton oil (1 μg/ear), capsaicin (250 μg/ear) and arachidonic acid (AA, 2 mg/ear) on the outside face of the right ear. The ear oedema was assessed 30 min, 60 min and 6 h after application of capsaicin, arachidonic acid or croton oil, respectively. The difference between the ear thickness from the baseline (before application of the phlogistic agents) and after induction of oedema was expressed in millimetre and taken as an indication of oedema.

2.5.2. Effect of *Chenopodium ambrosioides* (L) on paw oedema induced by different phlogistic agents

The paw oedema experiments were conducted in accordance with Busnardo et al. (2010) with some modifications. Oedema was induced in the right hind paw with a 20-μl intraplantar (i.pl.) injection of Cg (300 μg/paw), SP (20 nmol/paw), PGE2 (3 nmol/paw), or BK (3 nmol/paw). The contra-lateral paw (left paw) received the same volume of saline and was used as the negative control. In this experiment with bradykinin, the animals were pretreated with captopril (5 mg/kg s.c.) 1 h prior to BK induction to prevent BK degradation. Depending on the phlogistic agent used in the experiment, the measurement of oedema was made with a plethysmometer (UgoBasile, Italy) at different time points after its injection. Oedema was expressed as the difference between the right and left paws.

The paw oedema was assessed in mice previously treated by oral gavage (p.o.) with EE (150, 300 and 500 mg/kg) or indo-methacin (10 mg/kg, p.o.), 1 h before injection of the phlogistic agent.

2.5.3. Pleurisy

Pleurisy was conducted in mice previously treated by oral gavage (p.o.) with EE (150, 300 and 500 mg/kg) or indomethacin (10 mg/kg, p.o.), 1 h before carrageenan injection.

The animals were lightly anaesthetised with ethyl ether and after mild anaesthesia, Cg (300 μg/cavity) was injected into the right pleural cavity. The animals were sacrificed 4 h after the Cg injection. Immediately, the thorax was opened, the pleural cavity was washed with 1 ml saline (NaCl 0.9%) plus heparin (20 IU/ml), and the exudate was collected (Busnardo et al., 2010). Total cell counts were performed in Neubauer chambers by means of an optical microscope (magnification 40×) after diluting a sample of the collected fluid from the pleural space with Türk solution (1:40). Cellular smears were prepared with an aliquot of pleural lavage to determine the differential leucocyte count. The slides were stained with May-Grünwald-Giemsa dye, and the analysis was carried out under oil immersion objective.

Another sample of the pleural cavity fluid was used to evaluate the myeloperoxidase and adenosine desaminase activity and the NO and TNF-α levels.

2.5.4. Quantification of MPO and determination of ADA activities in Cg-induced pleurisy

MPO and ADA were performed in accordance with the methods described by Dalmarco et al. (2002). Pleural cavity fluid samples and standards were transferred to cuvettes, and the reaction was initiated with the addition of 360 μl of assay buffer, the reaction was stopped with 1% sodium azide. Afterwards, the samples were centrifuged at 50 × g for 5 min at room temperature (22–25 °C). The supernatants were separated, and the concentration of the enzyme was estimated by colorimetric measurement (absorbance at 450 nm) in an ELISA plate reader (Organon Technical, Roseland, NJ, USA). The results were expressed as mU/ml.

The test of ADA activity pleural cavity fluid samples were transferred to cuvettes, and the reaction was initiated with the addition of adenosine phosphate-buffered solution. After incubation for 1 h at 37 °C, the reaction was halted with the addition of a solution of phenol, nitroprusside and alkaline buffer. This solution was also added to the cuvettes with the different standard samples. The concentration of the enzyme was estimated by colorimetric measurements (absorbance, 620 nm) in an ELISA plate reader (Organon Technical, Roseland, NJ, USA). The results were expressed as U/L, and the experiments were done in duplicate.

2.5.5. Quantification of TNF-α and nitric oxide levels in carrageenan-induced pleurisy

The measurements of levels of TNF-α were evaluated using the method previously described by Dalmacco et al. (2002). After the 4-h pleurisy procedure, the animals were sacrificed, and the exudate samples were collected and stored at −70 °C. The pleural fluid recovered was centrifuged at 770 × g for 10 min (room temperature), and the cell-free supernatant was quantified for TNF-α contents by ELISA (NuncMaxsorp). Quantification was performed using a microplate reader at absorbance of 490 nm.

