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Antinociceptive effect of extract of *Emilia sonchifolia* in mice

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ABSTRACT

Aim of the study: Emilia sonchifolia (L.) DC. (Asteraceae) is a medicinal plant traditionally used in Brazilian folk medicine to treat asthma, fever, cuts, wounds and rheumatism. This study was conducted to establish the antinociceptive properties of hydroethanolic extract from aerial parts of *Emilia sonchifolia* in mice using chemical and thermal models of nociception.

Materials and methods: To evaluate the antinociceptive effect of *Emilia sonchifolia* hydroethanolic extract (EsHE) administered by oral route, peripheral (acetic acid-induced abdominal writhing and formalin), spinal (tail flick) and supra-spinal (hot plate) behavioral models of acute pain were used. High-performance liquid chromatography (HPLC) was used to determine the fingerprint chromatogram of the EsHE.

Results: The EsHE at test doses of 100 and 300 mg/kg, p.o. clearly demonstrated antinociceptive activity in all tests. The extract had a stronger antinociceptive effect than morphine. Administration of the opioid receptor antagonist, naloxone, completely inhibited the antinociceptive effect induced by EsHE (100 mg/kg). The presence of phenolic compounds in the extract of *Emilia sonchifolia* was confirmed using HPLC.

Conclusion: The extract of *Emilia sonchifolia* markedly exhibits opioid-mediated anti-nociceptive activity action in mice. Thus, may be useful in the treatment of inflammatory hyperalgesic disorders, which supports previous claims of its traditional use.

1. Introduction

Emilia sonchifolia is a traditionally used medicinal plant found in most tropical and subtropical regions worldwide. Various parts of the plant are used for the treatment of diseases (Lara et al., 2003; Matos et al., 1991; Singh et al., 2008). This weed is used in ethnomedicine against inflammation, eye sores, convulsion, cuts, wounds, rheumatism and insect bites (Agra et al., 2007; Matos et al., 1991; Muko and Ohiri, 1999; Perry, 1982; Shylesh and Padikkala, 1999; Shylesh et al., 2005). In Brazilian traditional medicine, aerial parts of *Emilia sonchifolia*, popularly known as "serralhinha" or "falsa-serralha" (Agra et al., 2007; Neto and de Morais, 2003).

The methanolic extract of *Emilia sonchifolia* has been reported to have anti-tumor property (Shhylesh and Padikkala, 2000; Shylesh et al., 2005), anti-inflammatory (Muko and Ohiri, 1999) and antioxidant activity (Gayathri Devi et al., 2006; Kawaree and Chowwanapoonpoh, 2009; Shylesh and Padikkala, 1999). In addition, anticonvulsant activity of aqueous extract has previously been reported (Asije et al., 2006). Phytochemical studies indicated that the aerials parts of *Emilia sonchifolia* contain alkaloids, and flavonoids and terpernes (Fu et al., 2002; Lija et al., 2006; Srinivasan and Subramanian, 1980). In addition, kaempherol-3-D-galactoside, quercitrin, quercetin, rutin, ursolic acid, senkirkine, doronine, β-sitosterol and stigmasterol are previous isolated from the *Emilia sonchifolia* (Srinivasan and Subramanian, 1980; Cheng and Röder, 1986; Gao et al., 1993).

Many medicinal plants provide relief of symptoms comparable to that of conventional medicinal agents. Therefore, the present study was aimed at evaluating the scientific basis for the traditional use of *Emilia sonchifolia* using chemical and thermal models of nociception.

2. Materials and methods

2.1. Plant material

Emilia sonchifolia (L.) DC. (Asteraceae) was collected in Alfenas, Minas Gerais, Brazil. Dr. G. Alves-da-Silva, Department of Pharmacy of Federal University of Alfenas, identified the plant, and the voucher specimen is deposited at the Herbarium of Federal University of Alfenas-MG (voucher number 0195).

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2.2. Preparation of the plant extracts and reference drugs

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

2.3. Analysis of EsHE by high performance liquid chromatography

High performance liquid chromatography (HPLC) analysis of the EsHE was performed in Shimadzu LC-100 HPLC using a Shimadzu CLC-ODS (250-4, 6 mm) C18 column with a 5 μm particle size. Mobile phases were composed of a (A) 0.5 mM/l aqueous acetic acid and (B) 0.5% acetic acid in methanol. The gradient of the mobile phases (A:B) used for separation were 0–30 min (10:90 to 0:100) and 45 min (0:100) with a solvent flow rate of 1.0 ml/min, an injection volume of 20 μl at a concentration of 1 mg/ml. The eluent was detected with a photodiode array detector (DAD) with UV light at 272 nm. LC solution software (Shimadzu) was used for data collection.

2.4. Assessment of antinociceptive activity of EsHE

Adult male Swiss mice (22–30 g), obtained from the Central Animal Facility of the Federal University of Alfenas, were housed under controlled light (12:12 h light–dark cycle; lights on at 06:00 am) and temperature conditions (23 ± 1 °C), with access to water and food *ad libitum*. The animals were allowed to habituate to the housing facilities for at least 1 week before the experiments began. All experiments were conducted in accordance with the Declaration of Helsinki on the welfare of experimental animals and with the approval of the Ethics Committee of the Federal University of Alfenas.

Animals were treated with the EsHE (30, 100 and 300 mg/kg) or with vehicle (1% sodium carboxymethylcellulose suspension in distilled water) given by the p.o. 1 h before the experiments. To assess the possible participation of the opioidergic system in the *Emilia sonchifolia* antinociception, mice were pre-treated with non-selective opioid receptor antagonist, naloxone (0.4 mg/kg), which was injected 15 min before the administration of the EsHE (100 mg/kg; p.o.) and morphine, and tested using the writhing, formalin, tail-flick and hot plate tests. Indomethacin (10 mg/kg) and morphine sulphate (1 or 10 mg/kg) in vehicle were used as reference drugs (Vilela et al., 2009). Test drugs were orally administered, except for morphine sulphate and naloxone, which was intraperitoneally administered in an equivalent volume of 10 ml/kg body weight of the animal.

2.4.1. Acetic acid-induced writhing in mice

Acetic acid (0.6%, v/v, 10 ml/kg) was injected into the peritoneal cavities of mice, placed in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection, as described earlier (Collier et al., 1968). Oral treatments (p.o.) with vehicle, indomethacin or EsHE were given 1 h prior to acetic acid injection ($n = 6$ per group). Morphine sulphate (1 mg/kg) was intraperitoneally administered (i.p.) 30 min before the test. The writhing response consists of a contraction of the abdominal muscle together with a stretching of the hind limbs. The antinociceptive activity was expressed as writhing scores over a period of 20 min.

2.4.2. Formalin test

A formalin solution (2.5% in 0.9% saline; 20 μl/paw) was injected into the hind paw plantar surface (i.pl.), and the animals were individually placed in transparent observation chambers, as previously described (Santos and Calixto, 1997). Oral treatments (p.o.) with vehicle, indomethacin, or EsHE were given 1 h prior to formalin injection ($n = 8$ per group). Morphine sulphate was administrated (10 mg/kg; i.p.) 30 min before the test. The time spent in licking the injected paw was recorded and expressed as the total licking time in the early phase (phase 1; 0–5 min) and late phase (phase 2; 20–30 min) after formalin injection.

2.4.3. Tail flick test

The lower two-thirds of the tail were immersed in a beaker containing water kept at 50 ± 0.5 °C (Wang et al., 2000). The time in seconds until the tail was withdrawn from the water was defined as the reaction time. The reaction time was then measured 0, 30, 60, and 120 min after the oral administration of vehicle, EsHE and morphine (10 mg/kg), whereupon the reaction time of 0 min is the start of the test. The mice ($n = 8$ per group) were exposed to hot water for no longer than 20 s to avoid tissue injury.