NO was measured as its breakdown products nitrite (NO2−) and nitrate (NO3−) using the Griess method. Pleural cavity fluid samples (0.1 mL) were transferred to cuvettes, and vanadium chloride 0.05 M (0.15 mL) in HCl 1.0 M (0.05 mL) was added to reduce nitrate to nitrite. Immediately, Griess reagent [150 μL of naphthyl ethylenediamide dihydrochloride (0.004 M) in H2O and sulphanilamide 0.06 M (150 μL) in H2PO4 0.03 M, vol. 1:1] was added. After incubation at 37 °C for 45 min, the reaction was transferred to a microplate. Nitrite concentrations were estimated by interpolation from a standard curve of sodium nitrite (0–150 IM) by colorimetric measurements at 540 nm in an ELISA plate reader (OrganonTecknika, Roseland, NJ, USA). Results were expressed as IM and the experiments were done in duplicate.

2.6. Determination of anti-nociceptive effect of *Chenopodium ambrosioides* (L)

2.6.1. Algogen-induced nociception in mice

The procedure for producing phlogogen-induced nociception in mice was similar to that described previously (Mattos et al., 2006). Twenty microlitres of PGE2 (3 nmol/paw), formalin (FORM, 2.5%), BK (10 nmol/paw), or CAP (1 nmol/paw), diluted in PBS solution, were injected intra-plantarly (i.pl.) under the ventral surface of the right hindpaw. The animals were treated with EE (150, 300 and 500 mg/kg, p.o.), or 0.9% saline solution (negative control, p.o.) 1 h before injection of phlogistic substances. A mirror was placed behind the chamber to enable unhindered observation of the phlogogen-injected paw. The animals were placed individually in transparent glass cylinders 20 cm in diameter, serving as observation chambers. They were observed individually for 15 min after PGE2 injection, 10 min after CAP and BK injection. The amount of time spent licking the injected paw, timed with a chronometer, was considered to be indicative of nociception. In the experiments with FORM, two distinct phases of intensive licking or biting activity were identified: an
early phase, neurogenic pain and a late phase, inflammatory pain (0–5 and 15–30 min after FORM injection, respectively).

2.7. Determination of wound healing effect of Chenopodium ambrosioides (L)

2.7.1. Induction of ulcerative process (skin wound healing)

The methodology adopted was based on Cross et al. (1996) with several modifications. On the day surgery, the rats were anaesthetised with a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) intra-peritoneally (i.p.). After confirmation of deep anaesthesia, the hair on the back of the animal was manually removed using a razor. The wound area was demarcated by a square (20 x 20 mm²) on the distal portion of the back of the animal.

Immediately after the complete withdrawal of the skin, the animals were placed in the ‘squat’ (‘crouching’) position, the perimeter of the wound was cleaned, and approximately 0.7 g of ointment base crude EE of Chenopodium ambrosioides (L) (PEG-EE) at concentrations of 1%, 3% and 5% was applied topically to one group. The ointment base was applied to one group, and one group (negative control) received no treatment. The animals were treated twice daily for 19 days, the wound area was measured from days 1–19 before each treatment until the wound healed completely in the negative control. Each wound was measured in triplicate using a transparent sheet, for which the extension of the wound and an average was calculated for each measurement. The drawings of the measures were scanned to a computer using a flatbed scanner. After capturing of the images, the area in mm² wounds was calculated with the help of the computer programme Image J® 1.3.1 (NIH, EUA) software.

2.8. Motor function assay: rota-rod

To evaluate the possible non-specific muscle-relaxant or sedative effects of EE, mice were tested in the rota-rod test, which was used to measure motor performance (Duham and Miya, 1957). The apparatus (Rota Rod® RS, LE-8500, series 1218/06, Leticia Scientific Instruments) consisted of a bar with a diameter of 2.5 cm, sub-divided into six compartments by disks, 25 cm in diameter. The bar rotated at a constant speed of 15 rpm. The mice were previously selected, eliminating those mice that did not remain on the bar for two consecutive periods of 60 s. On the day of the experiment, animals were treated with the EE (150, 300 and 500 mg/kg, p.o.) and vehicle (saline), 1 h before undergoing the test. The animals were assessed at the time 30, 60, 120 and 180 min after the treatment. The results were expressed as time (s) in which the animals remained on the rota-rod apparatus. The maximum time used was 60 s.

2.9. Body temperature measurement

Body temperature was measured using a commercially available digital thermometer (B & D, NJ, USA), which was gently inserted (0.5 cm) into the mouse rectum. Temperature measurements were performed in a temperature-controlled room (22 ± 2 °C), between 1500 and 1700 h. Temperatures were recorded before any treatment and 30, 60, 120 and 180 min after the treatment with EE of Chenopodium ambrosioides (L) (150, 300 and 500 mg/kg, p.o.) or vehicle (saline). This test was performed according to Ansonoff et al. (2006).

2.10. Statistical analysis

Statistical analyses of results were performed by middle analysis of variance (ANOVA) of the one-and two-way test for multiple comparisons, using the Dunnet, Newman-Keuls, Tukey and Bonferroni methods when appropriate. The results are presented as mean ± standard error of mean, except DI₅₀ (corresponds to doses of extracts that reduced the response to 50% in relation to the control group), which were presented with geometric means accompanied by their respective confidence limits (95%). The percentages of inhibition were cited as mean ± standard error of the mean difference (in %) between the areas on the curves obtained for each individual experiment relative to the corresponding control group. p-Values less than 0.05 (p < 0.05) were considered indicative of significance. All the analyses mentioned above were performed using the GraphPadInstat® or GraphPad PRISM® (GraphPad Software, San Diego, CA).

3. Results

3.1. Phytochemical analysis

The phytochemical analyses with EE of leaves and stems of Chenopodium ambrosioides (L) allowed the isolation of monoterpene ascaridole (1) (yield of 369 mg, 0.089% in relation to dry plant) and 1,2,3,4-tetrahydroxy-p-menthane (2) (yield of 188 mg, 0.045% in relation to dry plant) (Fig. 1). Other two monoterpene were detected but have not been identified yet. The compounds were identified by comparison of 1H and 13C NMR data with previously published data from Cavalli et al. (2004).

3.2. Determination of anti-inflammatory effect of Chenopodium ambrosioides (L)

3.2.1. Effect of Chenopodium ambrosioides (L) in ear oedema induced by different phlogistic agents

In the ear oedema assay, it was observed that treatment with cream containing the EE significantly (F(4,25)=104.6, p < 0.01) inhibited the ear oedema induced by croton oil in all concentrations when compared with control (base polawax; Fig. 2A). The EE extract and the positive control, dexamethasone, also inhibited the capsaicin-induced oedema (F(4,25)=106.6, p < 0.01; Fig. 2B). The same pharmacological profile was observed in ear oedema induced by AA (Fig. 2C). The treatment of animals with cream containing EE of plant (1%, 3% and 5%) and dexamethasone produced significant decrease of ear oedema (F(4,25)=272.4, p < 0.01).

3.2.2. Effects of Chenopodium ambrosioides (L) on paw oedema induced by different phlogistic agents

Fig. 3 shows the anti-oedematogenic activity of Chenopodium ambrosioides (L). The treatment with EE (150–500 mg/kg) significantly inhibited the paw oedema induced by carrageenan in a dose-dependent manner (F(4,24)=9.5, p < 0.01). This effect is prolonged by 48 h, indicating a long-lasting effect (Fig. 3A1 and A2). In addition, this extract at all doses was also able to inhibit the oedema induced by PGE₂ (Fig. 3B1 and B2) (F(4,12)=10.9, p < 0.01); SP (F(4,20)=4.0, p < 0.01) (Fig. 3C1 and C2), and BK

![Fig. 1. Structure of monoterpenes ascaridole (1) and 1,2,3,4-tetrahydroxy-p-menthane (2).](image-url)
3.2.3. Carrageenan-induced pleurisy

3.2.3.1. Effects of Chenopodium ambrosioides (L) on cellular infiltration. The treatment with EE (150–500 mg/kg) caused a significant decrease in leucocyte influx (Fig. 4A) in a dose-dependent manner ($F_{(5,36)}=643.8$, $p < 0.01$) when administered 1 h before carrageenan. This reduction was attributed to inhibition of mononuclear cells (Fig. 4B) of a dose-dependent manner ($F_{(5,36)}=9.8$, $p < 0.01$). The treatments also reduced the influx of neutrophils in a dose-dependent manner ($F_{(5,36)}=102.9$, $p < 0.01$) (Fig. 4C). The animals treated with indomethacin (10 mg/kg) significantly decreased leucocytes, monocytes and neutrophils ($p < 0.01$) (Fig. 4A–C).