2.4.4. Hot plate test

The hot plate was an electrically heated surface kept at a constant temperature of 50.0 ± 0.5 °C. Mice ($n = 8$ per group) were placed on the heated surface within the Plexiglas walls to constrain their locomotion on the plate, and the latency to a discomfort reaction (licking of the paws or jumping) was recorded 0, 30, 60, and 120 min after oral administration of vehicle, EsHE or morphine (10 mg/kg), whereupon the reaction time of 0 min is the start of the test. A cut-off time of 20 s was chosen to indicate complete analgesia and to avoid tissue injury. The latencies for paw licking or jumping were recorded for each animal (Yamamoto et al., 2002).

2.5. Evaluation of acute toxicity of the Emilia sonchifolia extract

EsHE was orally administered to a group of mice, both male and female. The behavior parameters observed after administration were convulsion, hyperactivity, sedation, grooming, and increased or decreased respiration during a period of 7 days. Food and water were provided *ad libitum*.

2.6. Open-field test

In order to discard the possible nonspecific muscle relaxants or the sedative effects of extract, the motor performance of the mice was evaluated on the open-field apparatus (Archer, 1973; Vilela et al., 2009). Groups of mice ($n = 8$) received vehicle and EsHE (30, 100 and 300 mg/kg) 1 h before the test. Each animal was placed in the center of the open arena and allowed to have free ambulation for 5 min observation of the locomotion frequency.

2.7. Statistical analysis

The data obtained were analyzed using the GraphPad software program Version 4.0 and expressed as mean ± S.E.M. Statistically significant differences between groups were calculated by the application of an analysis of variance (ANOVA) followed by the Newman–Keuls test. p-Values less than 0.05 ($p < 0.05$) were used as the significance level.

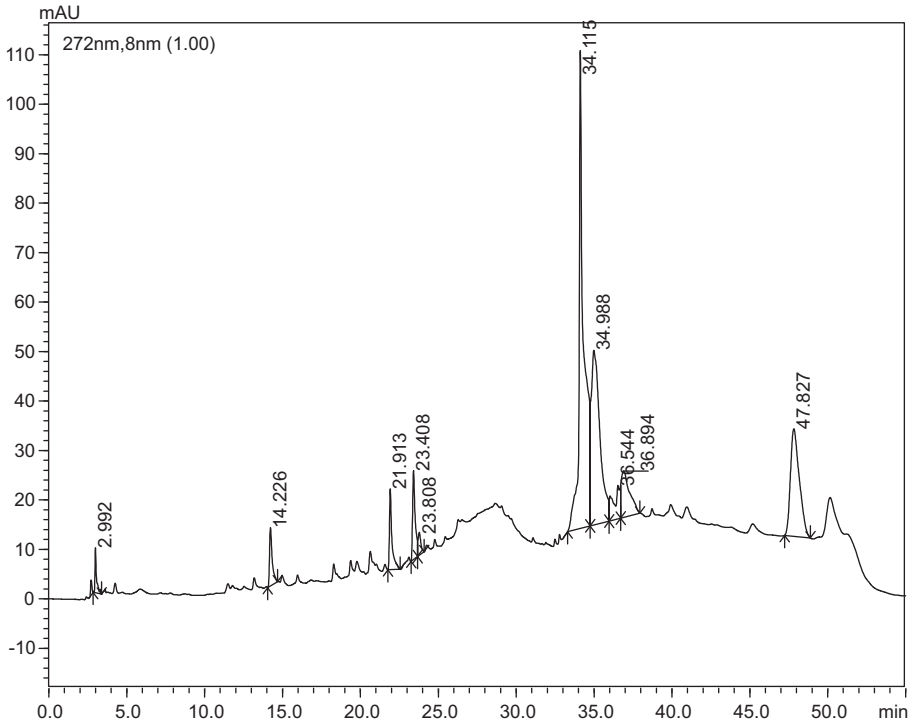

Fig. 1. High performance liquid chromatography profiles of *Emilia sonchifolia* hydroethanolic extract traced at 272 nm.

3. Results

3.1. HPLC analysis

Fig. 1 shows the HPLC analytical plot for *Emilia sonchifolia* extract at 272 nm, the peak at 21.91 min was identified as quercetin. The peaks at 14.23, 18.28, 19.37, 19.77, 20.63 and 47.83 min are compounds that have characteristics in the UV bands for flavonoids (Mabry et al., 1970). These flavonoids and others compounds have not been identified and are under investigation.

3.2. Acetic acid-induced writhing in mice

The oral administration of EsHE (at dose 100 and 300 mg/kg) caused a significant reduction in the number of writhing episodes induced by acetic acid compared to the control. The inhibition of writhes in percentage ranged from 63.0% and 80.2% after administration of indomethacin and morphine, respectively. Naloxone (opioid antagonist) given before the morphine or EsHE (100 mg/kg, p.o.) abolished the antinociceptive responses in this test (Fig. 2).

3.3. Formalin test

The EsHE at doses of 100 and 300 mg/kg, p.o. had a significant antinociceptive activity compared to the control in both the early and late phases ($p < 0.05$). The reference drug, indomethacin, suppressed only the second phase of the formalin test ($p < 0.05$). In order to compare the antinociceptive activity of EsHE, a group of mice was injected with morphine. This opioid significantly inhibited the total licking of both phases after formalin injection ($p < 0.05$). Pre-treatment of mice with naloxone completely prevented the morphine and EsHE (100 mg/kg) antinociceptive activity (Fig. 3).

3.4. Tail flick and hot plate tests induced nociception in mice

The EsHE administrated at doses of 100 and 300 mg/kg caused a significant increase in the response latency time as compared to the control animals. In the hot plate test, oral treatment with EsHE at doses of 100 and 300 mg/kg increased the latency time as compared

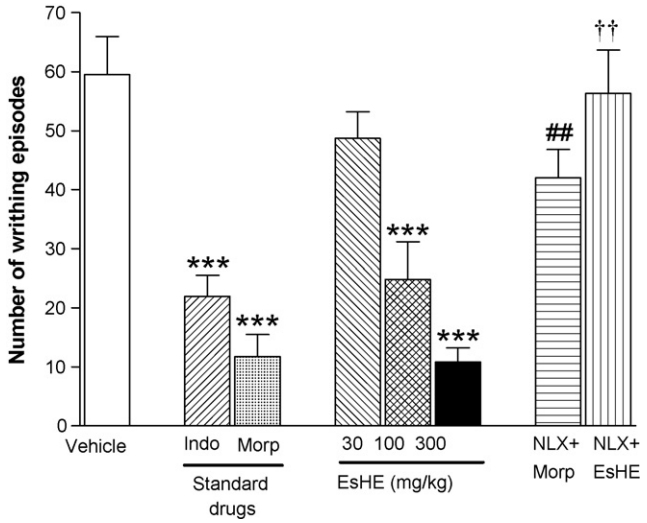

Fig. 2. Effects of *Emilia sonchifolia* hydroethanolic extract (EsHE) administered orally acetic acid-induced writhing movements in mice. Animals were treated orally with vehicle, EsHE (doses 30, 100, and 300 mg/kg), indomethacin (Indo; 10 mg/kg), morphine (Morp; 1 mg/kg), naloxone + morphine (NLX + Morp; 0.4 mg and 1 mg, respectively) or naloxone + EsHE (NLX + EsHE; 0.4 mg and 100 mg, respectively) prior to the acetic acid (0.6%, i.p.). Each column represents the mean with S.E.M. for six mice in each group. The symbols denote the significance levels (one-way ANOVA followed by Newman–Keuls test): $^{***}p < 0.001$ when compared with the control group (vehicle); $^{\#\#}p < 0.01$ when compared with the morphine group; $^{\dagger\dagger}p < 0.01$ when compared with the EsHE (100 mg/kg) group.