3.2.4. Effects of Chenopodium ambrosioides (L) on myeloperoxidase, adenosine-deaminase activities and the levels of NO and TNF-α

Fig. 5A shows that the treatment with oral EE 1 h before and 4 h after carrageenan injection statistically inhibited the activity of myeloperoxidase (MPO) ($F_{(5,18)}=28.3$, $p < 0.001$) in the doses of 300 and 500 mg/kg. A similar effect was also observed, in which ADA activity decreased at the dose of 500 mg/kg of EE ($F_{(5,18)}=35.5$, $p < 0.05$; Fig. 5B). Fig. 5C shows that EE (300 and 500 mg/kg) statistically promoted ($F_{(5,24)}=47.3$, $p < 0.001$) reduction in NO levels, and Fig. 5D demonstrates that the extract significantly inhibited ($F_{(5,18)}=45.4$, $p < 0.001$) the production of TNF-α in pleural fluid after carrageenan injection at the doses of 300 and 500 mg/kg. The animals treated with indomethacin were inhibited in MPO, ADA, NO and TNF-α, ($p < 0.001$) activities.

3.3. Determination of anti-nociceptive effect of Chenopodium ambrosioides (L)

3.3.1. Algogen-induced nociception in mice

The results demonstrate that the EE caused a pronounced anti-nociceptive effect (Figs. 6 and 7). The pre-treatment with the extract was able to significantly reduce both phases of nociception (neurogenic and inflammatory) induced by FORM in all doses used. In the first phase of nociception, the calculated ID$_{50}$ values were 284.1 (184.4–437.8) mg/kg. In the second phase of the nociception, EE and indomethacin produced inhibition of process pain induced by formalin (Fig. 6A and B). In this experiment, also observed the anti-oedematogenic effect of EE (Fig. 6C). The results also showed significant anti-nociceptive effects in the pain model induced by PGE$_2$, ($F_{(3,20)}=18.5$, $p < 0.01$). The calculated ID$_{50}$ as of 298.1 mg/kg (260.5–341.0 mg/kg) (Fig. 7A). We also observed a significant inhibition of the nociceptive response induced by CAP in the treatment at doses of 300 and 500 mg/kg (Fig. 7B), and ID$_{50}$ of 470.3 mg/kg (335.8–658.7 mg/kg). However, in nociception induced by BK, pre-treatment with EE inhibited the nociceptive response ($F_{(3,20)}=3.6$, $p < 0.05$) with only the highest dose tested (500 mg/kg).

3.4. Effects of Chenopodium ambrosioides (L) in the cutaneous wound healing

The macroscopic analysis of skin wounds in animals treated with EE showed the evolution of tissue repair and the absence of bleeding and purulent secretions on different days of evaluation, when compared to other groups. In the analysis of lesion size to evaluate wound healing and repair, a significant difference was observed between the groups treated with PEG ($p < 0.05$) and PEG+EE ($p < 0.05$) in relation to the control (untreated group) on days 7, 14 and 19 after injury induction, with recovery to the injured area (Table 1 and Fig. 8).
3.5. Additional evaluations

The treatment of animals with EE of *Chenopodium ambrosioides* (L) (150–500 mg/kg) did not show any significant alterations in motor performance or body temperature compared to the control group (p > 0.05) (results not shown).

4. Discussion

The search for new drugs that effectively interfere with inflammation and painful processes is currently of great relevance. Efforts have been made to discover and develop new and promising anti-inflammatory and analgesics from natural sources.
Among the plants widely used by the Brazilian population and in several regions of Central and South America for therapeutic purposes includes *Chenopodium ambrosioides* (L.). This plant is used in folk medicine as a topical anti-inflammatory (Lorenzi and Matos, 2002).