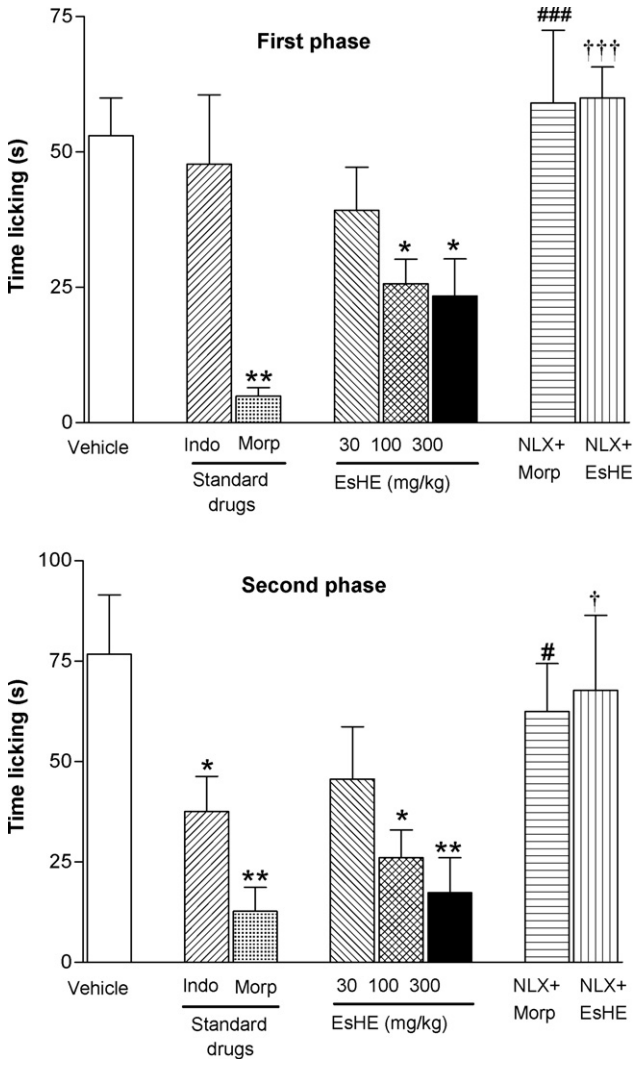

Fig. 3. Effects of *Emilia sonchifolia* hydroethanolic extract (EsHE) given by oral route on the nociceptive licking response induced by formalin in mice. Animals were treated orally with vehicle, indomethacin (Indo; 10 mg/kg), morphine (Morp; 10 mg/kg), EsHE (doses 30, 100, and 300 mg/kg), naloxone + morphine (NLX + Morp; 0.4 mg and 10 mg, respectively) or naloxone + EsHE (NLX + EsHE; 0.4 mg and 100 mg, respectively), prior to formalin. The total time spent licking the hindpaw was measured in the first (upon) and second (button) phases after intraplantar injection of formalin. Each column represents the mean with S.E.M. for eight mice in each group. The symbols denote the significance levels (one-way ANOVA followed by Newman–Keuls test): $^{*}p < 0.05$; $^{**}p < 0.01$ when compared with the control group (Vehicle); $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ when compared with the morphine group; $^{\dagger}p < 0.05$; $^{\dagger\dagger\dagger}p < 0.001$ when compared with the EsHE (100 mg/kg) group.