In the present study, ear oedema was induced by croton oil, AA and CAP. Ear oedema induced by croton oil is used to evaluate the anti-inflammatory activity of compounds that act on the acute phase of inflammation. This model allows inhibitors of the biosynthesis of prostaglandins and leukotrienes, acting on key enzymes in the cascade of biosynthesis of these mediators, cyclooxygenase(COX) and lipoxygenase(LOX) (Terraciano et al., 2006). The croton oil contains the compound 12-O-tetradecanoylphorbol-13-acetate, known as TPA, which induces an acute inflammatory response, characterised by vasodilatation and oedema formation. This response occurs in the first two hours, followed by increased thickness of the ear. The result is infiltration of polymorphonuclear leucocytes to the tissue that peaks in the sixth hour, and from then on tends to decrease, reaching basal values after 24 h of induction (Andújar et al., 2010). AA, when applied topically, produces an immediate increase in vascular permeability, resulting in oedema, which is associated with a marked increase in levels of prostaglandins (PGE$_2$, PGF$_2\alpha$), TXA$_2$, LTB$_4$, and LTC$_4$ (Nonato et al., 2011). CAP is an alkaloid found in the species *Capsicum annuum* that produces an immediate neurogenic inflammatory response characterised by vasodilation, increased blood flow and oedema formation by plasma leakage, in addition to pain (Kalil-Gaspar, 2003). The results of this study indicate that EE produced a pronounced inhibition of the ear oedema, similar to dexamethasone.

Phytochemical investigations on the species of the *Chenopodium* genus revealed a wide variety of terpenes, steroids, saponins and flavonoids (Kokanova-Nedialkova et al., 2009). *Chenopodium ambrosioides* (L.) has high levels of monoterpens, mainly in its essential oil (Monzote et al., 2009). In the analysed extract the monoterpens ascaridole and 1,2,3,4-tetrahydroxy-p-menthane were
isolated, confirming previously reported results (Cavalli et al., 2004). Ascaridole is the most abundant substance in the Chenopodium genus (Dembitsky et al., 2008). It was obtained as a major component of the essential oil of Chenopodium ambrosioides (L) (Cavalli et al., 2004). Ascaridole shows analgesic, sedative and anti-fungal effects (Olajide et al., 1997). The 1,2,3,4-tetrahydroxy-p-menthane has only been isolated from the extract from the leaves of Chenopodium ambrosioides (L) in Egypt and it was detected in the essential oil of this species (Cavalli et al., 2004). No records of activity were found for this compound. This data suggests that these compounds or other phytochemicals present in this extract may inhibit the formation of inflammation process mediators, and this effect is independent of the phlogistic agent used.

EE can produce its anti-oedematogenic effect by inhibiting the synthesis of eicosanoids and cytokines, in which PGE$_2$ and TNF-$\alpha$ would be the main oedematogenic mediators (Saraiva et al., 2011). These anti-inflammatory effects with cream containing the extract validate in part its popular use and demonstrate its efficacy when it is used topically.

Another model used to evaluate the anti-inflammatory activity of EE was the model of paw oedema induced by different phlogistic agents. Paw oedema induced by carrageenan in mice involves a biphasic inflammatory response. The first phase (0–1 h) is associated with the release of various mediators such the release of histamine, serotonin, SP and cytokines, as well as TNF-$\alpha$, IL-1$\beta$, IL-2 and IFN-$\gamma$ with the release of vascular permeability (Déciga-Campos et al., 2007). The second phase (1–6 h) is related to the elevated production of prostaglandins derived from release of free radicals and the induction of cyclooxygenase (Panthong et al., 2004). In this study, the results show that EE of Chenopodium ambrosioides (L) effectively inhibited the oedema induced by carrageenan at all doses (150–500 mg/kg, p.o.), corroborating the results obtained by Ibironke and Ajiboye (2007), who reported that treatment with the methanol extract of leaves of Chenopodium ambrosioides (L) inhibits the paw oedema induced by carrageenan. It can be inferred that the anti-inflammatory action of the extract may be due to inhibition of mediators involved in events such as histamine, BK, SP and prostaglandins. To investigate this hypothesis, we evaluated the effect of EE on the model of paw oedema induced by mediators released during the inflammation process induced by Cg.