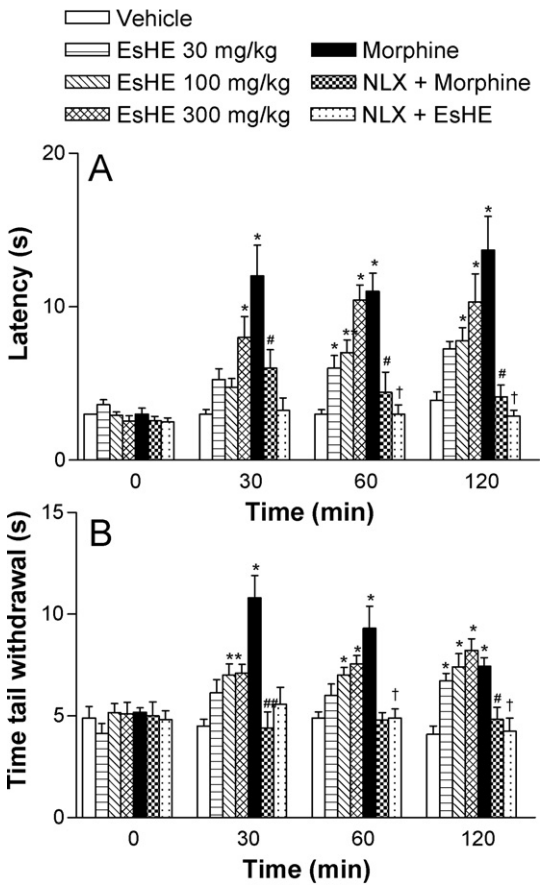

Fig. 4. Effects of *Emilia sonchifolia* hydroethanolic extract (EsHE) administered orally in the hot plate test (panel A) and in the tail immersion test in mice (panel B). Animals were pretreated orally with vehicle, morphine (Morp; 10 mg/kg), EsHE (doses 30, 100, and 300 mg/kg), naloxone + morphine (NLX + Morp; 0.4 mg and 10 mg, respectively) or naloxone + EsHE (NLX + EsHE; 0.4 mg and 100 mg, respectively) prior to the tests at 50 °C. Each column represents the mean with S.E.M. for eight mice in each group. The symbols denote the significance levels (one-way ANOVA followed by Newman–Keuls test): $^{*}p < 0.05$ when compared with the control group (Vehicle); $^{\#}p < 0.05$ when compared with the morphine group; $^{\dagger}p < 0.05$ when compared with the EsHE (100 mg/kg) group.

to the control group. As expected, administration of the vehicle did not induce any antinociceptive effect. Morphine significantly increased the latency time in both tests (Fig. 4).

Trying to elucidate the mechanism by which EsHE induces antinociception, animals were pre-treated with naloxone. Fig. 3 shows that naloxone completely prevented the morphine antinociceptive effect in tail flick and hot plate models. Naloxone also prevented the antinociceptive activity of the EsHE at 100 mg/kg dose. Effect of naloxone against EsHE was comparable with those obtained with naloxone against morphine on both spinal and supra-spinal models of analgesia.

3.5. Open-field test

A major concern in experiments designed to evaluate the analgesic action of new agents is whether pharmacological treatment causes other behavioral alterations, such as altering motor coordination or sedation, which could be misinterpreted as analgesia. Treatment with EsHE at 30–300 mg/kg did not cause a reduction in the numbers of crossings and rearings in the open-field test when compared to the control group (data not shown).

3.6. Acute toxicity

The EsHE at a dose of 0.5–5 g/kg, p.o. given to mice had no effect on their behavioral responses during the observation period of 7 days after administration. No mortality was observed up to 7 days of monitoring. The LD50 value of these extracts in mice was therefore estimated to more than 5 g/kg, p.o. As the effective dose used in the present study (100 mg/kg, p.o.) was 50-fold less than the dose used in the acute toxicity test, it was safe to assume that the normal doses of 100, and 300 mg/kg, p.o. given to mice in this study were safe.

4. Discussion

The present study showed the antinociceptive effect of the *Emilia sonchifolia* extract in different nociceptive responses generated by a chemical or thermal noxious stimulus. The antinociceptive effects of *Emilia sonchifolia* extract occurred at doses that

evoked no modification in the overall behavior of the animals. In this study, naloxone prevented the antinociception induced by EsHE, corroborating with the hypothesis that μ-opioid receptors must be involved.

The acetic acid-induced writhing is a sensitive test for assessment of analgesic drug. However, it can be seen as a general non-selective model for antinociceptive studies, since acetic acid indirectly induces the release of endogenous mediators, stimulating the peripheral nociceptor and sensitive neurons that were sensitive to the inflammatory mediators (Couture et al., 2001; Deraedt et al., 1980; Koster et al., 1959; Ribeiro et al., 2000). The results showed that EsHE significantly inhibited acetic acid-induced writhing responses. Therefore, one possible mechanism of antinociceptive activity of EsHE could be due to the blockade of the effect or the release of endogenous substances (arachidonic acid metabolites) that sensitize and activate peripheral nociceptors. The result of this test, however, does not ascertain whether the anti-nociceptive effect was mediated by central or peripheral process.

The neurogenic and inflammatory pain was evaluated using formalin test (Tjølsen et al., 1992). Subcutaneous injection of formalin into the animal hind paw evokes an array of stereotyped behaviors (Dubuisson and Dennis, 1977). The nociceptive response to formalin occurs in a biphasic pattern; there is an initial acute period (phase 1) and, after a short period of remission, phase 2 begins and consists of a longer period of sustained activity. The phase 1 corresponds to acute nociceptive neurogenic pain, and is sensitive to analgesic drugs that interact with opioid system. The phase 2 corresponds to an inflammatory pain, dependent of several inflammatory mediators release and action, and the expression of nociceptive behavior in this phase is very sensitive to non-steroid anti-inflammatory drugs as the cyclooxygenase inhibitors. Drugs that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the second phase (Abram and Olson, 1994; Manning, 1998; Rosland et al., 1990; Yamamoto et al., 2002; Yamamoto and Nozaki-Taguchi, 2002). Once both first and second phase behavioral hypernociception were affected by the EsHE previous treatment, it can be suggested that not only anti-inflammatory action (Muko and Ohiri, 1999) but also direct analgesic function can be present here. Therefore, the antinociceptive activity of EsHE in formalin test is strongly attributed to peripherally acting as well as centrally acting pain mediators. This would not be the first time that an anti-inflammatory agent had parallel and independent direct analgesic effect. In fact, the analgesic effect of both dipyrone and diclofenac involves not only their anti-inflammatory potential, but a peripheral antinociceptive effect is associated with ATP-sensitive K^+ channel (Alves and Duarte, 2002; Alves et al., 2004). The involvement of this channel may explain both spinal and peripheral analgesia, and the anticonvulsive use of the plant. To confirm the participation of central analgesic system in the antinociceptive activity of EsHE, hot plate test and tail-flick test were employed.

In the tail immersion test, which consists of a thermal stimulus, an increase in the reaction time is generally considered to be an important parameter for evaluating central antinociceptive activity (Rujjanawate et al., 2003). Hot plate test is predominantly a spinal reflex or behavioral reaction and used to test supra-spinal analgesia in compounds. The plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, paw licking and jumping. Both are considered to be supraspinally integrated responses. It is therefore, selective for centrally acting analgesic drugs, like morphine, while peripheral anti-inflammatory antinociceptive agents are found to be inactive on thermal stimulus. These tests also revealed that the antinociceptive effect of *Emilia sonchifolia* extract on mice remained present for at least up to 120 min after administration of the extract. The *Emilia sonchifolia* extract was found to have antinociceptive activity in the hot plate test, which is a specific central antinociceptive test. The antinociceptive effects of EsHE involve supraspinal as well as spinal components, as demonstrated by the use of the hot plate (Yaksh and Rudy, 1976; Yaksh and Rudy, 1977; Yeung et al., 1977) and tail immersion (Luttinger, 1985; Woolf et al., 1980) tests, respectively.

One of the main strategies in nociception studies has been the search for opioid analgesics acting at opioid receptors outside the central nervous system (CNS), with the prospect of avoiding centrally mediated side effects as tolerance and dependence (Benyhe, 1994; Vanegas and Tortorici, 2002). For the assessment of opioid system involvement in the analgesic activity the mice were pretreated with an opioid antagonist, naloxone. In this study, naloxone prevented the antinociceptive effect on both phases of the formalin test, as well in writhing, tail-flick and hot plate tests. Those results suggest that, at least part of the anti-hyperalgesic effect observed for the fractions is due to involvement of this system (μ-opioid) since naloxone reverted the antinociceptive activity.

In conclusions, the results presented in this study suggest that the hydroethanolic extract that was obtained from *Emilia sonchifolia* exhibited opioid-mediated antinociceptive activity in animal models. The precise mechanisms that are involved in the production of the antinociceptive response of *Emilia sonchifolia* extract are not completely understood, but they may be caused by the presence of flavonoids and others aromatic compounds. Thus, may be useful in the treatment of inflammatory hyperalgesic disorders, which supports previous claims of its traditional use.

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