SP, a neuropeptide, is involved in inflammatory processes and also in the regulation of pain threshold (Smith et al., 2010). During inflammation, the SP induces the release of mediators, cytokines, AA derivatives, histamine and oxygen radicals, which potentiate tissue damage and stimulate the recruitment of leucocytes, amplifying the inflammatory response (Bhatia, 2010). Another inflammatory mediator used was BK, which is one of the main representatives of the class of cytokine. BK plays an important role in inflammation, promoting events such as vasodilatation, increased vascular permeability and increased production of eicosanoids, and in pain, it promotes the sustainment of the painful stimulus (Ferreira et al., 2000).

The present study also investigated the effect of EE on the paw oedema induced by PGE$_2$. PGE$_2$ is synthesised in substantial amounts at the sites of inflammation, and it acts as a potent vasodilator, inducing the production of various chemo-attractants, such as leucocytes and pro-inflammatory cytokines including IL-1 and TNF-$\alpha$ (Kaur et al. 2004). This mediator also participates in acute pain, sensitising the nerve endings of nociceptive fibres A$\delta$ and C through its receptor EP1 (Rady et al., 2001). It can be observed in our study that treatment with
EE also reduced the paw oedema induced by SP, BK and PGE2, which indicates a broad spectrum of inhibition of various mediators involved in inflammation.

To obtain new results on the anti-inflammatory effect of *Chenopodium ambrosioides* (L) another classic model of inflammation was used: carrageenan-induced pleurisy. The mouse model of pleurisy involves the participation of several cell types.
Saleh et al. (1999) and mediators (Moore, 2003). Myeloperoxidase, adenosine-deaminase and products of pathways that are stimulated by nitric oxide synthase (iNOS) have been implicated in several aspects of the inflammatory cascade including plasma exudation and cell migration. In this context, Saleh et al. (1996) previously demonstrated in this same model of inflammation that MPO activity and nitrate/nitrite (NO) levels significantly increase 4 h after carrageenan-induction of inflammation in the mouse model of pleurisy. In our study, EE was effective in producing significant inhibition of the inflammatory response with respect to leucocyte migration in relation to neutrophils and mononuclear cells to inflammatory site.

ADA is essential for the proliferation and differentiation of lymphoid cells, particularly T cells and the maturation of monocytes, thus possessing an important function in the immune system in inflammatory processes (Antonioli et al., 2008). The results showed that EE was effective in producing significant inhibition of the inflammatory response with respect to leucocyte migration in relation to neutrophils and mononuclear cells to inflammatory site.

Nitric oxide is another important pro-inflammatory mediator involved in the exudation and leucocyte migration in inflammatory process (Abramson, 2008). The results show that EE significantly inhibited NO levels. This effect suggests that the anti-inflammatory effect of this plant may also be associated with the inhibition of reactive oxygen species including the superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (OH$^-$). This study also tested the effect of the extract on the cytokine TNF-α. TNF-α is a key cytokine in the development of pleural inflammation, which increases the production of chemokines by endothelial cells (Cailhier et al., 2006).

In summary, our results demonstrate that EE exhibits anti-inflammatory effects in important and different animal models of inflammation, and its effects extend to both vascular and cellular events of the inflammatory process.

Pain is also one of the signs of the inflammatory process; we assessed whether EE showed anti-nociceptive effects. Since EE was effective in inflammation induced by mediators such as BK and PGE$_2$, which also participate in the pain process, we assessed whether the treatment of animals with EE could inhibit the pain process induced by PGE$_2$ and BK and also by CAP and FORM. The results demonstrate that EE was statistically able to inhibit the pain induced by PGE$_2$ suggesting their participation in acute pain. In the model of nociception induced by BK, it was observed that only the dose of 500 mg/kg decreased pain threshold (Mattos et al., 2006). The anti-nociceptive effect of the extract was also observed in the model of pain induced by CAP. It is inferred that

| Table 1 | Cicatrization speed of the animals subjected to dorsal skin lesion in the days (1, 7, 14 and 19) after daily treatment with ointment 5% of EEB. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Time (days) (mm/d) | 1 | 7 | 14 | 19 |
| Control | 3108.40 ± 504.20 | 2054.20 ± 1180.01 | 933.00 ± 87.13 | 97.80 ± 4.36 |
| PEG | 2757.25 ± 825.74 | 1857.50 ± 1042.54 | 794.75 ± 211.96 | 61.75 ± 3.06 |
| PEG + EEB 5% | 2260.40 ± 720.12 | 1609.20 ± 182.69 | 611.00 ± 55.11 | 27.60 ± 4.17 |

Control—animals no treatment, PEG—ointment base, PEG + EEB—ointment with EEB of Chenopodium ambrosioides (5%). Asterisks denote statistical significance (*p < 0.05, **p < 0.01) compared with control. ANOVA, followed the Dunnett test.

Fig. 8. Macroscopic analysis of skin lesions in the animal, 1, 7, 14 and 19 days with daily treatment with PEG base and EEB ointment to 5% from the injury. Male Wistar rats (n = 5/group) underwent single lesion incisional of 20 mm.
CAP-induced pain is very similar to the first phase of the FORM model (Santos and Calixto, 1997), suggesting the possible action by the extract in pain of neurogenic origin.

The formalin-induced pain model causes nociceptor stimulation, resulting in typical animal behaviours such as licking and/or biting the paw injected with this agent. This model evaluates two distinct phase of nociception, the first of neurogenic origin (occurs chemical stimulation of nociceptors of sensory afferent fibres mainly type C) (Hunskaar and Hole, 1987) and the second of inflammatory origin [production of multiple events mediated by the extract in pain of neurogenic origin (Hunskaar and Hole, 1987)]. Our results show that the EE of Chenopodium ambrosioides (L) was able to significantly inhibit both phases (neurogenic and inflammatory) in the formalin test, in addition to inhibiting oedema. The reference drug, a COX inhibitor used for comparison, was effective only in the second phase. It has been documented that non-steroidal anti-inflammatory and analgesic medications are effective in the second phase of the formalin test, which is related to the inhibition of prostaglandin synthesis in the modulation of pain (Hunskaar and Hole, 1987). The plant under study is used in folk medicine as a healing agent. We attempted to analyse this effect using a wound healing method. This method is reliable and low cost (Martins et al., 2006). During the experiments, no signs of infection were observed in the group treated with the Chenopodium ambrosioides (L) ointment; this can be explained by its anti-microbial and anti-inflammatory properties, confirmed by this study and previous studies with this plant (Delespaul et al., 2000; Ibranone and Ajiboye, 2007), inhibiting both the inflammatory and infectious process of the event. The treatment with EE on the injured tissue produced an evolutionary tissue repair when compared to the control group (untreated). It is believed, based on previous data, that the injury was quickly filled with clots and fibrin, producing the formation of crust isolating tissue from the external environment (Faleiro et al., 2009), but more advanced studies are needed to verify such data. The results showed that treatment of animals with EE 5% ointment accelerated the process of repair and healing when compared to the control group.

In the present study, two additional pharmacological models were used to investigate possible adverse effects of EE that could interfere with the observed results or derail the use of the plant as an alternative therapy. The first model was the rota-rod test and was used to exclude the possibility that EE caused motor relaxation and sedation (Sulaiman et al., 2010). EE, in all doses used, did not induce any interference in the motor performance of the animals. This model used was the test of body temperature. It has been reported that analgesic substances can induce hypothermia (Alfonsi et al., 2004). In animals treated with EE, no changes in body temperature were observed. The literature on Chenopodium ambrosioides (L) reports that the essential oil of the plant exhibits toxicological effects at high doses and prolonged use (Montoya-Cabrera et al., 1996). However, reports in the literature are contradictory. In Brazil, the plant is used in food for the treatment of helminthic infestations, but no toxic effects have been associated with its use in this way (Kliks, 1985). In this study, the oral doses used were lower than those used in toxicological testing by Pereira et al. (2010). In addition, during the execution of the experiments, no behavioural or physiological changes were observed that may have indicate designs of intoxication.

5. Conclusions

The results of this study confirm and extend previous studies on the anti-inflammatory and anti-nociceptive effects of Chenopodium ambrosioides (L), identifying the mechanism of action as the inhibition of mediators (BK, NO, SP, PGE2 and TNF-α) and enzymes (MPO and ADA) involved in the inflammatory and pain processes. Together, the results partially validate the popular use of this plant for therapeutic use of inflammatory, pain and wound healing processes.

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